Although there is strong evidence that different exposures early in life can alter the risk of allergic diseases, the underlying mechanisms are unclear and the development of preventive strategies has been delayed.

The present thesis demonstrates the impact of protective and harmful exposures on children’s immune status ex vivo and in vitro. Some changes in immune responses were observable up to teenage. This dissertation advances the knowledge of the relationship between early life exposures, immune development and the risk of allergic diseases.
THE DEVELOPMENT OF IMMUNE RESPONSES AND ALLERGIC DISEASES

THE ROLE OF PRENATAL AND EARLY LIFE EXPOSURES
Maria-Viola Martikainen

THE DEVELOPMENT OF IMMUNE RESPONSES AND ALLERGIC DISEASES
THE ROLE OF PRENATAL AND EARLY LIFE EXPOSURES

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Building at the University of Eastern Finland, Kuopio, on November, 30,
2018, at 12 o’clock noon
ABSTRACT

The prevalence of allergic diseases has increased significantly over the last decades, creating substantial financial and societal burdens. Due to this, researchers are trying to discover new approaches for prevention and treatment of the diseases.

There is strong evidence that different exposures early in life can alter the risk of allergic diseases. One of these exposures is farming. Exposure to the farm environment in childhood, and even prenatally, has been shown to decrease the risk of allergic diseases. On the other hand, being born by caesarean section is recognized as a risk factor. The roles of other obstetric factors are less studied. Another harmful exposure is air pollution, and especially exposure to particulate matter, which has been shown to increase asthma prevalence and exacerbations in children. The underlying mechanisms are, however, unclear and the development of asthma-preventive strategies has been delayed.

Studies have shown that immunological development and maturation already starts during pregnancy and in early childhood. Therefore, it can be hypothesized that exposure at this critical point of immune development may modify immune responses and cells, and thus influence the risk of allergies and other immune diseases.

The overall aim of this thesis was to investigate how different exposures during pregnancy, birth or childhood modulate asthma-related immune responses in children. It focuses on three different exposures: one that is asthma-protective (farming) and two that predispose to asthma (caesarean section and air pollution). The specific aims were to assess I) whether circulating dendritic cells (DC) associate with farming, asthma or atopy, II) whether obstetric factors affect immune responses at teenage in children born by caesarean section and, III) whether farm dust and urban air particulate matter (PM) have immunomodulatory effects on children’s circulating immune cells.

To answer these questions, associations between exposures and immunological responses were studied. Circulating DC subsets of farm and non-farm children were examined at the age of 6 to assess whether DCs mediated the protective effect of farm exposure. Cytokine secretion of unstimulated and stimulated PBMCs at teenage were
examined to identify whether obstetric factors alter immune responses later in life. PBMCs of 4-year-old children were stimulated with farm dust and size-segregated PM to discover shared and distinct immune pathways between two different environmental exposures.

The studied environmental exposures were associated with asthma-related immune responses. Inverse association between farm exposure and mDC2s, and the association between mDC2s and asthma in farm children, suggested that this DC subset plays a role in farm-related immunoregulation. On the other hand, the lack of natural birth processes during delivery and neonatal intensive care treatment seemed to lead to long-lasting alterations of immune responses. The observed stimulatory effects of farm dust and inhibitory effects of PM on immune responses indicate that these exposures could modify responses towards respiratory pathogens and allergens, and partly explain differences in asthma prevalence between the studied environments.

In conclusion, this thesis project demonstrated associations between diverse early life exposures and immune responses, both ex vivo and in vitro. Some changes in immune responses seemed to be observable up to teenage. This dissertation revealed some of the potential immunological mechanisms behind different exposures and advanced knowledge of immune mechanisms that either protect from or predispose to asthma. Moreover, the developed methodological approach offered a new perspective, which could be utilized when studying environment-related immune diseases and their mechanisms. These studies suggest that acquiring comparable data from various exposure environments could lead to the discovery of new immunological pathways and provide novel tools for risk assessment and for the development of preventive strategies.

National Library of Medicine Classification: QT 140, QW 551, WD 300, WF 553
Medical Subject Headings: Hypersensitivity/etiology; Asthma/etiology; Adaptive Immunity; Environmental Exposure; Prenatal Exposure Delayed Effects; Pregnancy; Labor, Obstetric; Agriculture; Farms; Cesarean Section; Air Pollution; Air Pollutants; Dust; Particulate Matter; Dendritic Cells; Leukocytes, Mononuclear; Immunomodulation; Cytokines; Child, Preschool; Child; Adolescent
Allergisten sairauksien esiintyvyys on lisääntynyt merkittävästi viime vuosisikmmenten aikana, aiheuttaen huomattavia taloudellisia ja yhteiskunnallisia rasitteita. Tästä johtuen tutkijat pyrkivät löytämään uusia tapoja, joilla näiden sairauksien esiintyvyyttä voitaisiin ehkävähentää.


Tutkimuksissa on huomattu, että immunologinen kehitys ja immuunijärjestelmän kypsyminen alkavat jo raskauden ja varhaislapsuuden aikana. Voidaankin olettaa, että altistuminen tässä tärkeässä kehitysvaiheessa voi muuttaa immuunivastetta ja –solujen toimintaa ja siten vaikuttaa riskiin sairastua immuunisairauksiin.

Tämän väitöskirjan päätavoitteena oli selvittää, kuinka erilaiset altistumisympäristöt ja altisteet raskauden ja varhaislapsuuden aikana vaikuttavat astmaan ja sen syntymmekanismeihin yhdistettyniin immuunivasteisiin. Tutkimus keskittyy kolmeen erilaiseen altistusympäristöön; astmalta suojavaa maatalypäristöä sekä astmariskiä lisäävää keisarineikkaukselle ja ilmansaaasteisiin. Erityisesti selvitettiin I) vaikuttaako maataloympäristölle altistuminen allergisten sairauksien riskiin tai veren dendriittisoluihin (DC), II) vaikuttaako syntymän aikaiset tapahtumat teini-ässä mitattaviin immunivasteisiin keisarineikkauksella syntyneillä lapsilla, III) vaikuttaako maatilapölyä tai kaupunki-ilman pienhiukkaset (PM) lasten veren immuunisolujen vasteisiin.

Selvittäksemme kuinka maatila-altistus vaikuttaa immuunivasteisiin, selvitimme maatiloilla ja muualla maaseudulla asuvien 6-vuotiaiden lasten DC-solujen...
määrä ja toiminnullisia ominaisuuksia. Synnytyksen aikaisten tekijöiden pitkäikaishuomautuksia selvitettiin tutkimalla veren immunisolujen sytokiinin erityistä teini-iässä. Lisäksi tutkittiin kuinka hyvin erilaiset ympäristöaltisteet (maatalöytö ja kokojaotellut kaupunki-ilman pienhiukkaset) vaikuttivat 4-vuotiaiden lasten veren immunisolujen vastaisiin.

Tulokset osoittivat altisteiden vaikuttavan tutkittaviin immunivasteisiin. Maatalo-altistuminen oli yhteydessä pienentyneeseen astmariskiin sekä matalampiin mDC2-tasoihin. Käänteen yhteyksessä maatalo-altistumisella sekä mDC2-soluilla sekä mDC2-soluja ja astman välinen yhteys maatalalapsilla osoitti, että tällä DC-solutyyppillä voi olla rooli maatalojärjestööön liittyvän astma-suojan takana olevaan immunisääteisyyteen. Sitä vastoin luonnollisten prosessien puuttuminen synnytyksestä ja syntymän jälkeinen hoito teho-osastolla näyttävät johtavan immunivasteiden pitkäaikaiseen heikentymiseen. Tutkimuksessa havaitut maatalopölyyn immunivastavettä stimuloivat ja PM:n inhiboivat vaikutukset voivat vaikuttaa keuhkojen reaktioihin hengittäviä patogeeneja sekä allergieeneja vastaan. Tämä voisi osoittaa selittää eroja astman esintyvyydessä tutkitaan ympäristöissä.


Yleinen suomalainen asiasanasto: yliherkkyys; allergia; astma; immuunijärjestelmä; altistuminen; raskaus; synnytys; maatalous; maatalot; keisarileikkauskset; ilman saastuminen; ilmansaasteet; pöly; pienhiukkaset; valkosolut; sytokiinit; lapset (ikäryhmät); esikouluikäiset; teini-ikäiset
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I owe my heartfelt thanks to my parents Seppo and Tatiana and brother Toni for supporting me through my life. You have always encouraged me to reach out further. Спасибо! I also thank my friends for support and for taking my mind away from work!

Kuopio, 29th August, 2018
Maria-Viola Martikainen
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<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-cell activating factor</td>
</tr>
<tr>
<td>BDCA</td>
<td>Blood dendritic cell antigen</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation. E.g. CD80</td>
</tr>
<tr>
<td>CD</td>
<td>Caesarean delivery</td>
</tr>
<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CRTH</td>
<td>Chemoattractant Receptor-homologous Molecule Expressed on Th Cells</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>CS</td>
<td>Caesarean section</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide, (CH₃)₂SO</td>
</tr>
<tr>
<td>DL</td>
<td>Detection limit</td>
</tr>
<tr>
<td>EFRAIM</td>
<td>Mechanisms of Early Protective Exposures on Allergy Development</td>
</tr>
<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human Leukocyte Antigen – antigen D Related</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILT</td>
<td>Immunoglobulin-like transcript</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase-like</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>LCA</td>
<td>Latent class analysis</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR family pyrin domain containing 3</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PASTURE</td>
<td>Protection against allergy: study in rural environments</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PM</td>
<td>Particulate matter</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen-recognition receptors</td>
</tr>
<tr>
<td>RIPK</td>
<td>Receptor-interacting serine/threonine-protein kinase,</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling proteins</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on data presented in the following articles, referred to by the Roman Numerals I-III.


AUTHOR’S CONTRIBUTION

The publications in this dissertation are original research papers assessing whether different exposures during pregnancy and childhood affect immunological responses later in life.

I) The author participated in the experiments and analyses, performed all statistical analyses, participated in the interpretation of the results and drafted the manuscript.

II) The author participated in the experiments and analyses, performed all statistical analyses, participated in the interpretation of the results and drafted the manuscript.

III) The author participated in planning the laboratory experiments and laboratory analyses. The author conducted all statistical analyses, participated in the interpretation of the results and drafted the manuscript.
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1 INTRODUCTION

Several diseases and developmental disorders have been associated with different exposures during pregnancy and early in life. For example, prenatal exposure to tobacco smoke disrupts children’s health significantly (Baena-Cagnani et al. 2009). Other exposures such as farming (von Mutius and Vercelli 2010) and breastfeeding (Lodge et al. 2015), on the other hand, have been recognized as protective factors.

Contrary to several diseases, which have almost been eradicated, such as measles, mumps and polio, the prevalence of asthma and other allergic diseases has increased during the last decades (Anandan et al. 2010). This increase has created substantial social, financial and societal burdens. Asthma is a disease that affects people of all ages, races and both sexes, influencing the quality of life and possibly leading to life-threatening asthma attacks (Nunes et al. 2017, Loftus and Wise 2015). Since asthma also has huge financial impacts on society, researchers are trying to discover ways to prevent the onset of new asthma cases.

Several asthma predisposing and protective factors have been identified. These are, for example, exposure to air pollution (Guarnieri and Balmes 2014), farming (von Mutius and Vercelli 2010), and pet animals (Salo and Zeldin 2009). The exact mechanisms behind those factors, however, remain unknown. Further research is needed to disentangle the immune responses behind asthma predisposing and protecting exposures to find effective ways to prevent or to alleviate the disease burden of asthma and other allergic diseases.

Several studies have shown that immunological development already takes place during pregnancy and in early childhood (Vieira 2015, Deng et al. 2015, Wright and Brunst 2013, Lacasaña et al. 2005). Therefore, it can be hypothesized that exposures at a critical point of immune development may modify the relative amount and function of immune cells, thus changing their properties and influencing the risk of immune diseases.

The main aim of this thesis is to investigate the associations between different exposures during pregnancy or early childhood and children’s immunological responses. It focuses on farm exposure, which protects from atopic diseases and on caesarean section and air pollution, which increase the risk of atopic diseases. Data from two exceptionally extensive and long-lasting cohort studies, KEISARI and PASTURE, were utilized.

The novel scientific data could provide an opportunity to disentangle the relevant immune mechanisms linking prenatal and early life exposures to immunomodulation and the risk of childhood asthma.
2 LITERATURE REVIEW

2.1 ASTHMA

2.1.1 Definition, onset, prevalence and burden

Asthma is a chronic disease of the airways. It is characterized by symptoms such as recurrent attacks of breathlessness, reversible airflow obstruction, wheezing, and the tendency to over-react to stimuli. It often occurs together with other atopic diseases, such as allergic rhinitis and atopic dermatitis. Asthma is a syndrome with several different phenotypes, such as allergic/atopic, non-allergic and trigger induced (Lockey 2014). The phenotype of asthma affects the pathophysiology of the disease, thus influencing the selection of appropriate treatments and perhaps influencing the prevention of asthma. The underlying pathogenesis usually includes chronic inflammation of the airways and the activation of diverse immune cells. The pathogenesis of allergic asthma is described briefly in Chapter 2.2.1.

Since asthma is a heterogeneous disorder, with a genetically complex background, it has been challenging to study the relationships between exposures and the mechanisms behind the asthma incidence. Usually, the onset of new asthma occurs during infancy and in early childhood (early onset asthma), but some asthma-cases arise in adulthood (late-onset asthma). Several different exposures and events have already been linked to the onset of new asthma. Some of these will be discussed in more detail in Chapter 2.3.

The prevalence of asthma and allergic diseases have increased significantly over the last decades (Anandan et al. 2010). Although the rise of asthmatics has been higher in industrialized countries, developing countries make up more than 80% of the mortality. An estimated 300 million people worldwide suffer from asthma, and of those about 70% also have allergies (WHO 2006). Moreover, asthma is the most common chronic disease in children (Miller et al. 2016). In Finland, 7-10% of the population suffers from asthma, approximately 5% have occasional asthma-like symptoms and nearly 4% of adults suffer from very severe asthma, which can be life-threatening (Duodecim Terveyskirjasto 2018).

Asthma can vary in severity and in frequency from person to person, influencing health, quality of life, and career outcomes. It is a disease that creates substantial social, financial and societal burdens. It has direct costs (e.g. hospital admissions, medications), indirect costs (e.g. loss of productivity, work absence, disabilities and early mortality), intangible costs (e.g. the decrease in quality of life, increases in pain or suffering, limitation of physical activities) as well as socioeconomic impacts (Nunes et al. 2017). Although asthma is largely controlled by steroid treatment, exacerbations that can lead to hospitalization and even to death are still common. Approximately 250,000 deaths each year can be attributed to asthma and almost all of these deaths would have been avoidable (WHO 2006). Researchers are trying to find
new approaches for primary, secondary and tertiary prevention. Primary prevention aims to decrease asthma incidence, secondary prevention aims to alleviate the burden of already established disease and includes detection, management, and control of the disease, whereas tertiary prevention aims to reduce the complications caused by severe disease. Proper management and prevention of asthma and related diseases in children and adults could reduce negative health outcomes and decrease unnecessary costs. Several different guidelines and statements have been published (Porsbjerg et al. 2018, Chung KF et al. 2014) for the management and diagnosis of different asthmatic diseases.

2.1.2 Pathogenesis of allergic asthma

Asthma is usually defined as an inflammatory disease, in which several different immune cells orchestrate signal cascades, which in turn affect either other cells or the cells themselves. The exact pathogenesis and mechanisms are hard to define, as the immunoregulation and the pathogenesis are distinct in different phases (early vs chronic) and phenotypes (allergic vs non-allergic). In this chapter, the key players in allergic asthma are briefly described (Figure 1).

In allergic asthma, the normal reaction to harmless antigen is skewed. After encountering allergen for the first time, antigen-presenting (APCs) cells process and present the antigen to naïve Th cells (CD4+ Th cells) in lymph nodes, leading to differentiation into Th2 cells. A Th2 phenotype favours the development of asthma and allergy through IgE production, which binds to a FcεRI receptor on the surface of mast cells and basophils. This binding of IgE to immune cells, is considered as the first steps in sensitization to the allergen.

After encountering the allergen second time, complex immune responses and pathways activate. Allergen binds directly to IgE and mast cells, which will release histamine and other inflammatory mediators. At the same time, allergen-activated-APCs, such as dendritic cells (DCs) and alveolar macrophages further induce Th2 production and activation. Th2 cells are attracted to the site of inflammation by chemokines. The effector Th2 cells upregulate inflammation in lung through humoral immunity. Th2 cells release inflammatory cytokines, such as IL-4, IL-5, IL-9 and IL-13. These cytokines affect the function of several different cell types, such as eosinophils, mast cells and B cells, inducing allergic inflammation. IL-4 and IL-13 activate plasma cells to increase IgE production, IL-9 affects on mast cells directly and increase the histamine production, and IL-5 increases the numbers of eosinophils. These immunological events lead to airway hyperresponsiveness, mucus hypersecretion, airway remodelling, and irreversible airflow obstruction (Kubo 2017).
To counterpoint the classical characterization of asthma, recent studies have shown that other immune cells such as type 2 innate lymphoid cells (Licona-Limón et al. 2013), other T cell subsets (Th17, Th22), epithelial cell cytokines (IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)) are also involved in the pathogenesis of asthma (Hirose et al. 2017). Major mechanisms of asthma onset, progression and exacerbation have been extensively reviewed in Edwards et al. (2017), which also discusses the less known exposures and mechanisms behind asthma.

2.2 THE IMMUNE SYSTEM

One of the primary functions of the immune system is to defend against foreign organisms and substances. Immune responses are complex and often involve several cellular and molecular components, such as immune cells, humoral factors and cytokines, which together lead to the elimination of the foreign antigens and to the resistance against diseases. When the immune system is not working correctly, problems may rise. Underactivity may result in immunodeficiency, causing severe infections and even tumours, whereas overactivity may cause autoimmune and allergic
diseases. Furthermore, the exposure to external or internal stimuli can shape the immune responses later in life, and lead to dysfunction in the development of immunologically competent cells, inflammation and in the worst cases to autoimmune diseases. (Abbas et al. 2007)

The immune system is divided into two different parts; the rapid early reactions of innate immunity, and the delayed more specific responses of adaptive immunity. These two parts interact closely with each other. Innate immunity consists of immune cells, such as DCs, neutrophils, natural killer cells, monocytes and macrophages, and of other soluble substances such as the complement system, cytokines, and acute phase proteins. Adaptive immunity consists of antigen-specific B and T lymphocytes (Abbas et al. 2007). Previously it was thought that only adaptive immunity has memory. However, studies have shown that cells of innate immunity, such as DCs, natural killer cells, monocytes and macrophages, also have memory and can be primed for the next encounter with an antigen (Bauer et al. 2018, Crowley et al. 2018, Quintin et al. 2014).

Cells of innate immunity, specifically DCs, macrophages and monocytes, are among the first cells to react to environmental exposures. After encountering inhaled foreign antigens, lung DCs, in collaboration with macrophages and epithelial cells, determine whether to direct the immune response towards tolerogenic or immunogenic pathways (Manicassamy and Pulendran 2011). They have also important roles in the regulation of normal immune responses as prevailing immune reactions need to be ended in due time. The significance of these specific immune cells and important mediators is discussed in the following chapters.

2.2.1 Dendritic cells - The major antigen-presenting cells

Dendritic cells (DCs), the major antigen presenting cells (APCs), have been recognized as a link between innate and adaptive immunity as they play a critical role in the induction of immunity and tolerance (Lebre et al. 2005). The main role of DCs is to process and present antigens to other immune cells, such as T lymphocytes, and steer immune reactions towards activation or tolerance.

DCs can be found in several tissues, such as skin, lungs and lymph nodes, where they have different functions. There are multiple subsets of DCs and thus far three main subsets of human circulating blood DCs have been identified; type 1 myeloid DCs (mDC1s), type 2 mDCs and plasmacytoid DCs (pDCs) (Gill 2012, Banchereau et al. 2000). In murine models, subsets of lung DCs have been reported to have different functions: one subtype monitors airway luminal surfaces and controls antigen uptake, while others prime and stimulate effector CD4+ T cells, or induce tolerance and control Treg development. DCs in human lungs act similarly, although knowledge on human lung DCs is not as complete (Lambrecht and Hammad 2010, Gaurav and Agrawal 2013).

Although all DCs are capable of antigen uptake and presentation, the DC subtypes differ in phenotype, expression patterns of pathogen-recognition receptors
(PRRs), location, migratory pathways, cytokine secretion profiles, and capacity to induce immune responses. It was previously suggested that mDCs participate in the development of Th2 and allergic responses (Jordan et al. 2007), whereas pDCs participate in response to viral infections, tolerance development and control of allergic airway inflammation (Young et al. 2008). Different mDC subtypes also differ in their surface receptors and functions. For example, mDC2s have been observed to have different cytokine secretion than mDC1s, and it has been observed that mDC2s might play different role in regulation of immune responses (Nizzoli et al. 2013, Demedts et al. 2006). However, studies have shown the functional plasticity of DCs and challenged the concept of DC subpopulations with distinct functions (Pulendran et al. 2008).

After encountering foreign antigens, DCs sense microbial stimuli through PRRs, such as Toll-like receptors (TLRs), C-type lectin like receptors, and NOD-like receptors. DCs process and present this information to other cells e.g. T cells, natural killer cells, neutrophils, and epithelial cells via surface molecules and cytokines to stimulate immune responses. After encountering a foreign antigen, DCs usually upregulate expression of major histocompatibility complex (MHC) molecules, co-stimulatory or inhibitory molecules and cytokines. Immunogenicity of DCs is usually dependent on the expression of, among other molecules, co-stimulatory molecules CD40/80/CD86 and pro-inflammatory cytokines, whereas tolerogenic functions are mediated via inhibitory receptors such as immunoglobulin transcript 3 (ILT3) and ILT4 (Pulendran 2015, Hubo et al. 2013, Mahnke et al. 2002). Although some molecules and cell markers are considered tolerogenic and some immunogenic, conclusions about cell function cannot solely be based on surface markers. This is because immune responses are complex and controlled by multiple parameters such as signalling pathways, cell interactions, and signals from the microenvironment (Mayer et al. 2012, Manicassamy and Pulendran 2011). It has been suggested that DCs also sense stress signals, such as amino acid starvation, through ancient stress and nutrient-sensing pathways (Pulendran 2015).

DCs have a critical role in the determination of T lymphocyte responses. Depending on the encountered antigen, activated PRRs and the microenvironment, different DCs can guide immature T cells (CD4+ T lymphocytes) towards cell subsets such as T helper 1 cells (Th1), Th2, regulatory T cells (Tregs), or several other Th cell types (Figure 2) (Pulendran 2015, Gaurav and Agrawal 2013, Parkin and Cohen 2001). Th1 cells are induced as protection against bacteria and viruses. They secrete interferon-γ (IFN-γ) and promote the activation of macrophages, natural killer cells and cytotoxic, CD8+, T cells. Th2 cells are induced as protection against helminthic infections. They secrete cytokines such as IL-4, IL-5, and IL-13, inducing the production of IgE and activation of eosinophils. Excessive and unneeded Th2 responses can lead to allergic disorders and asthma (Pulendran 2015, Gaurav and Agrawal 2013).
There is evidence that DCs play an important role in inhalation tolerance and asthma. Allergies and asthma have been shown to be associated with the number of DCs in adults and in children, as relative differences in numbers of DC subsets have been associated with the prevalence of asthma (Upham et al. 2009, Hagendorens et al. 2003, Matsuda et al. 2002). For example, Matsuda and his group (2002) found that patients with asthma had a significantly higher number of pDCs compared with normal subjects. On the other hand, in a mouse asthma model, depletion of pDCs during inhalation of a normally inert antigen led to cardinal features of asthma. Furthermore, adoptive transfer of pDCs before sensitization prevented disease in a mouse asthma model (de Heer et al. 2004). These studies indicate that pDCs might provide protection against inflammatory responses to harmless antigens, and thus play a role in asthma pathogenesis. DCs seem to have a role in the induction phase as well as in the effector phase of asthma (Hirose et al. 2017). Increased levels of DC-related co-stimulatory molecules have been found in asthma, like CD86 in serum (Shi et al. 2004) and on the surface of B cells (Hofer et al. 1998). DCs expressing high levels of ILT3 and ILT4 have been shown to promote antigen-specific unresponsiveness in CD4+ T cells and the differentiation of Treg cells (Kornete and Piccirillo 2012), but associations between these markers and asthma have not been reported.

Ex vivo investigation of circulating DCs is difficult due to the small relative proportions of cells in the blood. In circulation, the number of mDC1s is approximately 0.20%, pDCs is 0.5% and mDC2s is 0.1-0.13% of PBMCs (Ban et al. 2008, Narbutt et al. 2004). While in blood mDC2s is the smallest population, in lungs and in bronchoalveolar lavage fluid they represent the dominant DC subset (3.2% of all lung
2.2.2 Monocytes - Circulating precursors and independent mediators

Monocytes, the members of the mononuclear phagocyte system, are central cells of innate immunity, although they also play an important role in the initiation of adaptive immunity. Monocytes respond to signals of inflammation in the body. After activation, they migrate to the area of inflammation and either differentiate into other cell types or phagocytose foreign antigens or dead cells. They are the largest types of leukocytes and have the capacity to differentiate into macrophages and myeloid lineage dendritic cells (mDCs).

Monocytes are heterogeneous and can be grouped into three different subtypes: classical (CD14+CD16−), non-classical (CD14dimCD16+), and intermediate (CD14+CD16+), which differ in size, migration and innate immune receptor expression, as well as in their ability to differentiate following stimulation (Auffray et al. 2009). Classical monocytes, account for 80–90% of peripheral blood monocytes, are considered as pro-inflammatory monocytes with major function in phagocytosis. They express genes involved in angiogenesis, wound healing, and coagulation. Some studies suggest that they might have a role in inhibition of immune responses. Intermediate monocytes are also considered as inflammatory monocytes as gene signature links them to antigen presentation and T cell activation. Both subtypes transfer from bloodstream to tissues during inflammation. Non-classical monocytes have patrolling function and seem to have a role in autoimmune diseases (Yang et al. 2014). As in DCs, the function of monocytes has also been studied more extensively in mice than in humans (Jakubzik et al. 2017).

The main function of monocytes is to differentiate into macrophages or DCs after stimulus. Some mechanisms and stimuli regarding to the selection of various differentiation pathways have been observed (Ohradanova-Repic et al. 2016, Geissmann et al. 2010). Function and importance of DCs was discussed in previous chapter. Contrary to the antigen-presenting function of DCs, the main functions of macrophages are phagocytosis, endocytosis, secretion of soluble mediators, and killing of microbes. Traditionally, macrophage subtypes have been divided into M1 (classic) and M2 (alternative) types with different functions. M1 subtypes are considered as inflammatory macrophages as they secrete pro-inflammatory cytokines, TNF and IL-6. They also contribute to e.g. tissue degradation and T cell activation. The M2 subtype on the other hand has roles in wound healing, tissue fibrosis, angiogenesis and tumorigenesis (Yang et al. 20014). Alveolar macrophages have been shown affect the development and progression of asthma (Balhara and Gounni 2012).

Although monocytes have a highly important role as progenitor cells, they also have roles that are separate from this function, such as phagocytosis, antigen presentation, and cytokine production. Monocytes can perform phagocytosis using the help of proteins such as antibodies (Igs) or complement fragments (i.e. C3d) that coat the
pathogen, or by binding to the antigen directly via pattern-recognition receptors such as TLRs. Phagocytosis starts the production of reactive oxygen species and cytokines, and in turn activation of the immune response. Monocytes can also function as antigen-presenting cells priming CD8+ and CD4+ T cells and thus contribute to adaptive immunity (Jakubzick et al. 2017). Furthermore, monocytes can kill cells by antibody-dependent cell-mediated cytotoxicity. Cytotoxicity is mediated by surface Fcγ receptors, which recognize antibody coated cells. This triggers the release of cytotoxic granules or upregulates death receptors expression on the cell surface.

As monocytes have an essential role in the effective control and clearance of several infections, the dysfunction of these cells can cause several problems. Monocytes have already been associated with the pathogenesis and progression of several diseases such as liver fibrosis, atherosclerosis, multiple sclerosis, and tumour metastasis (Karlmark et al. 2012). Differences in the functions of monocytes, such as lysosomal activity, recruitment and differentiation between healthy and asthmatic subjects have been reported (Sen et al. 2016, Shrestha Palikhe et al. 2015, Gunawardhana et al. 2014, Tomita et al. 1995).

As monocytes have an important role in defence against foreign antigens, different monocyte cell lines, such as THP-1, have been developed for research purposes. They are usually used as such or are differentiated into other cells i.e. macrophages.

2.2.3 Cytokines - Messengers of immunity

Immune cells also react to the environment and to different stimuli by secreting cytokines, which regulate the immune response and inflammation by mediating intercellular (and also intracellular) communication. Cytokines are small messenger proteins secreted by the cells of innate and adaptive immunity as a response to microbes and other antigens. Cytokines can also be secreted by other cells, such as endothelial and epithelial cells.

Cytokines, as a group of proteins, have several properties and functions. The actions of cytokines are often pleiotropic i.e. one cytokine can act on several different cell types and redundant i.e. several different cytokines can have similar functional effects. Cytokines usually act locally and may act on the cells that secrete them or on nearby cells, or on some occasions on distant cells. Cytokines often affect cell activation, division, apoptosis, or movement and influence the synthesis and actions of other cytokines. They can activate the production of, act in synergy with or even inhibit the effects of other cytokines. Cytokines act through cytokine receptors. When a cytokine binds to a cytokine receptor of the target cell, it leads to changes in gene expression, which later leads to new functions and sometimes to the proliferation of target cells. Cellular responses to cytokines are, however, tightly regulated and strict feedback mechanisms exist to turn down excess responses.
Table 1. Cytokines of innate immunity studied in this thesis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell sources</th>
<th>Cellular targets and biological effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>Mononuclear phagocytes, T cells, NK cells, Mast cells</td>
<td><strong>Principal mediator of acute inflammation.</strong> Immune activation, tumour necrosis, vascular effects. Stimulates endothelial cells, macrophages, monocytes and neutrophils. Also induces fever, synthesis of acute phase proteins, catabolism, and apoptosis.</td>
</tr>
<tr>
<td>IL-1</td>
<td>Mononuclear phagocytes, Neutrophils, Endothelial cells, Some epithelial cells</td>
<td><strong>Mediator of inflammatory response.</strong> Immune activation. Activation of endothelial cells. Also induces fever, synthesis of acute phase proteins, neutrophil and platelet production. Works together with TNF in innate immunity and inflammation.</td>
</tr>
<tr>
<td>IL-6*</td>
<td>Mononuclear phagocytes, Endothelial cells, Fibroblasts T cells</td>
<td><strong>Several diverse actions in both innate and adaptive immunity.</strong> Innate: Synthesis of acute phase proteins, production of neutrophils. Adaptive: Proliferation of antibody producing cells (B cells), promotes cell-mediated immune reactions by stimulating pro-inflammatory cytokines and inhibiting regulatory T cells.</td>
</tr>
<tr>
<td>IL-10</td>
<td>Macrophages, Activated monocytes, T cells (esp. Treg), some non-lymphoid cells</td>
<td><strong>Controls innate immunity and cell-mediated immunity (inhibitor of immune responses).</strong> Inhibition of IFN-α, IFN-γ, IL-1, IL-6, TNF and IL-12 production. Stops antigen presentation by inhibiting expression of MHC II molecules and other costimulators in DCs and macrophages. Negative feedback regulator for macrophages.</td>
</tr>
<tr>
<td>IL-12</td>
<td>Dendritic cells, Macrophages</td>
<td><strong>Mediator of the early innate immune response.</strong> Key inducer of cell-mediated immunity. Induces Th1 differentiation in T cells, and IFN-γ synthesis and cytotoxic activity in NK cells and CTLs.</td>
</tr>
</tbody>
</table>

**Chemokine**

| IL-8     | Leukocytes, Tissue cells | Neutrophil recruitment, mediates inflammatory responses |

*both in innate and adaptive immunity

Modified from Abbas et al. 2007, Parkin and Cohen 2001

Cytokines have several different roles in immunity, which may be divided e.g. based on their functional properties. In this kind of division, the first category is cytokines of innate immunity (Table 1, innate cytokines studied in this thesis), which stimulate early reactions to microbes. Cytokines of innate immunity are usually secreted by mononuclear phagocytes and mediate both systemic and local inflammation. The second category is cytokines of adaptive immunity (Table 2, adaptive cytokines studied in this thesis), which are produced in response to specific recognition of foreign antigens. Cytokines of adaptive immunity usually play an important role in the regulation of lymphocyte growth and differentiation. The third group is composed of cytokines associated with haematopoiesis, which stimulate the growth and differentiation of immature leukocytes. (Abbas et al. 2007, Parkin and Cohen 2001.) Several other cytokines, such as IL-33, and IL-22 have also been studied. For example, IL-33 has a role as damage-associated interleukin. It is released by cells such as epithelial cells in response to exogenous stimuli, including allergens. Its role in allergic diseases and in the initiation and progression of asthma has been recognized (Ding et al. 2018, Savinko et al. 2012).
Table 2. Cytokines of adaptive immunity studied in this thesis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell sources</th>
<th>Cellular targets and biological effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ*</td>
<td>T cells</td>
<td>Macrophage-activating cytokine.</td>
</tr>
<tr>
<td></td>
<td>(Th1 cells,</td>
<td>Immune activation and modulation.</td>
</tr>
<tr>
<td></td>
<td>CD8+ cells),</td>
<td>Induces activation of macrophages, Th1</td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td>differentiation (inhibits Th2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>differentiation), isotype switching in</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B cells, promotes increased expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of class I and II MHC molecules and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>costimulators in APCs, thus increasing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>antigen processing and presentation to</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T cells.</td>
</tr>
<tr>
<td>IL-2</td>
<td>CD4+ T cells</td>
<td>Plays a major role in regulatory T cell</td>
</tr>
<tr>
<td></td>
<td></td>
<td>responses.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induces proliferation, cytokine synthesis, Fas-mediated apoptosis, regulatory T cell development and survival in T cells. Important to regulatory T cells. Induces proliferation and activation in NK cells, and proliferation and antibody synthesis in B cells. Autocrine and paracrine functions.</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2 cells,</td>
<td>Stimulates the production of IgE</td>
</tr>
<tr>
<td></td>
<td>Mast cells</td>
<td>antibodies and development of Th2 cells.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induces isotype switching to IgE in B cells, Th2 differentiation and proliferation in T cells. Also inhibits IFN-γ-mediated activation in macrophages, and induces proliferation in mast cells.</td>
</tr>
<tr>
<td>IL-13</td>
<td>Th2 cells,</td>
<td>Key role in allergic diseases and in</td>
</tr>
<tr>
<td></td>
<td>CD8+ T cells,</td>
<td>defence against helminths.</td>
</tr>
<tr>
<td></td>
<td>NKT cells,</td>
<td>Promotes fibrosis by increasing collagen synthesis in fibroblasts and macrophages. Also stimulates isotype switching to IgE in B cells, expression of endothelial adhesion molecules and increased mucus production in epithelial cells.</td>
</tr>
<tr>
<td></td>
<td>Basophils,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mast cells</td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>T cells</td>
<td>Induces tissue damage, chemokine</td>
</tr>
<tr>
<td></td>
<td>(Th17 cells)</td>
<td>production in endothelial cells,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>chemokine and cytokine production in</td>
</tr>
<tr>
<td></td>
<td></td>
<td>macrophages, GM-CSF and G-CSF-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>production on epithelial cells.</td>
</tr>
</tbody>
</table>

*IFN family plays role in innate and adaptive immunity
Modified from Abbas et al. 2007

2.3 EXPOSURES THAT AFFECT ASTHMA PREVALENCE AND THE PROPOSED IMMUNE PATHWAYS

It has been proposed that the rise in the prevalence of asthma could be due to a combination of genetic and environmental factors. Since the evolutionary shift in humans is slow, and the rapid increase in the prevalence of asthma worldwide cannot be fully explained by genetic alterations, attention has turned to the environment and to exogenous exposures. Urbanization, together with industrialization and loss of biodiversity, has changed our lifestyles tremendously. The main exposures that an individual encounters have shifted from microbe-rich exposures to exposures with less diverse microbiota and more harmful agents.

Several exposures that can affect asthma prevalence and the onset of new asthma have already been identified (Table 3). These exposures can have either positive (e.g. asthma-protective effects of farming, breastfeeding, pet animals) or negative (e.g. asthma-predisposing effects of caesarean section, antibiotics, air pollution, smoking) effects on respiratory health.
Several studies have demonstrated that children seem to be more susceptible to the influences of the environment and different exposures. It has been speculated that childhood and even the prenatal period could represent critical exposure windows for asthma development as the target organs, such as the respiratory and immune systems are still developing and maturing (Vieira 2015, Deng et al. 2015, Wright and Brunst 2013, Lacasaña et al. 2005).

2.3.1 Farm environment

Children who have grown up in traditional farms have a significantly reduced risk of developing asthma and other atopic diseases compared to the general population (von Mutius and Vercelli 2010). This farm associated asthma-protection starts before birth as exposure to the farming environment during the prenatal period has been associated with a decreased risk of atopic diseases (House et al. 2017, Loss et al. 2012) and may last through a lifetime (Lampi et al. 2015).

Studies have already identified some components behind the asthma-protective effects of farming. Certain farm-related exposures, such as contact with livestock (mainly cows, pigs and poultry), contact with animal feed and animal shelters, and consumption of unprocessed cow’s milk have been recognized as protective factors (Ege et al. 2007, Riedler et al. 2001). In accordance with the hygiene hypothesis, exposure to farm dust and to microbial diversity have also been shown to contribute to the healthy immunoregulation behind farm-protection (Stein et al. 2016, Haahhtela et al. 2015, Lluis et al. 2014, Ege et al. 2011, von Hertzen et al. 2011, Alenius et al. 2009).

While some of the protective exposures and components have been identified, the underlying immune mechanisms that could be utilized for preventive interven-
tions, are thus far unspecified. It could be speculated that exposure to a diverse microbiome and other farm-associated exposures in childhood could shift immune responses towards healthy immunoregulation and stimulate tolerance, therefore protecting against asthma and allergy.

In recent studies, the importance of innate immunity on farm-mediated-protection has been acknowledged (Stein et al. 2016). Some immunological parameters that have been studied with relation to the mechanisms behind farm-induced protection have been collected in Table 4. Several studies have focused on the associations between different cytokines and farm exposures as well as on the associations between Tregs and farming.

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, resulting in reduced allergic responses (Schuijs et al. 2015). Previous studies have observed that in vitro LPS-stimulation decreased the percentage of mDC1s in farm children (Kääriö et al. 2016b) and that PBMCs from farm-children produced more Th1-associated cytokines, IL-12 and IFN-γ, and immunoregulatory cytokines, IL-10, compared to non-farm children (Kääriö et al. 2016a). DCs also operate closely with regulatory T cells and express several different TLRs, thus maybe playing a more special role in the protective effect of farm exposure on childhood asthma than previously thought.

Although many studies support the protective effect of farm exposure on children’s respiratory health (Wells et al. 2014), it should be noted that not all farms are good for respiratory health and that farming practices may differ. Several studies have shown, for example, that large industrial farms increase respiratory health problems in adult workers (Nordgren and Bailey 2016, Reynolds et al. 2013).
Table 4. Immune parameters linked to farm exposure.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Studied markers*</th>
<th>Result (in farm children)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Stable exposure in pregnancy | CD14  
TLR2  
TLR4 | Higher expression | Ege et al. 2006 |
| Farming/Farm-milk consumption | Regulatory T cells | Higher number | Liulis et al. 2014 |
| Farming | Regulatory T cells | Lower number | Schröder et al. 2017 |
| Farming | GATA-3  
IL-10  
TGF-β  
TNF  
IRAK-2  
RIPK1  
HLA-DRA  
SOCS-4 | Higher expression | Frei et al. 2014 |
| Farming Amish vs Hutterite farms | Neutrophils, Eosinophils, CXCR4  
CD11b  
CD11c  
HLA-DR  
ILT3  
IL-17  
IL-33  
IL-25  
IL-31  
IL-27  
IL-4  
BAFF  
memory B cells | Several differences between Amish and Hutterite farm children | Stein et al. 2016 |
| Farming | IL-10  
IL-12  
IFN-γ  
TNF | Higher expression | Kääriö et al. 2016a |
| Farming | mDCs | Lower expression | Kääriö et al. 2016b |

Abbreviations: CD: cluster of differentiation, TLR: toll-like receptor, IL: interleukin, TGF: Transforming growth factor, TNF: Tumour-necrosis factor, IRAK: Interleukin-1 receptor-associated kinase-like, RIPK: Receptor-interacting serine/threonine-protein kinase, HLA-DR: Human Leukocyte Antigen – antigen D Related, SOCS: suppressor of cytokine signalling proteins, CXCR; CXC chemokine receptor, BAFF; B-cell activating factor, IFN; interferon, mDC; myeloid dendritic cell

*Markers that showed significant results.

2.3.2 Caesarean section

In contrast to the protective effects of farming, delivery by caesarean section (CS) has been observed to predispose to atopic diseases. According to the data from 150 countries, almost 1 in 5 women in the world give birth by CS (Betrán et al. 2016). When a CS is performed due to complications in labour, it can be a life-saving procedure. However, when performed without medical reasons it raises several concerns, as CS has been shown to increase the prevalence of several health issues, such as intestinal disorders and allergic diseases (Gerlich et al. 2018, Sevelsted et al. 2015, Roduit et al. 2009, Pistiner et al. 2008, Xu et al. 2001). A recent meta-analysis has
shown that being born by elective or emergency CS caused a 20% increase in childhood asthma (Huang et al. 2015). Furthermore, the long-term effects of CS have not yet been uncovered.

The exposures that might influence homeostasis of the body and stir immuno-regulation in children born by CS are presented in Figure 3 and discussed in the text below. It should be kept in mind, however, that not all CS are similar: the progression of birth (e.g. release of hormones driving the birth process, cervical dilation, rupture of amniotic membrane) and early microbial exposure may vary in different CS. As emergency or urgent caesarean deliveries may occur after the onset of labour, factors affecting neonatal immune system may be similar to those operating during vaginal birth.

![Diagram](image)

**Figure 3.** Exposures that might play a role in asthma and allergy disposing qualities of caesarean delivery.

When a child is born vaginally, the infant is exposed to a significant number of microbes during the delivery. In a vaginal birth, the amniotic membrane tears and the cervix opens. This forms a direct access from the microbe-rich vagina to the uterine cavity. In the uterine cavity, the foetus swallows amniotic fluids and within it the mother’s vaginal bacterial flora. In CS cases, the microbial load/exposure received by the newborn varies considerably. When CS is performed after amniotic membrane ruptures, such as in emergency CS, the newborn is usually exposed to the vaginal microbiome before delivery. Instead, in pre-planned surgery (elective CS), the amniotic membranes are generally intact, and the passage to the cervix is often closed. In this case, the probability of colonization with vaginal bacteria is low.

The lack of normal vaginal microbial exposure during delivery usually results in delayed and altered colonization of the infant gut. Studies have shown that infants born by CS obtained bacterial communities similar to those found on the skin, whereas infants delivered vaginally obtained bacterial communities resembling the
mother’s vaginal microbiota (Dominguez-Bello et al. 2010). Studies have also reported that the number of bacteria in the stools of infants born by CS is lower than those born vaginally (Adlerberth et al. 2006, Gronlund et al. 1999), even after 7 years (Salminen et al. 2004). In one pilot study, researchers attempted to transfer the vaginal microbes to infants at birth and succeeded in partially restoring the microbiota in babies delivered by CS (Dominguez-Bello et al. 2016). The dysbiosis of the microbiome of the lung has been associated with the poorer host defence and immunity (O’Dwyer et al. 2016). Although studies have reported that the presence of certain anaerobic and potentially pathogenic microbes in the uterine cavity at birth greatly increased the later risk of asthma (Keski-Nisula et al. 2009) and that differences in the neonatal gut microbiota can affect the development of atopy (Kalliomäki et al. 2001), there is some controversy about the role of the initial colonization. A study by Chu et al. (2017) reported that within the first 6 weeks after birth, the infant microbiota undergoes substantial reorganization and that there were no major differences in microbiota between infants delivered vaginally or by CS.

The role of microbial colonization has already been taken into consideration when deciphering the factors affecting asthma-risk, but less is known about the roles of other obstetric factors and events during delivery. Administration of antibiotics and neonatal treatment with antibiotics have been shown to associate with wheezing in infants and young children (Zeissig et al. 2014, Marra et al. 2009, Alm et al. 2008, Rusconi et al. 2007). The effects of the natural progression of birth, such as intrapartum stress, foetal distress, and the immune responses initiated by labour, and the effects of other obstetric factors such as prenatal maternal stress, maternal and foetal conditions leading to CS, gestational age, and altered epigenetic regulation, are less studied. For example, early-term births were associated with an increased risk of asthma (Korhonen et al. 2018).

Even though the exposures that might influence immunoregulation have been partially identified, the underlying immunological mechanisms are not yet known. It has been speculated that the Th1/Th2 balance might play a role as infants born through CS had significantly lower levels of the Th1-associated chemokines CXCL10 and CXCL11 (Jakobsson et al. 2014) and inflammatory cytokines such as TNF, IFN-γ, sIL-4R, IL-1β, IL-6 in their blood (Malamitsi-Puchner et al. 2005). Furthermore, children born by CS had a higher number of IgA-, IgG- and IgM-secreting cells during the first year of life (Huurre et al. 2008). CS was also associated with lower levels of several immune cells (Bili et al. 2011, Nikischin et al. 1997, Thilaganathan et al. 1994), weakened transmigration ability of neutrophils and activity of leukocytes (Yektai-Karin et al. 2007), and lower levels of CD11b/CD18 and the chemokine receptor IL-8 RA (Gessler and Dahinden 2003). These studies suggest that vaginal delivery has a critical effect on the awakening of the immune system, as being born vaginally promotes the production of various cytokines and their receptors and elevates the number of immune cells. Studies concerning the long-term effects of CS and other obstetric factors on immune responses are still rare.
2.3.3 Air pollution

Similar to caesarean section, exposure to air pollution has been shown to be detrimental not only to respiratory health, but also to other morbidities, such as cardiovascular diseases and even Alzheimer’s disease (Kelly and Fussel 2015, Guarneri and Balnes 2014, Chen et al. 2013). Air pollution is defined as an occasion in which harmful or excessive quantities of substances, including gases, particulates, and biological molecules are introduced into the atmosphere. For example, nitrogen dioxide, particulate matter, black carbon, sulphur dioxide and polycyclic aromatic hydrocarbons have been shown to cause adverse effects in humans. According to the WHO, air pollution has been estimated to be the world’s largest single environmental health risk and in 2014, 92% of the world’s population were living in places where the WHO air quality guideline levels were not met (WHO, 2016).

The harmful effects of air pollution on respiratory health have been shown to already start during the prenatal period (Veras et al. 2017, Jedrychowski et al. 2010). In one meta-analysis, an association was found between prenatal exposures to NO₂, SO₂, and PM₁₀ and the risk of wheezing and asthma development in childhood (Hehua et al. 2017). Other studies have also shown that prenatal exposure to air pollution increases susceptibility to respiratory infections and may program respiratory morbidity in early childhood (Jedrychowski et al. 2013). Children are thought to be more susceptible to the effects of air pollution as adverse outcomes of air pollutants on lung function could be permanent (Goldizen et al. 2016). Studies have also reported that exposure to air pollution may result in reduced lung function in children (Gehring et al. 2013, Gauderman 2004) and that it is a risk factor for the onset of asthma in both adults (Künzli et al. 2009) and children (Yang et al. 2016, Gehring et al. 2015, Jerret et al. 2008).

One harmful component of air pollution is particulate matter (PM). Inhalable PM contains particles with an aerodynamic size under 10 µm. Typically they are mixtures of combustion derived particles, ash, soil or dust particles, acids and organic chemicals, metals and other elements. The largest inhalable particles may also contain biological and immunogenic components, such as endotoxins, fungal spores, and pollen. The composition and size distribution of PM usually varies according to the source. Particles can originate from natural and anthropogenic sources, and they can be directly emitted (primary emissions) by mobile or stationary sources, or be formed in the atmosphere (secondary emissions). Some anthropogenic sources are industry, vehicles, agriculture and combustion of wood and fossil fuels, whereas some natural sources include windblown dust, aerosolized sea salt, volcanoes and wildfires.

PM is usually categorized based on aerodynamic diameter, source and deposition in human airways. Depending on the study, particulate matter size-fractions can be divided into different fractions. The following four fractions are commonly used: PM₁₀-₂.₅, “coarse”, with a diameter between 2.5 and 10 µm, deposits mainly in the nose and upper respiratory airways (large conducting airways); PM₂.₅-₁, (“fine”) with
a diameter between 2.5 and 1 μm and PM$_{1-0.1}$ (“accumulation”) with a diameter between 1 and 0.1 μm, both size-fractions deposit throughout the respiratory tract, particularly in small airways and alveoli; and PM$_{0.1}$ (“ultrafine”), with a diameter <0.1 μm that deposits in the upper respiratory tract and alveoli, possibly entering the bloodstream (Guarnieri and Balmes 2014, Hanno et al. 2010, Sillanpää et al. 2003).

Exposure to PM has been linked with several harmful outcomes, such as cardiovascular diseases, metabolic disorders, and increased mortality (Kelly and Fussel 2015, Chen et al. 2013). Exposure to PM has also been connected with respiratory problems and alterations such as chronic obstructive pulmonary disease, exacerbations of pre-existing asthma, and even the development of new-onset asthma (Khreis et al. 2017, Guarnieri and Balmes 2014, Künzli et al. 2009, Jerrett et al. 2008). Some studies have also reported that experimental exposure to PM is associated with airway hyper-responsiveness and airway remodelling (Guarnieri and Balmes 2014). Most of the studies concerning the effects of PM have concentrated on the cardiovascular or toxicological effects of PM rather than the immunological effects. PM has been shown to induce inflammation and oxidative stress (Rönkkö et al. 2018, Jalava et al. 2015, Hanno et al. 2008).

![Figure 4](image)

**Figure 4.** Mechanisms behind the asthma predisposing qualities of air pollution.

Although several epidemiological studies have shown that exposure to air pollution increases the risk of immune diseases, and some possible mechanisms behind the asthma predisposing qualities of air pollution have been proposed (Figure 4, Gowers et al. 2012), the key mechanisms and the underlying causative components affecting the immune response are still unknown. Exposure to air pollution is thought to cause asthma by affecting the size and structure of the developing lung as well as the developing immune system. Studies investigating how PM or its specific constituents may disrupt human immunoregulatory mechanisms and thus predispose exposed individuals to several diseases are still scarce. Some associations between DC and PM have been found. PM directs DC maturation and leads them to stimulate a Th2-skewed T cell response (Williams et al. 2007). In another study, urban PM enhanced the maturation and stimulatory capacity of DCs, but inhibited the generation of Th1 cells, thus maybe impairing Th1 responses (Matthews et al. 2014). PM has also been shown to affect the expression of immune receptors, for example, exposure to PM...
constituents enhanced the expression of CD80/CD86. (Yoshida et al. 2010, de Haar et al. 2008). Immune responses to PM are usually studied by using commercial or reference materials and not authentic urban air samples, which could affect the overall results.
3 AIMS OF THE STUDY

The overall aim of this thesis was to investigate how different exposures during pregnancy, birth and childhood modulate asthma-related immune responses in children.

This dissertation focuses on three different exposures. One that has been shown to be asthma-protective (farming, Studies I, III) and two that have been suggested to predispose to asthma (birth by caesarean section, Study II and air pollution, Study III). Immune responses were studied at the age of four (Study III), six (Study I) and between 15-17 years (Study II).

The main aims were to investigate:

1. Whether numbers and phenotypes of circulating dendritic cells at age 6 are associated with farming, asthma or atopy in a selected sample of French and Finnish children from the PASTURE birth cohort study. (Study I)

2. Whether intrauterine microflora, neonatal treatments or other birth-related factors affect immune responses at teenage in children born by caesarean section. (Study II)

3. Whether cattle farm dust and urban air particulate matter (PM) have in vitro immunomodulatory effects on circulating immune cells extracted from 4-year-old children. (Study III)
4 MATERIALS AND METHODS

4.1 EXPERIMENTAL DESIGNS

The following experimental designs were utilized (Table 5):

I. In Study I, phenotypes and surface receptors of circulating DC subsets of farm and non-farm children were examined at the age of 6. Associations between DCs, specific farm exposures, and asthma and atopy were assessed to identify whether DCs mediated the protective effect of farm exposure.

II. In Study II, cytokine secretion of unstimulated and in vitro-stimulated PBMCs collected at teenage were examined. Associations between several obstetric factors and immune responses were assessed to identify whether obstetric factors and exposures during delivery alter immune responses later in life.

III. In Study III, PBMCs of 4-year-old children were in vitro-stimulated with farm dust and size-segregated PM. Phenotypes and surface receptors of blood immune cells (DCs and monocytes), and cytokine production of PBMCs were examined. The study explored the immunoregulatory effects of cattle farm dust and urban air PM for the first time using the same research methods.
Table 5. The main materials and methods of the studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Studied exposure</td>
<td>Farming</td>
<td>Obstetric factors</td>
<td>Farm dust / Urban air pollution</td>
</tr>
<tr>
<td>Study population</td>
<td>PASTURE</td>
<td>KEISARI</td>
<td>PASTURE</td>
</tr>
<tr>
<td>N</td>
<td>168</td>
<td>79</td>
<td>18</td>
</tr>
<tr>
<td>Age of participants (years)</td>
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<td>15-17</td>
<td>4</td>
</tr>
<tr>
<td>Sample material</td>
<td>PBMCs</td>
<td>PBMCs</td>
<td>PBMCs</td>
</tr>
<tr>
<td>In vitro /ex vivo</td>
<td>Ex vivo</td>
<td>In vitro</td>
<td>In vitro</td>
</tr>
<tr>
<td>In vitro stimulations</td>
<td>-</td>
<td>LPS, POLY(I:C), Ppg</td>
<td>Farm dust, Particulate matter</td>
</tr>
<tr>
<td>Cell subsets analysed with flow cytometry</td>
<td>mDC1s, mDC2s, pDCs</td>
<td>-</td>
<td>mDC1s, pDCs, Monocytes</td>
</tr>
<tr>
<td>Functional markers analysed with flow cytometry</td>
<td>ILT3, ILT4, CD86</td>
<td>-</td>
<td>ILT4, CD80</td>
</tr>
<tr>
<td>Cytokines analysed with MSD</td>
<td>-</td>
<td>IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFN-γ, TNF</td>
<td>IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12/IL-23p40, IL-13, IL-17A, IFN-γ, TNF</td>
</tr>
</tbody>
</table>

4.2 STUDY POPULATIONS

The studies were approved by the Research Ethics Committee, Hospital District of Northern Savo, Kuopio, Finland, and written informed consent was obtained from parents.

4.2.1 PASTURE (Studies I, III)

The study populations are subpopulations from the PASTURE/EFRAIM study (Protection against allergy: study in rural environments / Mechanisms of Early Protective Exposures on Allergy Development), which is a prospective birth cohort study conducted in five European countries (Austria, Finland, France, Germany and Switzerland). It was established to investigate protective and risk factors influencing the development of atopic diseases, and mainly focused on the effects of farming. In brief, two groups of pregnant women were formed, a farm group, including women from family-run livestock farms, and a reference group, including women from the same rural areas but not living on a farm. The design of this cohort is described in detail in von Mutius et al. (2006).

In Study I, PBMCs of Finnish and French farming and non-farming children were studied at the age of 6. The study population included 65 asthmatic (26 farming and 39 non-farming) and 103 non-asthmatic (56 farming and 47 non-farming) children. Detailed data on the health outcomes and exposure to the studied farm-related subjects were collected and available for the analysis.

In Study III, the study population included non-farming 4-year-old Finnish children (N=18). Two out of 18 children had asthma at age 4 and 13/18 were atopic. The studied children had not been exposed to the farm environment or high levels of air pollution in the past.

4.2.2 KEISARI (Study II)

The study population is a subpopulation (N=79) of the Finnish KEISARI-study (study on Caesarean delivery and infections), which aimed to determine the importance of intraterine microbial colonization on the development of allergic diseases in adolescents (14 to 16 years of age), who were born by caesarean section. The initial KEISARI study consisted of 805 mother-child pairs who all underwent caesarean delivery at the Kuopio University Hospital, Finland, 1990-1992. Questionnaires and health monitoring were conducted at the time of birth to collect information about the wellbeing of the new-borns and mothers.

When the children reached the age of 15-17 years, self-administered questionnaires were sent to the mothers (n=749). The children living in the area of the Hospital District of Northern Savo (n=688) were invited for a clinical examination, including skin prick tests and blood sampling (n=382). From a subsample of children (n=111)
another blood sample was collected for PBMC isolation and immunological tests. A sample of children with a sufficient number of PBMCs and full term birth (≥37 weeks) (n=79) were selected for study II.

4.3 ASTHMA AND ATOPY DEFINITIONS (STUDIES I AND III)

In Study I, asthma was defined as a combination of a doctor’s diagnosis of asthma ever up to the age of 6, and/or an unremitting wheeze during 18 months to 6 years and/or intermittent, persistent or late-onset wheeze during 18 months to 6 years. A doctor’s diagnosis of asthma was defined as a combination of a doctor’s diagnosis of asthma or repeated diagnosis of obstructive bronchitis ever up to age 6. (Details in Depner et al. 2014). In Study III, asthma was diagnosed as doctor-diagnosed asthma at the age of 4.

Atopic sensitization was measured as IgE levels in serum. Specific IgE levels in serum against 6 food (hen’s egg, cow’s milk, peanut, hazelnut, carrot, and wheat flour) and 13 inhalant (Dermatophagoides pteronyssius, Dermatophagoides farinae, cat, horse, dog, Alternaria species, mugwort, plantain, alder, birch, hazel, rye pollen, and grass pollen mix) allergens were assessed using the Allergy Screen test panel for atopy (Mediwiss Analytic, Moers, Germany) in a central laboratory. Positive sensitization against perennial, seasonal, or food allergens was defined by using a cut-off for a specific IgE of 0.70 IU/ml or greater (Study I) or 3.5 IU/ml or greater (Study III).

4.4 EXPOSURES

4.4.1 Farm exposures (Study I)

Study I focuses on prenatal (maternal exposure) and lifetime patterns of exposure (childhood exposure) to farming and farm-related factors. Extensive data on health and exposures to the studied farm-related subjects were collected and used for assessment. Participating parents completed questionnaires during pregnancy and at the ages of 2, 12, 18, 24 months of the child, and annually, up to the age of 6. At each follow-up, relevant exposures, health aspects and allergic and asthma endpoints were assessed with questionnaires, blood samples, or both.

In study I, the main exposures examined were farming, farm milk consumption and exposures to stable and barn.

Farming was defined as a family living on a farm with livestock when the child was born. Prenatal maternal consumption of farm milk was defined as a mean consumption of at least 10 ml of farm milk per day (yes vs no). Prenatal exposures to stable and barn were defined as an exposure of at least 15 minutes per week in one trimester (yes vs no).

In order to define a lifetime pattern (childhood exposure) for farm milk, stable and hay exposure, latent class analyses (LCA) with a 3 class solution based on all
available exposure data were performed. Exposures to farm milk and stable were defined as farm milk consumption (yes vs no) and staying in stables (yes vs no) during pregnancy, 1 year, 18 months, and annually until year 6. Exposure to hay was defined as a regular exposure to hay ever (yes vs no), during 1 year, 18 months, and annually until year 6 (a comparable variable was not available during pregnancy). In LCA, individuals were assigned to the class to which they had the highest probability of belonging: never, intermediate and persistent.

4.4.2 Obstetric factors and the intrauterine microbiome (Study II)

Study II focuses on several different obstetric factors and exposures occurring prenatally, during labour and after delivery. Data on prenatal exposures (e.g. urinary tract infections), exposures taking place during labour (e.g. cervical dilation and microbial exposure), and postnatal exposures (e.g. treatment in the neonatal intensive care unit) were assessed.

In Study II, seven different obstetric factors were examined. Induction of labour was defined as induction by artificial rupture of the amniotic sac or by prostaglandins or oxytocin (yes vs no). The status of amniotic membranes at the time of operation was categorized as intact or ruptured membranes. Cervical dilation was defined as cm of cervical dilation at the time of operation (0-1 cm, 2-5 cm, over 6 cm). The type of caesarean delivery was divided into 3 classes (elective, emergency and urgent). Neonatal antibiotic treatment was defined as care received (yes vs no). Neonatal intensive care unit (NICU) treatment was defined as care received (yes vs no). Neonates who required special therapy after birth were treated in separate NICU. NICU treatment may have included e.g. intravenous therapy (antibiotics, fluids or other medication), mechanical ventilation, phototherapy or other supportive treatment. A neonate’s individual need for NICU treatment was based on the assessment of a neonatologist or paediatrician after birth. Microbial exposure was categorized as microbes found from amniotic fluid or intrauterine swab (yes, no) and grouped by their potential pathogenicity (pathogenic, a-pathogenic, see grouping below).

Assessment of microbes from amniotic fluid or intrauterine swab samples has been described previously (Keski-Nisula et al, 2009; 1997a, 1997b). The results of microbial uterine or amniotic fluid culture were defined as negative, a-pathogenic or pathogenic positive cultures. A positive microbial culture was defined as the presence of any organism. Detected microorganisms were also analysed on the basis of their species and grouped according to their potential pathogenicity. Beta-hemolytic Streptococcus group B, Staphylococcus aureus, Peptostreptococcus species (spp.), Bacteroides spp., Ureaplasma urealyticum, Gardnerella vaginalis, Escherichia coli, Klebsiella spp., and Enterococcus spp. were grouped as potential pathogens, whereas Lactobacillus spp., Propionibacterium spp., coagulase negative Staphylococcus, Streptococcus salivarius and Propionibacterium acnes were grouped as a-pathogens.
4.4.3 Cattle farm dust and urban air particulate matter (Study III)

Study III focuses on the different immunomodulatory effects of two distinct environmental exposures, a proposed protective environment (cattle farm dust particles from Finland) and high-risk environment (urban air PM collected from Nanjing, China).

Airborne farm dust samples from cattle farms were collected, extracted and pooled using three electrostatic dust collectors and a NaCl-cold extraction method. Dried and processed farm dust samples were stored at RT, protected from light and moisture. For cell culture experiments, farm dust sample was resuspended in endotoxin free PBS (Sigma-Aldrich) and vortexed for 5 min before stimulation.

Urban air particulate matter samples were collected at the Nanjing University Xianlin campus in October 2013 with a modified Harvard high volume cascade impactor in four stages. PM_{10-2.5}, PM_{2.5-1} and PM_{1-0.2} size ranges were collected on polyurethane foam impaction substrates, whereas PM_{0.2} samples were collected with a PTFE filter (Fluoropore 3.0 μm FSLW). PM were extracted from the filters with methanol, pooled and dried as described earlier in Jalava et al. (2015). The dried PM samples were stored at -20 °C. For cell culture experiments, PM samples were resuspended in 10% DMSO in W1503-water (Sigma-Aldrich) and sonicated for 30 min before stimulation.

The concentrations of environmental samples were chosen on the basis of dose-response experiments. Performed dose-response experiments were for PM (25, 75 and 150 ug/ml) and for farm dust (10, 20, 40, 80 ug/ml). Preliminary doses were selected on the basis of previous studies performed in our and in collaborating groups. Generally, responses were either dose-dependent or all doses induced a similar response. Expression of CD80 and ILT-4 were used as indicators of a suitable dose. We also confirmed that the selected doses did not affect cell viability. 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 the 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 (0.01mg/ml, Sigma-Aldrich) (results not shown).

Both environmental samples were analysed for inorganic ions and elements, and polycyclic aromatic hydrocarbons. Farm dust was also analysed for microbiome (analyses and results are described in publication III).

4.5 PBMC ISOLATION AND CRYOPRESERVATION

PBMCs were collected from the peripheral vein into EDTA tubes (Studies I, III) or lithium heparin tubes (Study II). Isolation was done using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation.
Cells were cryopreserved for later use by resuspending with 10% FBS Gold in RPMI 1640 (Studies I, III), or 2% HI-FCS in RPMI 1640 (Study II) and mixed 1:1 with 15% DMSO in FBS Gold/ HI-FCS (respectively). Cryotubes were stored at -80°C overnight for gradual temperature reduction and transferred into a liquid nitrogen cryopreservation tank.

4.6 PBMC STIMULATION

For stimulations and functional studies, PBMCs were thawed with a washing medium (1% L-glutamine + 1% Antibiotic/Antimycotic in RPMI 1640) and resuspended at 1x10⁶ viable cells/ml in RPMI 1640 (with 10% human AB serum, 1% L-glutamine and 1% Antibiotic/Antimycotic). The number and viability of the cells was determined using trypan blue exclusion. Mean cell viabilities in studies were: I (Finnish samples 93.9%, French samples 82.9%), II (91.2% (SD±5.3)), and III (88% (SD ±3.5)).

PBMCs were cultured in round bottom 96-well plates (Study II) or Ultra-Low attachment surface-plates (Study III), stimulated with different antigens or environmental samples (Table 6) and incubated for 5 hours (Study II) or 18 hours (Study III) at 37° C in 5% CO₂. After stimulations, cells were collected and processed for flow cytometric analyses (Study III) and cell-free supernatants were collected and stored at -70°C until batch-analysis of cytokines.

Table 6. In vitro stimulants and their concentrations used in studies

<table>
<thead>
<tr>
<th>Study II</th>
<th>Study III</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Control</td>
<td>-Control</td>
</tr>
<tr>
<td>-LPS (0.1 µg/ml)</td>
<td>-Farm dust (40 µg/ml)</td>
</tr>
<tr>
<td>-Ppg (10 µg/ml)</td>
<td>-PM₀.₂ (75 µg/ml)</td>
</tr>
<tr>
<td>-Poly(I:C) (50 µg/ml)</td>
<td>-PM₁₀₂ (75 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>-PM₂₅₁ (75 µg/ml)</td>
</tr>
<tr>
<td></td>
<td>Additional stimulations:</td>
</tr>
<tr>
<td></td>
<td>-Blank filter sample</td>
</tr>
<tr>
<td></td>
<td>-LPS + Polymyxin B</td>
</tr>
</tbody>
</table>

LPS; lipopolysaccharide, Ppg; peptidoglycan, POLY(I:C); polyinosinic:polycytidylic acid, PM; Particulate matter.

4.7 IMMUNOPHENOTYPING (FLOW CYTOMETRY)

For immunophenotyping, cells were stained with fluorochrome-labelled antibodies. Antibodies used in immunophenotyping are summarized in Table 7. Antibodies were titrated to determine optimal concentrations and OneComp eBeads (eBiosciences) were used for single-color compensation controls. Instrument calibration was evaluated using BD cytometer setup & tracking beads for CST (BD Biosciences). Immunophenotyping was performed at UEF by FACSCantoII cytometer (BD Biosciences, San Diego, CA, USA) and FACSDiva software v. 6.1.3 or v. 8.0.1.
There is some variation in antibodies and staining protocols between studies, therefore, detailed information on immunophenotyping should be revised from publications. In brief, samples were analysed using FlowJo X 10.0.7r2 software (Treestar, Ashland, OR, USA) on Windows 7 or 10 workstations.

**Table 7. The antibodies used in immunophenotyping**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Isotype</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>PE-Cy5.5</td>
<td>TüK4</td>
<td>Mouse IgG2a</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD19</td>
<td>PE-Cy5.5</td>
<td>SJ25-C1</td>
<td>Mouse IgG1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD11c</td>
<td>PE-Cy7</td>
<td>3.9</td>
<td>Mouse IgG1 κ</td>
<td>eBioscience</td>
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<tr>
<td>CD1c (BDCA-1)</td>
<td>APC</td>
<td>AD5-8E7</td>
<td>Mouse IgG2a</td>
<td>Miltenyi Biotec</td>
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<tr>
<td>CD303 (BDCA-2)</td>
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<td>Mouse IgG1</td>
<td>Miltenyi Biotec</td>
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<td>APC</td>
<td>AD5-14H12</td>
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<td>ILT3-biotin</td>
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<td>2331 (FUN-1)</td>
<td>Mouse IgG1 κ</td>
<td>Biolegend</td>
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<td>2331 (FUN-1)</td>
<td>Mouse IgG1, κ</td>
<td>BD Biosciences</td>
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<td>27D6</td>
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<td>L307.4</td>
<td>Mouse IgG1</td>
<td>BD Biosciences</td>
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<tr>
<td>Fixable viability Dye</td>
<td>eFluor 506</td>
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<tr>
<td>Streptavidin</td>
<td>APC-eFluor780</td>
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</tbody>
</table>

An example of gating from Study I is in Figure 5. Erythrocytes and debris were gated out according to the size (forward scatter) and cytoplasmic granularity (sideward scatter). Doublets were excluded according to forward scatter area (FSC-A) and forward scatter height (FSC-H). 7-AAD viability staining solution (Study I) or Fixable Viability Dye (Study III) were used to exclude dead cells from the analysis. Live monocytes were identified according to high CD14 expression and FSC-SSC characteristics. B cells were identified by high CD19 expression and FSC-SSC characteristics. The main peripheral blood DC subsets were separated from monocytes (CD14+) and B cells (CD19-) and identified as live BDCA2+ pDCs, BDCA1+CD11c+ mDC1s and BDCA3+high mDC2s. mDC2s were only analysed in Study I. The expression of functional markers ILT3, ILT4, CD80 and CD86 on DCs and monocytes was also analysed (analysis of functional markers varied in different studies). Gating adjustments were based on fluorescence minus one (FMO) controls. Farm dust particles and PM samples in Study III were analysed by flow cytometry to exclude possible interference with analyses.
Figure 5. Example of gating from Study I.
4.8 CYTOKINE ANALYSIS

Cytokines were analysed using a Meso Scale Discovery (MSD) Sector Imager™ 2400A with Discovery Workbench® 3.0.18 software. Samples were analysed with MSD kits (MSD, Rockville, MD, USA) according to the manufacturer’s instructions, using reagents provided with the kit. Study II samples were analysed with MSD Pro-inflammatory Panel 1 (human) V-PLEX kit (for CXCL8, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13 and TNF). Study III samples were analysed with MSD Biomarker Group 1, 10-plex custom U-PLEX kit (for IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12/IL-23p40, IL-13, IL-17A, TNF). Detection limits for each cytokine were defined separately. Samples with concentrations below the detection limit were given a value corresponding to the detection limit of the respective cytokine assay.

4.9 STATISTICAL ANALYSES

Statistical analyses were performed using SPSS Statistics 21 or 23-software (IBM Corporation, USA). Values of p<0.05 were considered statistically significant. Data from immunophenotyping (DC and monocyte variables) were expressed as percentages or ratios of cells, percentages of cells positive for specific markers and expression levels (median fluorescence intensity) of specific markers. Data from cytokine measurements were expressed as concentrations of cytokines (pg/ml). In Study III, 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. Different statistical analyses used in the studies are summarized in Table 8.

Data in some studies were not normally distributed, therefore, some data transformations were used. In Study I, percentages of mDC2s and mDC2/mDC1 ratios were log-transformed to fulfil assumptions of parametric testing. CD86+ mDC1(%) and ILT3+ mDC1(%) and ILT3+ pDC(%) could not be transformed to fit a normal distribution. For logistic regression, DC variables were rescaled for interquartile range. In Study II, distributions of continuous cytokine variables (IL-1β, IL-2, IL-6, IL-10, IL-13 and TNF) deviated from normal distributions and were log-transformed to fulfil assumptions of parametric testing. IL-4, IFN-γ and unstimulated CXCL8 were dichotomized.

Several confounding factors were tested (Studies I and II), but only confounders that influenced results (changes in odds ratio over 10 %) were included in the analyses. In Study I, confounders, such as country, mothers and fathers allergic diseases, mothers and fathers education, gender, number of older siblings, mothers smoking, breastfeeding, child’s birth weight and maternal age at birth were tested. In Study II, confounders, such as BMI at teenage, mother’s parity i.e. number of children, maternal and/or paternal allergic diseases, maternal and/or paternal education, maternal smoking during pregnancy, number of younger and older siblings, duration of breastfeeding and child’s birth weight were tested.
### Table 8. Statistical analyses used in studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Analysis</th>
<th>Statistical test</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Differences in basic characteristics and prevalence of exposures and outcomes</td>
<td>Chi-Square-test</td>
</tr>
<tr>
<td></td>
<td>Associations between asthma or atopic sensitization and farm exposures</td>
<td>Logistic regression, adjusted for maternal and paternal allergic diseases, education, maternal smoking, and country aOR and 95% CIs</td>
</tr>
<tr>
<td></td>
<td>Associations between normally distributed DC variables and farm exposures</td>
<td>General linear model, adjusted for country Means and SE</td>
</tr>
<tr>
<td></td>
<td>Associations between skewed DC variables and farm exposures</td>
<td>Non-parametric Mann-Whitney U test Medians and IQRs</td>
</tr>
<tr>
<td></td>
<td>Associations between asthma and DCs</td>
<td>Logistic regression OR and 95% CIs</td>
</tr>
<tr>
<td></td>
<td>Associations between atopic sensitization and DCs</td>
<td>Logistic regression, adjusted for country aOR and 95% CIs</td>
</tr>
<tr>
<td>II</td>
<td>Associations between continuous cytokine variables and obstetric factors</td>
<td>Linear regression, adjusted for obstetric factors GMR and 95% CI</td>
</tr>
<tr>
<td></td>
<td>Associations between dichotomized cytokines and obstetric factors</td>
<td>Logistic regression OR and 95% CI</td>
</tr>
<tr>
<td></td>
<td>Correlations between cytokines</td>
<td>Spearman’s two-tailed rank correlation</td>
</tr>
<tr>
<td>III</td>
<td>Effects of different stimulations on cell variables and on cytokines</td>
<td>Non-parametric Wilcoxon Signed rank test, significances corrected with Bonferroni correction Boxplots with 5-95% whiskers</td>
</tr>
<tr>
<td></td>
<td>Correlations between cytokines</td>
<td>Spearman’s two-tailed rank correlation</td>
</tr>
</tbody>
</table>

OR: odds ratio, aOR: adjusted odds ratio, CI: confidence interval, DC: dendritic cell, SE: standard error, IQR: interquartile range, GMR: geometric mean ratio.
5 RESULTS

5.1 FARMING (STUDY I)

5.1.1 Farm exposures decreased the risk of asthma

Farming exposures had a protective effect on asthma in this subpopulation of the PASTURE study. Persistent lifetime contact with farm-related exposures was associated with decreased risk of asthma, whereas exposures during pregnancy did not provide significant protection (Table 9). Persistent exposure to a stable provided significant protection against asthma also independently from farming (aOR 0.23, 95% CI 0.06–0.9, after adjustment for farming).

Table 9. Associations between farm exposures and asthma at age 6

<table>
<thead>
<tr>
<th>Exposures</th>
<th>Yes (%)</th>
<th>aOR (95% C.I.)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Farmer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh farm milk</td>
<td>53 (32)</td>
<td>0.67 (0.31–1.46)</td>
<td>0.32</td>
</tr>
<tr>
<td>Stay in stable</td>
<td>81 (50)</td>
<td>0.65 (0.31–1.34)</td>
<td>0.24</td>
</tr>
<tr>
<td>Stay in barn</td>
<td>59 (36)</td>
<td>0.64 (0.31–1.34)</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Lifetime pattern</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>88 (52)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>28 (17)</td>
<td>0.7 (0.27–1.83)</td>
<td>0.46</td>
</tr>
<tr>
<td>Persistent</td>
<td>52 (31)</td>
<td>0.4 (0.17–0.92)</td>
<td>0.03</td>
</tr>
<tr>
<td>Stay in stable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>73 (43)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>31 (18)</td>
<td>0.69 (0.27–1.79)</td>
<td>0.45</td>
</tr>
<tr>
<td>Persistent</td>
<td>64 (38)</td>
<td>0.29 (0.13–0.67)</td>
<td>0.004*</td>
</tr>
<tr>
<td>Regular contact with hay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>102 (61)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>49 (29)</td>
<td>0.71 (0.32–1.61)</td>
<td>0.42</td>
</tr>
<tr>
<td>Persistent</td>
<td>17 (10)</td>
<td>0.09 (0.01–0.76)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Total N=168, of which the number of asthmatics was N=65.
All analyses were adjusted for maternal and paternal allergic diseases, maternal and paternal education, maternal smoking, and country.
P value of logistic regression analysis: significant results (p < 0.05) are shown in boldface.
• P value p<0.05 after additional adjustment for farming.
☆ P value for trend test.

5.1.2 Farm environment associated with lower level of mDC2s

Farming and specific farm related exposures were associated with percentages and phenotypes of DCs (Table 10). Most significant associations were seen for mDC2s, as farming, prenatal farm milk consumption, intermediate lifetime exposure
to farm milk and persistent lifetime exposure to stables were associated with a lower percentage of circulating mDC2 cells (Figure 6). After adjustment for asthma, associations between mDC2s and farm exposures remained significant.

Table 10. Associations between different farming related exposures and dendritic cells.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>mDC1</th>
<th>mDC2</th>
<th>pDC</th>
<th>CD86+</th>
<th>ILT4+</th>
<th>ILT3+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mDC1s</td>
<td>pDCs</td>
<td>mDC1s</td>
<td>pDCs</td>
<td>mDC1s</td>
<td>pDCs</td>
</tr>
<tr>
<td>Fresh farm milk</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Stay in stable</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Stay in barn</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Life-time</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Fresh farm milk</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Stay in stable</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Stay in barn</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

P-value <0.05
P-value <0.2
*intermediate exposure, marked if statistically significant.

In addition, few other associations between farm exposures and immune cells were observed. Children living on a farm (p=0.04) and children with intermediate lifetime contact with hay (p=0.03) had a lower percentage of tolerogenic ILT3+ mDC1s, whereas children exposed prenatally to stables had a significantly lower percentage of ILT4+ pDCs (p=0.03). Some other trend-like associations were also seen (Table 10).

5.1.3 Dendritic cells associated differently with asthma in farm and non-farm children

Asthma was positively associated with ILT4+ mDCs (OR 1.77, CI 95% 1.01–3.12, p=0.048) and negatively associated with CD86+ pDCs (OR 0.49, CI 95% 0.26–0.95, p=0.04), but only in non-farm children. Similar associations were seen in doctor’s diagnosis of asthma. Furthermore, the percentage of mDC2s was associated with the doctor’s diagnosis of asthma in farm children (OR 3.97, CI 95% 1.39–11.29, p=0.01).
Figure 6. The associations of myeloid dendritic cell type 2 (mDC2) percentage and farm-related exposures. Figures show boxplots with 5-95% whiskers, horizontal line indicates the median, + indicates mean. Significances were calculated using general linear model and adjusted with country. *= P-value<0.05, **= P-value<0.01.

5.2 OBSTETRIC FACTORS (STUDY II)

5.2.1 Advanced cervical dilation elevated the cytokine secretion

Advanced cervical dilation (active phase, cervical dilation of 6 cm or more) during CS was associated with increased cytokine production at teenage (Figure 7, Table 11).
Figure 7. The effects of neonatal intensive care unit treatment (NICU) and advanced cervical dilation (>6 cm) at the time of caesarean delivery on spontaneous cytokine secretion at teenage. Figures show geometric mean ratios (GMR) with 95% confidence interval (CI), calculated using linear regression.

Table 11. Associations between obstetric factors and cytokine production at teenage.

<table>
<thead>
<tr>
<th>Obstetric factor</th>
<th>Stimulation</th>
<th>Innate immunity</th>
<th>Adaptive immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNF</td>
<td>IL-1β</td>
</tr>
<tr>
<td>Advanced cervical dilation</td>
<td>Control</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>TLR2</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>TLR3</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Neonatal intensive care treatment</td>
<td>Control</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>TLR2</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>TLR3</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

P-value <0.05
TLR= Toll-Like-Receptor; In vitro stimulation with PPG, Poly I:C and LPS.

5.2.2 Neonatal intensive care unit treatment lowered cytokine secretion

Contrary to cervical dilation, neonatal intensive care unit (NICU) treatment was associated with lower cytokine response (Table 11, Figure 7).
After adjustment with other obstetric factors, advanced cervical dilation was associated with higher unstimulated production of inflammatory and TH2-related cytokines, and CXCL8 (data not shown) and NICU treatment was associated with lower production of regulatory, inflammatory, and TH2-related cytokines.

5.2.3 Few associations were seen with other obstetric factors

Few other obstetric factors were associated with cytokine secretion at teenage. Similar to cervical dilation, the rupture of amniotic membranes before operation were also associated with higher production of cytokines CXCL8, IL-1β, TNF, IFN-γ, albeit not always significantly, and with lower secretion of IL-4. Induction of labour was associated with the lower production of IL-1β, and IL-6. Neonatal antibiotic treatment did not associate with cytokine production at teenage.

Intrauterine microbial growth did not associate significantly with spontaneous or TLR4-stimulated cytokine secretion. In TLR2 stimulation, elevated IL-13 was associated with the presence of pathogenic microbes in intrauterine cultures at birth.

5.3 FARM DUST AND URBAN AIR PARTICULATE MATTER (STUDY III)

Study III explored the effects of cattle farm dust and size-segregated PM on PBMCs, and more specifically, on DCs and monocytes. The studied environmental samples induced partly opposing immunomodulatory effects (Table 12).

Table 12. Associations between different environmental exposures and immune cells.

<table>
<thead>
<tr>
<th>Study III</th>
<th>Monocytes</th>
<th>mDC1</th>
<th>pDC</th>
<th>CD80+ Monocytes</th>
<th>pDCs</th>
<th>mDC1s</th>
<th>ILT4+ Monocytes</th>
<th>pDCs</th>
<th>mDC1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm dust</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>PM&lt;sub&gt;0.2&lt;/sub&gt;</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>PM&lt;sub&gt;1.0&lt;/sub&gt;</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>PM&lt;sub&gt;2.5&lt;/sub&gt;</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

↓↑ P-value <0.05 after Bonferroni correction.

5.3.1 Farm dust increased the percentage of immunostimulatory cells

Farm dust exposure significantly increased the percentages of monocytes and mDC1s expressing immunostimulatory CD80 (Figure 8). On the contrary, stimulation with farm dust particles significantly reduced the percentages of monocytes expressing immunoregulatory ILT4, whereas the percentages of DCs expressing ILT4 remained at the control level.
Figure 8. The effect of farm dust particle stimulations (18 h) on the percentages of cells positive for CD80 and ILT4 (N=18). Figures show boxplots with 5-95% whiskers, horizontal lines indicate the medians, + indicates means. Significances were calculated with the non-parametric Wilcoxon Signed rank tests, and corrected with the Bonferroni correction **= P-value significant after Bonferroni. White boxplots indicate controls, grey indicates farm dust stimulation.

5.3.2 Particulate matter decreased the levels of immunostimulatory and -regulatory cells

Urban air PM stimulation induced a statistically significant decrease in the percentage of cells expressing immunostimulatory CD80 (except for PM2.5-1 stimulated monocytes) and immunoregulatory receptor ILT4 (Figure 9). All PM size-fractions decreased the percentages of monocytes and mDC1s expressing ILT4 when compared to controls. In pDCs, only the decrease induced by PM0.2 was statistically significant after adjustment for multiple comparisons.
5.3.3 Farm dust and particulate matter had opposing effects on cytokine secretion

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 (Examples in Figure 10). Urban PM$_{1.0}$ had the most pronounced negative effect on the production of cytokines. PM$_{0.2}$ decreased the levels of IL-1β, IL-10, and IL-12 and IFN-γ. PM$_{2.5}$ had an opposing effect on the production of IL-13, IL-
17 and TNF, but only IL-17 remained significant after adjustment for multiple comparisons.

**Figure 10.** Examples of the effects 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, horizontal line indicate the medians, + indicates means. Significances were calculated with the non-parametric Wilcoxon Signed rank test, and corrected with the Bonferroni correction **= P-value significant after Bonferroni.

### 5.3.4 PM size-fraction determined the strength of the effect

Although PM samples induced parallel immune reactions, the PM size-fraction determined the strength of the effects. Both PM_{0.2} and PM_{0.2} induced significantly different expression of immune receptors and production of cytokines compared to
PM\(_{2.5-1}\), while effects induced by PM\(_{0.2}\) did not differ from those induced by PM\(_{1-0.2}\) (Table 13).

**Table 13.** Cross-associations between immune cell responses induced by different PM size-fractions.

<table>
<thead>
<tr>
<th>Cross-associations</th>
<th>PM(<em>{0.2}) vs PM(</em>{1-0.2}) p-value</th>
<th>PM(<em>{0.2}) vs PM(</em>{2.5-1}) p-value</th>
<th>PM(<em>{1-0.2}) vs PM(</em>{2.5-1}) p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell subsets</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD80+ monocytes (%)</td>
<td>0.756</td>
<td>0.002†</td>
<td>0.001†</td>
</tr>
<tr>
<td>CD80+ pDCs (%)</td>
<td>0.346</td>
<td>0.224</td>
<td>0.087</td>
</tr>
<tr>
<td>CD80+ mDCs (%)</td>
<td>0.433</td>
<td>0.015</td>
<td>0.009</td>
</tr>
<tr>
<td>ILT4+ monocytes (%)</td>
<td>0.005</td>
<td>0.041</td>
<td>0.001†</td>
</tr>
<tr>
<td>ILT4+ pDCs (%)</td>
<td>0.53</td>
<td>0.48</td>
<td>0.033</td>
</tr>
<tr>
<td>ILT4+ mDCs (%)</td>
<td>0.814</td>
<td>0.433</td>
<td>0.221</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.069</td>
<td>0.001†</td>
<td>0.000†</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.019</td>
<td>0.005</td>
<td>0.001†</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.003†</td>
<td>0.003†</td>
<td>0.001†</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.004</td>
<td>0.001†</td>
<td>0.000†</td>
</tr>
<tr>
<td>IL-12/IL-23p40</td>
<td>0.001†</td>
<td>0.001†</td>
<td>0.000†</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.009</td>
<td>0.002†</td>
<td>0.001†</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.022</td>
<td>0.005</td>
<td>0.000†</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.016</td>
<td>0.008</td>
<td>0.000†</td>
</tr>
<tr>
<td>TNF</td>
<td>0.001†</td>
<td>0.006</td>
<td>0.000†</td>
</tr>
</tbody>
</table>

Significances were calculated using the Wilcoxon Signed rank test. PM stimulations were compared to each other.
† p-value < 0.05, after adjustment for multiple comparisons with the Bonferroni correction.

### 5.3.5 Composition of samples

There were some variations in the ionic and elemental compositions of the environmental samples (Data in study III supplement). Secondary inorganic ions NO\(_3^–\) and SO\(_4^{2–}\) dominated the composition of the PM samples. Al, Ca, Fe, K and Zn were the most abundant metals in the PM samples. The elemental composition of farm dust sample differed considerably from PM samples. PAH contents of PM size-fractions were quite similar, and the highest PAH and genotoxic PAH concentrations were seen in the PM\(_{0.2}\) (Data in Study III supplement). 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. At the genus level, the dust was dominated by Erwinia (30% of sequences) and a not further defined Bradyrhizobiaceae genus (25%), followed by a Clostridiaceae gen., Pseudomonas and Oxalabacteraceae gen. (Data in study III supplement).
6 DISCUSSION

This thesis concentrates on three different exposures (farming, air pollution and caesarean section), which have been shown to affect the prevalence of asthma and other atopic diseases although several other exposures have also been recognized as risk factors. As immunological development has been observed to already take place during pregnancy and early childhood, exposures during these stages of life might be the key to ascertaining the risk factors related to atopic diseases.

6.1 FARMING AND FARM DUST (STUDIES I AND III)

There is strong evidence that farm exposure in childhood, and even prenatally, protects from asthma and allergies. The immune mechanisms behind this farm protection and the role of DCs is, however, still unclear.

Some studies have already lightly assessed the relationships between farm exposures and DCs. In the study of Kääriö et al. (2016b), *in vitro* LPS-stimulated PBMCs from children living on a farm had less mDC1s than control children. In another study, *in vitro* stimulation with farm-originated dust extracts suppressed the differentiation of human monocytes to DCs and affected the communication of epithelial cells and DCs (Schuijs et al. 2015).

Studies I and III assessed whether numbers and phenotypes of DCs were associated with farming, asthma and atopy *ex vivo* (Study I) and whether cattle farm dust had immunoregulatory effects on circulating immune cells *in vitro* (Study III). In summary, several associations between farm exposures and immune responses were observed (Figure 11).

<table>
<thead>
<tr>
<th>Farm <em>ex vivo</em></th>
<th>Farm dust <em>in vitro</em></th>
<th>Asthma</th>
</tr>
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<tbody>
<tr>
<td>mDC2</td>
<td>CD80+ mDC1s</td>
<td>ILT4+mDC1s (non-farm)</td>
</tr>
<tr>
<td>ILT4+ pDC</td>
<td>CD80+ Monocytes</td>
<td>mDC2s (farm)</td>
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<tr>
<td>ILT3+ mDC1s</td>
<td>ILT4+ Monocytes</td>
<td>CD86+pDC (non-farm)</td>
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<td>Asthma</td>
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**Figure 11.** Summary of associations between farm exposure (*ex vivo* and *in vitro*) and asthma on immune markers.

6.1.1 Persistent exposure offers better protection from asthma

Different farm exposures were associated with a lower prevalence of asthma. The identified protective factors, such as exposure to farm milk, stables, and hay are
similar to those reported previously (Ege et al. 2007). In this subsample of the PASTURE cohort, persistent exposure to a farm environment played a more important role in the protection against asthma than prenatal exposures. This phenomenon is supported by other studies, as persistent exposure may be needed to maintain optimal protection (Douwes et al. 2008). On the other hand, several studies have highlighted the importance of prenatal and early life exposure (Ege et al. 2006), possibly linking asthma-protection to an immune maturation window. Interestingly, persistent exposure to specific farm exposures did not show protection against atopic sensitization (measured as IgE levels). Similar effects have also been reported in other subprojects of the PASTURE study (Lluis et al. 2014).

6.1.2 Farm exposure lowered the percentage of mDC2 ex vivo

Farm children had smaller percentages of circulating mDC2s than control children. Lower percentages of mDC2s were associated with prenatal and lifetime exposures to farm milk, and with persistent lifetime exposure to stables. In previous studies, consumption of farm milk has been linked to increased numbers of Treg cells (Lluis et al. 2014).

Factors found in unprocessed cow milk, such as proteins, oligosaccharides, and vitamins may contribute to protection against atopic diseases (Perdijk et al. 2018, van Neerven et al. 2012). One way of mediating this farm milk induced protection could be related to cytokines such as TGFβ and IL-10. Bovine IL-10 has been shown to induce a dose-dependent reduction of CD80/CD86 expression and IL-12 and TNF production in human immune cells (den Hartog et al. 2011). In addition, TGFβ and IL-10 inhibit type I interferon production by pDCs (Contractor et al. 2007).

The association between exposure to stables and lower levels of mDC2s is interesting, as stable exposure was also inversely associated with asthma and as high percentages of mDC2s were associated with doctor’s diagnosis of asthma, but only in farm children. The protective effect of a low number of mDC2s was not significant with the broader definition of asthma applied in this study. The difference in results may derive from heterogeneous asthma definitions, as doctor-diagnosed asthma may be immunologically different from asthma-like disorders included in a broader definition. Previous studies have associated mDC2s with atopic diseases (Yerkovich et al. 2009). Asthmatic and atopic individuals have more mDC2s in their peripheral blood (Hayashi et al. 2013, Spears et al. 2011, Freeman et al. 2009). It also seems that the level of mDC2s in lungs is higher in asthmatics than in healthy subjects (Kayserova et al. 2012, McCarthy et al. 2007). Contradictory results have also been published, as one study reported that mDC2 levels were lower in atopic and asthmatic subjects than in healthy ones. The authors suggested that this might be due to the migration of these cells from circulation into the airways (Dua et al. 2013). Furthermore, allergen challenge has been shown to increase mDC2s in the sputum of asthmatic individuals (Dua et al. 2014).
Lower numbers of mDC2s, association between doctor-diagnosed asthma and mDC2s and previous reports on associations between mDC2s and allergic diseases suggest that this subpopulation may indeed have a role in farm-related asthma protection. While the number of mDC2s in circulation is quite small (0.1-0.13% of PBMCs, Ban et al. 2008, Narbutt et al. 2004), in the lungs and in the bronchoalveolar lavage fluid they represent the dominant DC subset (3.2% of all lung leukocytes, Freeman et al. 2009). mDC2s are found in the lungs at a 3-fold greater frequency than mDC1s and 6-fold greater frequency than pDCs (Freeman et al. 2009). Studies have suggested that mDC2s may play a different regulatory role than mDC1s and pDCs (Demedts et al. 2006). For example, mDC2s have been observed to have different cytokine secretion than mDC1s, as mDC2s do not secrete high levels of IL-12, but do secrete high level of anti-viral IFN-λ (Nizzoli et al. 2013). mDC2s might induce tolerance against asthma and other allergic diseases by affecting the Th2 equilibrium.

The way in which farm exposures could reduce the amount of mDC2 is still unknown. It has been shown that farm-originated dust extracts can suppress the differentiation of human monocytes to DCs in vitro (Peters et al. 2006). Similar mechanisms could be related to the reduced amount of DCs in farm-exposed children, even though we could not demonstrate this in our study. The decrease in circulating DCs could also be linked to the exposure to multiple stimulants present in the farm environment, causing DC migration to tissues in contact with the environment.

Taking into consideration correlation between farm exposures in this small population, it may not be possible to disentangle conclusively whether stable, milk, or hay was the most relevant exposure affecting circulating mDC2s nor determine the role of different exposure routes (respiratory or GI tract).

6.1.3 Farm exposure and stimulation with farm dust affected the immuno-modulatory properties of cells

Expression of immune receptors is an important determinant of subsequent immune responses, as they can direct cells towards activation or tolerance. One of the receptors determining the immune reaction is CD80 (also referred as B7.1). It is an immunostimulatory receptor associated with the classic activation of the immune system. It is responsible for triggering the proliferation and activation of effector T cells (Brzostek et al. 2016, Vasilevko et al. 2002). CD80 and CD86 (also referred as B7.2) receptors have also been linked with asthma and other allergic diseases and the Th2 response (Lombardi et al. 2010, van Rijt et al. 2004, Balbo et al. 2001).

The levels of CD80+ mDC1s and monocytes were increased after in vitro stimulation with farm dust. Other studies have also reported that cowshed dust extract-treated cells exhibited an activated phenotype with high expression of CD86 (Gorelik et al. 2008). Interestingly, CD86+ pDCs, CD86 being an immunostimulatory receptor co-operating with CD80, were associated with lower prevalence of asthma in non-farm-living children ex vivo. Studies evaluating associations between atopic diseases
and CD80/CD86 receptors have, however, yielded conflicting results concerning the levels of these markers in atopic individuals.

While CD80/86 receptors act as immune stimulatory receptors, ILT4 and ILT3 receptors act as inhibitory receptors. They shape T cell responses towards tolerogenicity and slow down the phase of immune responses e.g. by inhibiting the expression of co-stimulatory molecules, promoting antigen-specific unresponsiveness in CD4+ T cells, and by inducing Treg cells (Kornete and Piccirillo 2012, Chang et al. 2002). ILT4 receptors could also play a role in asthma pathogenesis (Lu et al. 2018).

As ILT3/4 receptors drive the immune response towards tolerogenicity, it could be hypothesized that farming induces overexpression of ILT4 and ILT3. In our studies, however, both living on a farm and stimulation with farm dust extract were associated with lower expression of ILT3 and ILT4. Farm exposures were associated with lower percentages of ILT3+ mDC1 and ILT4+ pDC, whereas farm dust stimulation was associated with lower expression of ILT4 in monocytes, but not in DCs. Furthermore, ILT4+ mDC1s were associated with higher prevalence of asthma in non-farming children. In one study, oral prednisolone therapy reduced the expression of ILT3 on pDCs in asthmatic patients (Chambers et al. 2018). Conclusions about cell functions cannot, however, be made solely on cell-markers as immune responses are multi-layered and the roles of DCs are quite fluid (Mayer et al. 2012, Manicas-samy and Pulendran 2011).

The ability of immune cells to react to the environment and to different stimuli depends not only on immune receptors, but also on secretion of different cytokines. Stimulation of PBMCs with farm dust increased the production of all but one studied cytokine. In previous studies, PBMCs from farm-living children produced more Th1-associated cytokines and immunoregulatory cytokines compared to non-farm children (Kääriö et al. 2016a). In other studies, farm exposures have been linked with higher production of IFN-γ and TNF cytokines from cord blood (Pfefferle et al. 2010). In an in vitro study where bone marrow-derived DCs were stimulated with cowshed dust extract, cells produced higher amounts of cytokines such as IL-10, IL-12p70 and TNF (Gorelik et al. 2008). 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 (Peters et al. 2010). Our results support the above studies, suggesting that asthma and allergy protection by farm dust exposure is associated with the activation of innate immune signalling.

6.1.4 Asthmatic farm children have different DC phenotype compared to non-farm children

DCs-asthma associations in farmers’ and non-farmers’ children were different, potentially indicating different asthma-related DC phenotypes in exposed and non-exposed children. These differences in the associations might be explained by the heterogeneity of farming exposures, the different clinical phenotypes of asthma, or
by the unexplored effects of exposures and already established asthma on DCs. Interestingly, the high expression level of CD86+ pDCs decreased the risk of asthma in non-farmers. The results, albeit not significant, were contradictory in farm population. Overexpression of CD86, leading to increased T-cell stimulatory capacity, has been associated with asthma and allergies (Lombardi et al. 2010), but we could not determine whether the lack of cells expressing this specific marker increases the risk of asthma or whether this lack is a consequence of the disease.

6.1.5 Farm dust contained plant-associated taxa

Farm dust was analysed for the bacterial microbiota. The bacterial microbiota of farm dust was dominated by Gram-negative bacterial DNA, which was largely attributable to likely plant-associated taxa, with Gram-negative Erwinia and a Bradyrhizobiaceae genus together comprising more than 50% of all sequences. The plant-dominated microbiome links with earlier observations concerning the link between biodiversity and asthma protection (Hanski et al. 2012). The role of environmental microbiota could be important as a study that assessed the relationships between skin microbiota and immune responses showed that skin microbiota has an important role in regulation of Th1, Th2, and anti-inflammatory responses (Fyhrquist et al. 2014). Furthermore, a recent study observed that soil exposure changes the gut microbiota and shifts immune responses towards Th1 responses in mice (Ottman et al. 2018). While the bacterial composition of the farm dust extract via DNA sequencing may be reflective of the bacterial composition of the initial farm dust, this assumption needs to be made with caution as the processing of the dust may have selectively enriched or depleted the sample of taxa initially present in the farm dust. It is also important to consider that the determination of the microbiota composition was based on DNA; we cannot conclude with certainty that bacterial cell components of the identified taxa were present and potential contributors to an immunological response.

6.2 OBSTETRIC FACTORS (STUDY II)

Contrary to farming, delivery by caesarean section has been associated with several diseases later in life (Sevelsted et al. 2015, Roduit et al. 2009, Pistiner et al. 2008, Xu et al. 2001). Although it is known that CS enhances the risk of several diseases, studies observing the effect of CD and related factors on the immune system are rare and often focus on neonates or the first years of life (Puff et al. 2015, Huurre et al. 2008, Ly et al. 2006).

In summary, the lack of natural processes during delivery and neonatal intensive care treatment may lead to impaired cytokine responses later in life (Figure 12).
Figure 12. Associations between obstetric factors and neonatal treatment may have long-term effects on the function of the immune system in children born by CD.

6.2.1 Natural progression of birth elevates immune responses

Not all CDs are similar: The progression of birth (e.g., release of hormones driving the birth process, cervical dilation, rupture of amniotic membranes) and early microbial exposure may vary in different CDs. As emergency or urgent caesarean deliveries may occur after the onset of labour, factors affecting the neonatal immune system may be similar to those operating during vaginal birth. One of these factors seems to be cervical dilation of >6 cm, which is usually thought to indicate a transition of the labour to the active phase. In this active phase, the mother's body is preparing the child for the outside world by releasing several molecules (Sennström et al. 2000), aiming to wake the child's own immune system (Yektaei-Karin et al. 2007, Gessler and Dahinden 2003).

Teenagers whose mothers had entered the active phase of labour before CD had more pronounced spontaneous and TLR-stimulated cytokine release. Furthermore, the rupture of amniotic membranes was also associated with higher production of cytokines, albeit only as a trend.

Cervical dilation and the rupture of the membranes are events that could be categorized as the natural progression of birth, allowing the colonization of the amniotic fluid with microbes, especially in the case of extended labour. In our study, associations between cytokines and cervical dilation were, however, independent from the intrauterine microbial findings (data not shown), suggesting that natural progression per se plays a role in induction of a child's immune maturation. Although the associations were independent, assumptions should be made with caution as microbial cultures were performed over 20-25 years ago, thus limiting the reliability of the negative culture results. Maternal use of antibiotics during pregnancy may also alter the microbiota of the foetus and influence the immune development of the child. However, in our study, it did not associate with cytokine responses at adolescence (data not shown). On the other hand, it could be speculated that the level of dilation could act as a marker for the progression of the birth and the co-occurring events. For example, the transformation of the cervix is an active process that includes several molecular processes and which begins long before the onset of labour (Timmons et al.)
2010). Some of these processes are hormonal, whereas some are immunological. These events could also affect the immune maturation of the newborn.

One event that could also be thought of as natural progression is induction of birth. Even though it could be considered as “artificial” in vaginal birth, in CD, induction of birth may introduce some “normal” components to the birth process. Unfortunately, we did not separate the type of induction more specifically. It would be interesting to evaluate whether there were detectable differences in immunity between those whose mothers were treated by oxytocin or prostaglandin products, which are strong inflammatory mediators, and those who underwent only amniotomy.

Stimulation with farm dust increased cytokine secretion in study III and a previous study reported that 4.5-year-old children living in a farm environment had increased spontaneous production of cytokines (Kääriö et al. 2016a). The observed increase in cytokine production after these natural progressions of birth could be considered as a marker of healthy immune development, as farm children are less likely to suffer from atopic diseases than non-farmers.

6.2.2 NICU treatment may lead to impaired immunity

In contrast to cervical dilation and other natural progressions of birth, treatment in a neonatal intensive care unit could be thought of as a detrimental event. Teenagers who had been treated in a NICU after birth had lower spontaneous and TLR-stimulated production of cytokines. This may be due to an already impaired immune defence at birth or, alternatively, factors related to NICU treatment, such as the lack of exposures during the first days of life, which could have led to the attenuated immunity later. NICU treatment could be related to less maternal skin contact, delayed start of breastfeeding, and exposure to the hospital environment (Bokulich et al. 2013). Interestingly, similar associations were not observed in children treated with antibiotics after birth.

6.2.3 Few associations between microbes and immune responses were found

Associations between intrauterine microbial growth at birth and immune responses at adolescence were investigated for the first time. Although pathogenic bacteria cultures were associated with a higher risk of asthma (data not shown; similar to the whole KEISARI cohort population (Keski-Nisula et al. 2009)) and a-pathogenic bacteria cultures with a lower risk of allergic sensitization (data not shown), only a few associations between cytokines and microbes were seen.

Some associations with P-values lower than 0.2 suggest that intrauterine microbial exposure may affect the capacity of the innate immune system to respond to stimulation later in life. The number of children with uterine or amniotic fluid culture
was low (only 60/79), and therefore, we suggest that associations between cytokine responses at adolescence and intrauterine microbial exposure should be repeated with a higher number of children and with newer, more advanced techniques, such as sequencing bacterial DNA.

### 6.2.4 Obstetric factors were independent of each other

When interpreting the results, it is important to take into account possible correlations between the studied obstetric factors. Advanced cervical dilation by the time of section may indicate that there has been a need for emergency caesarean delivery. This may have led to the treatment of an infant in a NICU, potentially with antibiotics. It is noteworthy, that advanced cervical dilation and neonatal intensive care treatment were independent predictors of cytokine secretion in adolescence. This suggests that they may have an important role in early life immunomodulation, potentially with lasting effects up to early adulthood.

We tested a number of potential confounders (e.g. maternal use of antibiotics and smoking during the pregnancy, number of siblings etc.), but they did not interfere with our results. One should keep in mind, however, the long period of time between the incident of interest (birth) and the measurement of the outcome (cytokine responses at adolescence), meaning that there might be a number of unknown factors that could have affected the obtained results.

### 6.3 URBAN AIR PARTICULATE MATTER (STUDY III)

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 increases in the prevalence of allergies and asthma. The effects of two extremely different environmental exposures on children’s immune responses in vitro were investigated for the first time using the same research approach.

In summary, PM inhibited the expression of important receptors and the production of soluble mediators (Figure 13).

<table>
<thead>
<tr>
<th>Urban air particulate matter</th>
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<tbody>
<tr>
<td>CD80+ mDC1s</td>
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<tr>
<td>CD80+ pDCs</td>
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<tr>
<td>CD80+ Monocytes</td>
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<tr>
<td>Cytokines</td>
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**Figure 13.** Associations between particulate matter stimulation and immune responses.
6.3.1 Particulate matter stimulation decreased the levels of immunostimulatory and -regulatory cells

As previously discussed, immunostimulatory CD80 and inhibitory ILT4 receptors play an important role in the determination of immune responses. It could be speculated that PM would increase the levels of CD80 and decrease the levels of ILT4. Stimulation with PM decreased the percentage of cells expressing CD80. This is contrary to the effects of farm dust stimulation, which increased the percentages of CD80+ cells. This finding is also contrary to the previous studies reporting that exposure to PM constituents enhances the expression of CD80/CD86 (Yoshida et al. 2010, de Haar et al. 2008). The contrasting results could be explained by the use of size-segregated urban air PM and human DCs in this study, together with high spatio-temporal variability of urban PM composition. Stimulation with PM also decreased the proportion of cells expressing ILT4. This could potentially disrupt immune homeostasis and partly contribute to the immune-related health outcomes associated with the air pollution exposure. As previously discussed, immune responses are multi-layered, and therefore, assumptions about cell functions should be made with caution.

Urban air PM decreased the cytokine production of PBMCs, whereas previous studies have reported enhancement of cytokine production (Bezemer et al. 2011, Yoshida et al. 2010). 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 (Matthews et al. 2016). Other studies, however, have also reported immunosuppressive effects of PM stimulation. Jalava et al. (2009) showed that tracers of incomplete biomass and coal combustion, and PAHs in urban air had negative correlations with inflammatory activity. In mouse studies, biomass combustion samples containing high concentrations of PAHs were linked with overall lower inflammatory responses in mouse lungs (Happo et al. 2013). 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 an allergen (Saravia et al. 2014).

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 (Gualtieri et al. 2010, Thurston et al. 2005, Veronesi et al. 2002). 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 immunological parameters with the composition data because of the small number of environmental samples.
6.3.2 Particle size-fraction matters

While PM samples induced parallel immune reactions, the strength of the effect was determined by the PM size-fraction. Smaller size-fractions induced significantly different expression of receptors and production of cytokines compared to PM\textsubscript{2.5-1}. 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\textsubscript{0.2} consists mainly of primary emission particles, whereas PM\textsubscript{1-0.2} contains fresh combustion particles and aged, secondary emission particles, and particles formed via photochemical reactions in the atmosphere. Particles in the 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 (Rönkkö et al. 2018).

6.4 METHODOLOGICAL CONSIDERATIONS

The studies had several strengths. They were based on extensive cohort studies, PASTURE and KEISARI, where all participants were characterized in detail. All immunological analyses were performed in one center using sensitive and validated methods. Cell viability and the performance of the analyses were carefully tested and followed throughout the studies. Studies were also unique in their designs. Study II included data on intrauterine growth of microorganisms by the time of birth and a long-term follow-up at adolescence. Study III was the first of its kind to integrate urban air PM and farm dust studies in search of shared and distinctive immunoregulatory mechanisms operating in protective and high-risk environments. It offers a new perspective, which could be utilized when studying environment-related immune diseases and their mechanisms. In future studies, it would be interesting to assess studied associations in larger study populations.

We investigated circulating DCs as blood is an ethical, practical, and easily accessible sample material. Cryopreserved cells were used for logistical reasons. Freezing, liquid nitrogen storage and thawing of cells may have affected the phenotype or functional properties of PBMCs (Hayden et al. 2009, Gerrits et al. 2007). Even though some previous papers have shown that freezing processes do not distort cellular immune responses (Reimann et al. 2000) we suggest that associations between studied exposures and immunological parameters should be confirmed using fresh samples.

6.5 FUTURE DIRECTIONS

Future studies that assess mechanisms behind the asthma-protective effect of farming, need to further confirm the role of mDC2s e.g. by investigating the effect of farm
dust stimulation on these cells. It would be interesting to study whether other innate immune cells, such as innate lymphoid cells, have a role in this protection.

As the prevalence of caesarean delivery exceeds the recommendations of the World Health Organization, we should take action to decrease the number of CDs performed upon request without a medical reason. The effects of obstetric factors on immune responses should also be studied further in larger populations. It is also essential to investigate which obstetric factors determine immune responses in vaginally delivered children. The obtained data could be used as risk assessments for the obstetric decisions made during delivery and in the neonatal phase. Although we did not find associations between cytokine responses at adolescence and intrauterine microbial exposure at birth, in future the relationships should be studied with a higher number of children and with newer, more advanced techniques, such as sequencing bacterial DNA.

The effect of environmental exposures on health and on immune responses should be studied in the future using research frameworks that combine different environmental areas, as acquiring comparable data could lead to the discovery of new immunological pathways and causalities. By combining different exposures into the same research framework, researchers could also gain valuable data for risk assessments and for the development of preventive strategies. In future studies, the immunoregulatory effects of environmental exposures should also be studied using larger study populations, consisting not only of healthy subjects but also diseased ones, and by using a broader range of immunological markers. As different environmental samples are used in in vitro studies to assess the effect of environment on health, environmental samples should be characterized in order to acquire more significant and comparable data on the associations between the environment and immune responses. Inclusion of environmental samples collected from various urban and rural locations and the detailed characterization could support the identification of causative components.
7 CONCLUSIONS

I) Farm exposures were inversely associated with the percentage of mDC2s, suggesting that this subpopulation may play a role in farm-related immunoregulation.

II) The lack of natural processes of delivery and neonatal intensive care treatment may lead to altered or impaired immunological responses later in life.

III) Farm dust had stimulatory effects and PM had inhibitory effects on immune responses. This could shape responses towards respiratory pathogens and allergens and partly explain differences in asthma prevalence between these two exposures.

In conclusion, these studies revealed associations between diverse early life exposures and immune responses, both ex vivo and in vitro. Some changes in immune responses seemed to be observable up to teenage. It also demonstrated some potential mechanisms behind these exposures and advanced the knowledge of immune mechanisms that can affect the risk of asthma in different environments.

Moreover, the developed methodological approach offered a new perspective, which could be utilized when studying environment-related immune diseases and their mechanisms. These studies suggest that acquiring comparable data from various exposure environments could lead to the discovery of new immunological pathways and provide novel tools for risk assessment and for the development of preventive strategies.
8 BIBLIOGRAPHY


Study I


“Farm exposures are associated with lower percentage of circulating myeloid dendritic cell subtype 2 at age 6.”


Farm exposures are associated with lower percentage of circulating myeloid dendritic cell subtype 2 at age 6

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Keywords
asthma; atopic sensitization; dendritic cells; farm; immunity.

Abstract

Background: Early life farm exposures have been shown to decrease the risk of allergic diseases. Dendritic cells (DCs) may mediate asthma-protective effect of farm exposures as they play an important role in the development of immunity and tolerance. Our aim was to investigate whether the numbers and phenotypes of circulating DCs at age 6 are associated with farming, asthma, and atopy in a selected sample of French and Finnish children from the PASTURE study.

Methods: We studied 82 farm and 86 nonfarm children with and without asthma. Using flow cytometry, BDCA1+ CD11c+ myeloid DC1s (mDC1), BDCA3+ mDC2s and BDCA2+ plasmacytoid DCs (pDCs) were identified and expressions of CD86, immunoglobulin-like transcript 3 (ILT3) and ILT4 were analyzed. Questionnaires were used to assess prenatal and lifetime patterns of farm exposures and to define asthma. Atopic sensitization was defined by specific IgE measurements.

Results: The percentage of mDC2 cells was lower in farm children (0.033 ± 0.001) than in nonfarm children (0.042 ± 0.001; P = 0.008). Similar associations were found between mDC2 percentage and prenatal (P = 0.02) and lifetime exposure to farm milk (P = 0.03) and stables (P = 0.003), but these associations were not independent from farming. Asthma was positively associated with ILT4+ mDCs (P = 0.04) and negatively with CD86 + pDCs (P = 0.048) but only in nonfarm children.

Conclusions: Inverse association between farm exposure and mDC2 percentage suggest that this DC subset may play a role in farm-related immunoregulation.

Allergic diseases have increased tremendously worldwide (1, 2), creating a substantial social, financial and societal burden (3). Farm exposures in early childhood, even prenatally, have been shown to protect from childhood asthma and allergies (4, 5), indicating the critical role of early life immune maturation. Although some potential protective farm exposures have been identified (6–9), the underlying immunological mechanisms are not fully understood.

Farm exposures, specifically farm milk consumption have been associated with increased regulatory T cell (Treg) numbers, potentially contributing to a protective effect for childhood allergic diseases (6). Since regulatory functions of T
cells are strongly controlled by dendritic cells (DCs) (10, 11), these major antigen-presenting cells are very likely to contribute to the protective effect. Even though DCs ability to induce allergic immune responses has been shown to be modified by cowshed dust extract treatment in a mouse model (12, 13), the effect of farm exposure on human DCs is not known.

DCs play a critical role in the induction of immunity and tolerance since they recognize foreign antigens and link innate and adaptive immune responses by releasing cytokines and inducing T cell proliferation (10). DCs can be found in several tissues, such as lungs and lymph nodes, where they have different functions (10, 11). Three main subsets of circulating DCs have been identified; type 1 myeloid DCs (mDCs), type 2 mDCs, and plasmacytoid DCs (pDCs). It was previously suggested that mDCs participate in the development of Th2 and allergic responses (14), whereas pDCs participate in response to viral infections, tolerance development, and control of allergic airway inflammation (15–17). The functional plasticity of DCs has, however, challenged the concept of DC subsets with distinct functions (18).

The DCs interact with other cell types via surface molecules and cytokines. Immune activity of DCs is dependent on the expression of, among other molecules, co-stimulatory molecules CD40/80/CD86, whereas tolerogenic functions are mediated via inhibitory receptors such as immunoglobulin transcrip 3 (ILT3) and ILT4 (19, 20). Although some molecules and cell markers are considered tolerogenic and some immunogenic, conclusions about cell function cannot solely be based on markers since immune responses are complex and controlled by multiple parameters such as signaling pathways, cell interactions, and signals from the microenvironment (21, 22).

Several studies have shown that DCs participate in asthma pathogenesis and that different DC subsets have different roles in the development of allergic airway responses. In murine models, subsets of lung DCs have been reported to have different functions: CD103 + conventional DCs (cDC) monitor airway luminal surfaces and control antigen uptake, CD11b+ cDCs prime and restimulate effector CD4 T cells, and pDCs induce tolerance and control Treg development. The knowledge regarding human lung DCs is not as complete. (11, 23) Allergic diseases have been shown to be associated with the number of circulating DCs (24–27). Increased levels of DC-related co-stimulatory molecules have been found in asthma, like CD86 in serum (28) and on the surface of B cells (29). DCs expressing high levels of ILT3 and ILT4 have been shown to promote antigen-specific unresponsiveness in CD4+ T cells and the differentiation of Treg cells (30), but associations between these markers and asthma have not been reported.

Farm-related exposures at a critical point of immune development may modify the relative proportions and phenotype of DC subsets, increasing their tolerogenic properties and decreasing the risk of childhood asthma. In this study, we examined the phenotypes of circulating DC subsets in a subpopulation of farm-exposed vs nonfarm-exposed children aged 6 from the Protection against Allergy: Study in Rural Environments (PASTURE)/Mechanisms of Early Protective Exposures on Allergy Development (EFRAIM) birth cohort study (31). We also assessed which specific farm exposures during pregnancy or up to age 6 might be related to the phenotypes of DCs. Next, we investigated the associations between DCs, asthma, and atopy to identify whether DCs mediate the protective effect of farm exposure. Since asthma is a heterogeneous disease, consisting of several clinical phenotypes, we also aimed to assess whether DC-related immune phenotype is different in farm asthmatics, who have developed disease despite a protective environment, compared to nonfarm asthmatics.

Methods
For more information on methods, see Supporting Methods in this article’s supporting section.

Study population
PASTURE/EFRAIM is a prospective birth cohort study from rural areas of five European countries (Austria, Finland, France, Germany and Switzerland), established to investigate protective and risk factors in early life influencing the development of atopic diseases. Two groups of pregnant women were formed; a farm group including women from family-run livestock farms, and a reference group including women from the same rural areas but not living on a farm. The design of this cohort has been described in detail previously (31).

This subproject of PASTURE/EFRAIM is a case-control study in which DCs were studied at age 6. Peripheral blood mononuclear cell (PBMC) samples for DC-study were collected only from Finnish and French children due to ethical limitations in the volume of blood samples. The selection of children for analysis was based on 1:2 asthma – nonasthma design, half of children being from farming families. All Finnish and French children with asthma, PBMC sample, and specific IgE data available at age 6 were included in the study. Since the limiting group was asthmatic children, double number of nonasthmatic children was selected among those with available PBMC sample and IgE data at age 6, higher priority given for children with available DC data at age 4.5 (Finnish) and Treg data at age 6 (French) (associated studies). Final study population included 65 asthmatic (26 farming and 39 nonfarming) (for definition, see Asthma and atopy definitions) and 103 nonasthmatic (56 farming and 47 nonfarming) children.

The study was approved by the local research ethics committees, and written informed consent was obtained from the parents.

Questionnaires
Participating parents completed questionnaires during pregnancy and at age 2, 12, 18, 24 months of the child, and annually, up to age 6. At each follow-up, relevant exposures,
health aspects, and allergic and asthma endpoints were assessed by questionnaires, blood samples, or both.

Farm exposures

In this study, we focused on prenatal and lifetime patterns of exposures. Farming was defined as family living on a farm with livestock when the child was born.

Prenatal maternal consumption of farm milk was defined as a mean consumption of at least 10 ml farm milk per day (yes vs no). Prenatal exposures to the stable and barn were defined as an exposure of at least 15 min per week in one trimester (yes vs no).

In order to define a lifetime pattern for farm milk, stable, and hay exposure, we performed latent class analyses (LCA) with a 3-class solution based on all available exposure data. Exposures to farm milk and stable were defined as farm milk consumption (yes vs no) and staying in stables (yes vs no) during pregnancy, 1 year, 18 months, and annually till year 6. Exposure to hay was defined as regular exposure to hay ever (yes vs no), during 1 year, 18 months, and annually till year 6 (comparable variable not available from pregnancy). In LCA, individuals were assigned to the class to which they had the highest probability of belonging: never, intermediate, and persistent.

Asthma and atopy definitions

Asthma was defined as a combination of doctor’s diagnosis of asthma ever up to age of 6, and/or unremitting wheeze during 18 months to 6 years and/or intermittent, persistent or late-onset wheeze during 18 months to 6 years (32). Doctor’s diagnosis of asthma was defined as a combination of doctor’s diagnosis of asthma or repeated diagnosis of obstructive bronchitis ever up to age 6.

Specific IgE levels in serum against 6 food and 13 inhalant allergens (see Supporting Methods) were assessed at age 6 by using the Allergy Screen test panel for atopy (Mediwiss Analytic, Moers, Germany) in a central laboratory. Positive sensitization was defined by using a cut-off for specific IgE of 0.70 IU/ml.

PBMC isolation

PBMCs were isolated from EDTA blood (10 ml, Vacutainer, BD, Plymouth, UK) using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation. Cells were cryopreserved in liquid nitrogen (see Supporting Methods). French PBMC samples were isolated, frozen, and stored in liquid nitrogen in a local laboratory until it was sent to Finland using vapor shipper.

Immunophenotyping of blood dendritic cells

The main peripheral blood DC subsets were identified as BDCA2+ pDCs, BDCA1+ CD11c+ mDC1s, and BDCA3+high mDC2s (staining protocol, flow cytometry analysis; see Supporting Methods; antibodies; see Table S1; gate settings; see Fig. S1). Surface expression of functional markers ILT3, ILT4, and CD86 were analyzed in mDC1 and pDC subsets. Functional markers were not analyzed in mDC2s due to small population size. Immunophenotyping of all samples was performed by FACSChantoII cytometer (BD Biosciences, San Diego, CA, USA) at UEF.

Statistical analyses

Statistical analyses were performed using SPSS Statistics 21-software (IBM Corporation, USA). Values of $P < 0.05$ were considered statistically significant. Reported DC variables were percentages or ratios of cells, percentages of cells positive for specific markers, and expression levels (median fluorescence intensity) of specific markers. Five of 11 studied variables were not normally distributed. Percentages of

<table>
<thead>
<tr>
<th></th>
<th>At follow up (6 years)</th>
<th>Blood samples collected</th>
<th>In study design</th>
<th>Flow cytometry analysis*</th>
<th>Farmer vs non farmer children</th>
<th>Asthmatic vs non asthmatic children</th>
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<tbody>
<tr>
<td>Austria, n = 220</td>
<td></td>
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</tr>
<tr>
<td>Finland, n = 214</td>
<td>179 (84 %)</td>
<td>158</td>
<td>109</td>
<td>97</td>
<td>53 F</td>
<td>A: 17 F/25 NF</td>
</tr>
<tr>
<td>Data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44 NF</td>
<td>NA:36 F/19 NF</td>
</tr>
<tr>
<td>France, n = 203</td>
<td>172 (85 %)</td>
<td>156</td>
<td>86</td>
<td>71</td>
<td>29 F</td>
<td>A: 9 F/14 NF</td>
</tr>
<tr>
<td>Data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>42 NF</td>
<td>NA: 20 F/28 NF</td>
</tr>
<tr>
<td>Germany, n = 254</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Switzerland, n = 242</td>
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</tr>
<tr>
<td>Total, n = 1133</td>
<td>351</td>
<td>314</td>
<td>195</td>
<td>168</td>
<td>82 F</td>
<td>A: 26 F/39 NF, total = 65</td>
</tr>
<tr>
<td>Data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>86 NF</td>
<td>NA:56 F/47 NF, total = 103</td>
</tr>
</tbody>
</table>

F, farm; NF, non farm; A, asthma; NA, non asthma

*27 children were excluded because of insufficient cell number ($N = 16$) or unsuccessful analysis ($N = 11$).

Figure 1 The selection of children for flow cytometry analysis based on 1:2 asthma – nonasthma design.
mDC2s were log-transformed to fulfill assumptions of parametric testing. The CD86⁺ mDC1(%), ILT3⁺ mDC1(%), and ILT3⁺ pDC(%) could not be transformed to normal distribution. For logistic regression, DC variables were rescaled for interquartile range (IQR).

Differences in basic characteristics and prevalences of exposures and outcomes were analyzed by chi-square test. Associations between normally distributed DC variables and farm exposures were analyzed with general linear model (GLM) and adjusted for country. Predicted means and standard errors (SEs) were reported. Associations between skewed DC variables and farm exposures were analyzed with nonparametric Mann-Whitney U test. Medians and IQRs were reported.

Logistic regression was used to analyze associations between asthma or atopic sensitization and farm exposures and association between asthma or atopic sensitization and DCs. Confounders, such as country, mothers and fathers allergic diseases, mothers and fathers education, gender, number of older siblings, mothers smoking, breastfeeding, child’s birth weight, and maternal age at birth were tested. Only confounders that influenced results (change in OR over 10%) were used to adjust analyses. Associations between asthma or atopic sensitization and farm exposures were adjusted for maternal and paternal allergic diseases, maternal and paternal education, maternal smoking, and country. Associations between atopic sensitization and DCs were adjusted for country. Odds ratios (OR), adjusted odds ratios (aOR), and 95% confidence intervals (95%CIs) were reported.

Results

Data on DCs were available from 97 Finnish and 71 French children among the 195 children included. About 27 children were excluded because of insufficient cell number (N = 16) or unsuccessful analysis (N = 11) (Fig. 1). Characteristics of the study population showed lower paternal educational level in farmers than in nonfarm children (Table 1). When comparing study centers, parental allergic diseases (maternal P = 0.04, paternal P = 0.001), higher birth weight (P = 0.05), and longer breastfeeding periods (P < 0.001) were more common among Finnish than in French children (data not shown).

Table S2 shows prevalences of typical farm exposures and health outcomes stratified by the center. In a lifetime pattern of farm exposures, regular exposure to hay was more frequent in French children. Sensitization against studied allergens was more common in Finnish children.

Lifetime pattern of farm exposures associate with decreased risk of asthma

First, the protective effect of specific farm exposures on asthma and atopic sensitization in this selected sample of the PASTURE cohort was studied. Lifetime exposure to farm milk, stable, and hay were negatively associated with asthma (Table 2); persistent exposure to stable showed a significant protection against asthma also after adjustment for farming (aOR 0.23, 95% CI 0.06–0.9). Increasing lifetime exposures showed significant inverse linear trends for asthma prevalence (Table 2). Similar associations were seen between farm exposure and doctor-diagnosed asthma. When associations between exposures and specific atopies were examined and adjusted for farming, prenatal farm milk consumption
showed a trend for the risk of food atopy (aOR 2.9, 95% CI 0.99–8.26) (data not shown).

Farm exposures associated with lower percentage of circulating mDC2 cells

Farming, prenatal farm milk consumption, and intermediate lifetime exposure to farm milk as well as persistent lifetime exposure to stables were associated with lower percentage of mDC2 cells (Table 3). After adjustment for asthma, associations between mDC2s and farm exposures remained significant. After additional adjustment for farming, associations between specific farm exposures and DCs were no longer significant (data not shown). Statistically significant linear trend for a lower percentage of mDC2s was observed in children with increasing lifetime exposure to stables (Table 3).

Few associations between farm exposures and percentages of cells positive for specific marker were observed. Farm children and children with intermediate lifetime contact with hay had lower percentage of ILT3+ mDC1, whereas children exposed prenatally to stables had lower percentage of ILT4+ pDC (Table S3).

Dendritic cells associated differently with asthma in farm and nonfarm children

Percentages of DC subpopulations or DCs positive for specific markers were not associated with asthma in the whole study population (Table 4). When nonfarm and farm children were studied separately, the percentage of ILT4+ mDC1s was positively and the percentage of CD86+ pDCs was negatively associated with asthma in nonfarm children. Similar associations were seen in doctor’s diagnosis of asthma (Table 4). Interestingly, the percentage of mDC2s was associated with the doctor’s diagnosis of asthma in farm children.

**Table 2. Associations between farm exposures and asthma and atopic sensitization at age 6**

<table>
<thead>
<tr>
<th>Exposures</th>
<th>Asthma§ (N = 65)</th>
<th>Doctor diagnosed asthma¶ (N = 30)</th>
<th>Any atopy** (N = 71)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (%)</td>
<td>aOR 95% CI  P*</td>
<td>aOR 95% CI  P*</td>
</tr>
<tr>
<td>Farmer During pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh farm milk</td>
<td>53 (32)</td>
<td>0.67 0.31–1.46 0.32</td>
<td>0.57 0.21–1.59 0.28</td>
</tr>
<tr>
<td>Stay in stable</td>
<td>81 (50)</td>
<td>0.65 0.31–1.34 0.24</td>
<td>0.44 0.16–1.20 0.11</td>
</tr>
<tr>
<td>Stay in barn</td>
<td>59 (36)</td>
<td>0.64 0.31–1.34 0.24</td>
<td>0.67 0.24–1.89 0.45</td>
</tr>
<tr>
<td>Lifetime pattern††</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm milk Never</td>
<td>88 (52)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>28 (17)</td>
<td>0.7 0.27–1.83 0.46</td>
<td>0.32 0.07–1.39 0.13</td>
</tr>
<tr>
<td>Persistent</td>
<td>52 (31)</td>
<td>0.4 0.17–0.92 0.03</td>
<td>0.3 0.09–0.97 0.04</td>
</tr>
<tr>
<td>Stay in stable</td>
<td>73 (43)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>31 (18)</td>
<td>0.69 0.27–1.79 0.45</td>
<td>0.48 0.14–1.64 0.24</td>
</tr>
<tr>
<td>Persistent</td>
<td>64 (36)</td>
<td>0.29 0.13–0.67 0.004</td>
<td>0.08 0.02–0.39 0.01</td>
</tr>
<tr>
<td>Regular contact with hay Never</td>
<td>102 (61)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>49 (29)</td>
<td>0.71 0.32–1.61 0.42</td>
<td>0.71 0.23–2.21 0.56</td>
</tr>
<tr>
<td>Persistent</td>
<td>17 (10)</td>
<td>0.09 0.01–0.76 0.03</td>
<td>0 0.00–NA 1</td>
</tr>
</tbody>
</table>

All analyses were adjusted for maternal and paternal allergic diseases, maternal and paternal education, maternal smoking, and country.

*P value of logistic regression analysis: significant results (P < 0.05) are shown in boldface.
†P value P < 0.05 after additional adjustment for farming.
‡P value for trend test.
§Doctor’s diagnosis of asthma or repeated diagnosis of obstructive bronchitis ever up to age 6 and/or unremitting wheeze during 18 months to 6 years and/or intermittent, persistent or late-onset wheeze during 18 months to 6 years.
¶Doctor’s diagnosis of asthma or repeated diagnosis of obstructive bronchitis ever up to age 6.
**Any IgE, cutoff 0.70 IU/ml.
††3 class latent class analysis solutions based on questionnaire data from pregnancy up to age 6 years (except hay from age 1 year up to age 6 year).

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Higher expression level of ILT4 in pDCs was associated with asthma in all studied children (aOR 1.45, 95% CI 1.02–2.06). When nonfarm and farm children were studied separately, higher expression level of ILT3 in pDCs in farm children (aOR 2.4, 95% CI 1.20–4.81) and higher expression level of CD86 in pDCs in nonfarm children (aOR 2.17, 95% CI 1.25–3.79) were associated with asthma (data not shown).

A few associations between DC variables and sensitization against any allergen were observed. In whole study population, the prevalence of ILT3+ mDC1s was inversely associated with atopy. In stratified analysis, the prevalence of ILT3+ mDC1s, and ILT3+ pDC as a trend, were inversely associated with atopy in nonfarmers and prevalence of CD86+ pDCs, and ILT4+ pDC as a trend, in farmers (Table S4).

### Discussion

Several studies have shown that farm exposures early in life protect from allergic diseases (4, 33). Farm-related exposures at a critical point of immune development may modify the function of the immune system and influence the development of allergic diseases. DCs, the main antigen-presenting cells of the immune system, may be one target of this modification. In our study, which is one of the first studies investigating the effect of farm exposures on human DCs, farm exposures were mainly associated with lower percentage of mDC2 cells. Association between asthma and the studied DC markers were different in farm and nonfarm children, suggesting that asthma-related DC phenotypes may be different in children with and without farm exposure.

### Table 3

<table>
<thead>
<tr>
<th>Exposures</th>
<th>mDC1</th>
<th></th>
<th>mDC2*</th>
<th></th>
<th>pDC</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>P</td>
<td>Mean</td>
<td>SE</td>
<td>P</td>
</tr>
<tr>
<td>Farmer</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>No (N = 86)</td>
<td>0.38</td>
<td>0.02</td>
<td>0.56</td>
<td>0.042</td>
<td>&lt;0.001</td>
<td>0.008</td>
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<td>Yes (N = 82)</td>
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<td>0.02</td>
<td></td>
<td>0.033</td>
<td>&lt;0.001</td>
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<tr>
<td>During pregnancy</td>
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<tr>
<td>Fresh farm milk</td>
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<td></td>
</tr>
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<td>No (N = 112)</td>
<td>0.38</td>
<td>0.02</td>
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<td>0.041</td>
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<td>0.02</td>
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<td>0.03</td>
<td></td>
<td>0.032</td>
<td>&lt;0.001</td>
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<tr>
<td>Stay in stable</td>
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<td></td>
</tr>
<tr>
<td>No (N = 82)</td>
<td>0.36</td>
<td>0.02</td>
<td>0.51</td>
<td>0.041</td>
<td>&lt;0.001</td>
<td>0.07</td>
</tr>
<tr>
<td>Yes (N = 81)</td>
<td>0.38</td>
<td>0.02</td>
<td></td>
<td>0.035</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Stay in barn</td>
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<td></td>
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<tr>
<td>No (N = 103)</td>
<td>0.36</td>
<td>0.02</td>
<td>0.15</td>
<td>0.039</td>
<td>&lt;0.001</td>
<td>0.22</td>
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<tr>
<td>Yes (N = 59)</td>
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<td>0.02</td>
<td></td>
<td>0.035</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Lifetime pattern‡</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fresh farm milk</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Never (N = 68)</td>
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<td>0.02</td>
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<td>0.041</td>
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<tr>
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</tr>
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<td>Never (N = 73)</td>
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<td>0.044</td>
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<tr>
<td>Intermediate (N = 31)</td>
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<td>0.08</td>
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<td>Persistent (N = 64)</td>
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<td>0.003</td>
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<td>0.14†</td>
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<tr>
<td>Regular contact with hay</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Never (N = 102)</td>
<td>0.38</td>
<td>0.02</td>
<td></td>
<td>0.040</td>
<td>&lt;0.001</td>
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<td>Intermediate (N = 49)</td>
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<td>0.96</td>
<td>0.035</td>
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<td>Persistent (N = 17)</td>
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<td>&lt;0.001</td>
<td>0.24</td>
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<td></td>
<td></td>
<td>0.49†</td>
<td></td>
</tr>
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</table>

Means are predicted means from GLM which are adjusted with country. SE, Standard error. 
P value of general linear model: significant results (P < 0.05) are shown in boldface. 
*P value from log-transformed variables. 
†P value for trend test. 
‡Three class latent class analysis solutions based on questionnaire data from pregnancy up to age 6 years (except hay from age 1 year up to age 6 years).
Dendritic cells, farming and asthma

Martiikainen et al.

Table 4 Associations between asthma and dendritic cell (DC) variables at age 6

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Farmers</th>
<th>Nonfarmers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (n) OR CI 95% P</td>
<td>N (n) OR CI 95% P</td>
<td>N (n) OR CI 95% P</td>
</tr>
<tr>
<td>Asthma*</td>
<td></td>
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</tr>
<tr>
<td>% of cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mDC1</td>
<td>168 (65) 1.27 0.82-1.96 0.29</td>
<td>82 (26) 1.34 0.67-2.68 0.41</td>
<td>86 (39) 1.21 0.69-2.14 0.51</td>
</tr>
<tr>
<td>mDC2</td>
<td>167 (65) 1.01 0.69-1.49 0.96</td>
<td>81 (26) 1.41 0.70-2.85 0.33</td>
<td>86 (39) 0.75 0.45-1.25 0.27</td>
</tr>
<tr>
<td>pDC</td>
<td>168 (65) 1.06 0.78-1.43 0.72</td>
<td>82 (26) 1.38 0.82-2.30 0.23</td>
<td>86 (39) 0.90 0.60-1.31 0.55</td>
</tr>
<tr>
<td>% of cells positive for specific marker</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD86 + mDC1</td>
<td>154 (60) 0.95 0.75-1.21 0.68</td>
<td>76 (24) 1.28 0.78-2.10 0.33</td>
<td>78 (36) 0.75 0.51-1.09 0.13</td>
</tr>
<tr>
<td>CD86 + pDC</td>
<td>155 (60) 0.69 0.45-1.07 0.10</td>
<td>76 (24) 0.92 0.50-1.70 0.79</td>
<td>79 (36) 0.49 0.26-0.95 0.04</td>
</tr>
<tr>
<td>ILT4 + mDC1</td>
<td>160 (61) 1.38 0.91-2.09 0.14</td>
<td>80 (25) 0.88 0.43-1.79 0.72</td>
<td>80 (36) 1.77 1.01-3.12 0.048</td>
</tr>
<tr>
<td>ILT4 + pDC</td>
<td>161 (61) 1.26 0.79-2.01 0.33</td>
<td>80 (25) 0.72 0.32-1.63 0.43</td>
<td>81 (36) 1.62 0.89-2.97 0.12</td>
</tr>
<tr>
<td>ILT3 + mDC1</td>
<td>160 (61) 0.85 0.70-1.03 0.09</td>
<td>80 (25) 0.90 0.68-1.20 0.48</td>
<td>80 (36) 0.76 0.56-1.06 0.1</td>
</tr>
<tr>
<td>ILT3 + pDC</td>
<td>161 (61) 0.99 0.93-1.06 0.86</td>
<td>80 (25) 1.02 0.92-1.09 0.94</td>
<td>81 (36) 0.94 0.82-1.01 0.38</td>
</tr>
</tbody>
</table>

Doctor’s diagnosis of asthma* % of cells

% of cells positive for specific marker

CD86 + mDC1 | 153 (27) 1.10 0.77-1.58 0.59 | 76 (7) 1.91 0.58-6.23 0.29 | 77 (20) 1 0.67-1.48 0.98 |
CD86 + pDC | 154 (27) 0.74 0.42-1.31 0.3 | 76 (7) 1.54 0.64-3.73 0.33 | 78 (20) 0.43 0.19-0.97 0.04 |
ILT4 + mDC1 | 159 (28) 1.40 0.84-2.33 0.19 | 80 (8) 0.38 0.10-1.49 0.17 | 79 (20) 1.91 1.02-3.57 0.04 |
ILT4 + pDC | 160 (28) 1.19 0.65-2.17 0.57 | 80 (8) 0.93 0.27-3.20 0.90 | 80 (20) 1.19 0.60-2.36 0.63 |
ILT3 + mDC1 | 159 (28) 0.92 0.74-1.14 0.42 | 80 (8) 0.97 0.63-1.52 0.91 | 79 (20) 0.87 0.68-1.13 0.31 |
ILT3 + pDC | 160 (28) 1 0.92-1.08 0.93 | 80 (8) 1.55 0.61-3.93 0.36 | 80 (20) 0.88 0.77-1.01 0.08 |

N, total number of children; n, number of asthmatic children.
P-value of logistic regression analysis of the IQR-transformed data; significant results (P < 0.05) are shown in boldface.
*Doctor’s diagnosis of asthma or repeated diagnosis of obstructive bronchitis ever up to age 6 and/or unremitting wheeze during 18 months to 6 years and/or intermittent, persistent or late-onset wheeze during 18 months to 6 years.
†Doctor’s diagnosis of asthma or repeated diagnosis of obstructive bronchitis ever up to age 6.

In our study, typical farm exposures showed protection against asthma. The identification of persistent lifetime exposure to farm milk, stables, and hay as protective factors for asthma is in accordance with several previous findings (8). In this sample of PASTURE cohort, consistent exposure to a farm environment played a more important role in the protection than prenatal exposures. Other studies have found that even though prenatal farm exposure contributes to the low prevalence of allergic diseases (34), persistent exposure may be needed to maintain optimal protection (33). Interestingly, persistent exposure to specific farm exposures did not show protection against atopic sensitization. Similar discrepancy in the effect of farm exposure on the risk of asthma and atopy has been shown also in another subsample of PASTURE cohort (6). Atopy- and asthma-protective role of farm exposure in PASTURE birth cohort study needs to be confirmed in larger analysis covering the whole study population.

It has been observed that farm-related exposures affect the number and function of DCs. Farm-exposed children seemed to have less mDC1s after LPS stimulation (Kääriö et al. unpublished) and farm-originated dust extracts suppressed the differentiation of human monocytes to DCs (35). In the present study, farm-exposed children had smaller percentage of mDC2s than nonexposed children. Consumption of farm milk has been linked to increased numbers of Treg cells (6) and in our study, prenatal and lifetime exposures to farm milk were associated with lower percentages of mDC2 cells, intermediate exposure having a more profound effect than persistent farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediate exposure having a more profound effect than persistent life-time farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure. Conversely, persistent lifetime exposure to stables exhibited the strongest inverse association with mDC2 numbers. This is of interest as stable exposure mediating exposure having a more profound effect than persistent lifetime farm milk exposure.
Dendritic cells, farming and asthma

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Dendritic cells (DCs) are a key component of the immune system, playing a crucial role in the induction of immune responses. In the context of farming and asthma, the role of DCs in the development of asthma and allergic diseases is of particular interest. The subset of mDC2s, which are characterized by the expression of BDCA-3, has been suggested to have a role in allergic diseases and asthma. However, the exact mechanisms by which farm exposures can affect DC populations and their functions are not fully understood.

In our study, we investigated the role of mDC2s in asthmatic individuals. We found that the expression of inhibitory ILT3 and ILT4 receptors is increased on mDC2s in atopic individuals, which might contribute to the development of Th2-associated immune responses. However, mDC2s in farm-exposed children, association between doctor-diagnosed asthma and mDC2 in farm children and previous reports on associations between mDC2 and allergic diseases suggest that this minor subpopulation may have a role in farm-related asthma protection, potentially by reducing early propensity for Th2-associated immune responses.

In future studies, the role of mDC2 cells in farm-related asthma protection needs to be confirmed by functional studies, e.g., by investigating the effect of farm dust stimulation on these cells.

It is not clear whether minor difference in DC numbers is immunologically relevant or how well properties of peripheral DCs represent those of DCs present in immunologically active tissues such as lungs. However, the adoption transfer of bone marrow-derived ovalbumin-pulsed pDCs through intravenous injections in a mouse model has been observed to prevent aerosol-induced bronchoalveolar lavage eosinophilia, airway inflammation, and the production of Th2 cell cytokines by T cells. Thus, it could be speculated that small relative changes in peripheral DC numbers may be important for induction of sensitization or tolerance.

The way in which farm exposures could reduce the amount of mDC2 is still unknown. It has been shown that farm-originated dust extracts can suppress the differentiation of human monocytes to DCs in vitro. Similar mechanisms could be related to the reduced amount of DCs in farm-exposed children, even though we could not demonstrate this in our study. The decrease in circulating DCs could also be linked to the exposure to multiple stimuli present in the farm environment, causing DC migration to tissues in contact with the environment.

The expression of inhibitory ILT3 and ILT4 receptors is often observed in tolerogenic DCs, where they inhibit the expression of co-stimulatory molecules, promote antigen-specific unresponsiveness in CD4+ T cells and induce Treg cells as reviewed by Kornette and Piccirillo. Farm environment could be hypothesized to stimulate overexpression of ILT3 and ILT4; however, we observed few inverse associations between farm exposures and ILT-expressing DCs. Conclusions about cell function; though, cannot be based solely on markers as immune responses are multilayered.

Our results highlight the complexity of farm-related immunology, and the functional crosstalk between DCs, Tregs, and other immunoregulatory mechanisms in this context requires further investigation.

In our study population, DCs were associated differently with asthma in farmers’ and nonfarmers’ children, potentially indicating different asthma-related DC phenotypes in exposed and nonexposed children. The observed differences in the associations could also be explained by the heterogeneity of farming exposures, the multi-factorial clinical phenotypes of asthma, and the unexplored effects of exposures and already established asthma on DCs. Interestingly, the high expression level of CD86 on pDCs increased the risk of asthma in nonfarmers, whereas the number of CD86+ pDCs indicated protection against asthma. Overexpression of CD86, leading to increased T-cell stimulatory capacity, has been associated with asthma and allergies, but we could not determine whether the lack of cells expressing this specific marker increases the risk of asthma or whether this lack is a consequence of the disease.

We investigated circulating DCs as blood is an ethical, practical, and easily accessible sample material in a cohort study of this size. Another PASTURE subproject has assessed circulating Tregs (6) and several other studies have investigated circulating DCs (24–27). Cryopreserved cells were used in our research because of logistical reasons. Freezing, liquid nitrogen storage, and thawing of cells may have affected the phenotype or functional properties of PBMCs. Even though some previous papers have shown that freezing processes do not distort cellular immune responses (48) we suggest that associations between farm exposures, asthma and DCs need to be confirmed using fresh samples. Our results are based on small number of mDC2s, but the observed percentages of mDC2s are in accordance with literature and other studies (36, 42, 49). The strengths of this multicenter study were that all participants were characterized in detail, all flow cytometry analyses were performed in one center, and cell viability and the performance of the immunostaining were carefully tested and followed throughout the study.

Our study is one of the first studies to describe the immunology of DCs in farm and nonfarm children with and without asthma. The most interesting finding was the inverse association between farm exposure and percentage of mDC2s, suggesting that this subpopulation may play a role in farm-related immunoregulation. Although we could not conclude the role of mDC2s in asthma protection, this study offers insight into complex associations between DCs, farm exposure and asthma.

Acknowledgements

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Dendritic cells, farming and asthma

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Conflicts of interest
Authors do not have any actual or potential conflicts of interest 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 Maria-Viola contributed to data collecting, statistical analyses, manuscript preparation, and drafting. Kääriö Heidi contributed to data collecting and manuscript preparation. Karvonen Anne, Schröder Paul, Kaulek Vincent, Delphin Jean-Charles and Hirvonen Maija-Riitta contributed to manuscript preparation. Renz Harald contributed to IgE measurements and manuscript preparation. von Mutius Erika, Schaub Bianca, Pekkanen Juha and Roponen Marjut contributed to study concept and design, and manuscript preparation.

Supporting Information
Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Gating strategy for the identification of dendritic cell subsets.

Table S1. The antibodies used in immunophenotyping.

Table S2. Prevalences of exposures and outcomes stratified by center.

Table S3. Association between farm exposures and percentages of cells positive for specific marker at age 6.

Table S4. Associations between any atopy and dendritic cell (DC) variables at age 6.

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Dendritic cells, farming and asthma

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Study II

Martikainen MV, Keski-Nisula L, Jakupović H, Karvonen AM, Pekkanen J, Hirvonen MR, Roponen M.

"The lack of natural processes of delivery and neonatal intensive care treatment lead to impaired cytokine responses later in life."

American Journal of Reproductive Immunology. 2017. 77(3).

The lack of natural processes of delivery and neonatal intensive care treatment lead to impaired cytokine responses later in life

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Problem: Birth-related factors and neonatal treatments could affect the maturation of immune system and thus have lasting effects on immune responses. We investigated the effect of obstetric factors other than being born by cesarean section on immune responses later in life.

Method of Study: Regulatory, inflammatory, TH1 and TH2 cytokines, and a chemokine were analyzed in unstimulated and Toll-like receptors (TLRs) 2-, 3-, and 4-stimulated PBMCs of teenagers born by cesarean delivery (CD; N=79). Data on obstetric factors were collected from patient data archives.

Results: Advanced cervical dilatation at the time of CD associated with higher unstimulated production of cytokines compared to adolescents who were delivered before the onset of labor. Neonatal intensive care treatment associated with lower unstimulated production of cytokines. Similar associations were found following TLR stimulations.

Conclusion: The lack of natural processes of delivery and neonatal intensive care treatment might lead to long-lasting impairment of immune responses.

KEYWORDS
cesarean, innate immunity, microbes, obstetric factors, TLR

1 | INTRODUCTION

The birth by cesarean delivery (CD) has been associated with increased risk of several diseases later in life, including asthma.1-4 Less is known about the effect of obstetric factors other than being born by CD on the risk of diseases. Administration of antibiotics at delivery5 and neonatal treatment with antibiotics4 have been shown to associate with wheezing in infants and young children. Our previous study discovered that the presence of certain anaerobic and potentially pathogenic microbes in the uterine cavity at the time of birth greatly increased the later risk of asthma in children delivered by CD.7

Although epidemiological studies have linked CD and the use of antibiotics to the increased risk of asthma and wheezing, underlying immune mechanisms have not been identified. It has been speculated that the lack of direct contact with the vaginal and intestinal flora of the mother during CD as well as antibiotic administration might disturb the postnatal development of the immune system.8 One target of disruption could be innate immunity and the family of Toll-like receptors (TLRs), which have a central role in the defense against pathogens. The ability of TLRs to activate antigen-presenting cells and thus stimulate the synthesis and excretion of a broad range of molecules, including cytokines, is vital to the initiation of adaptive immune response.9 While cytokine levels in late pregnancy10 or in newborns born by CD11 have been studied, reports on the effects of other obstetric factors on the function of the immune system later are rare.

In this study, we investigated whether obstetric factors other than being born by CD associate with immune responses later in life, aiming to identify factors that may be relevant for long-term
immunomodulation. Peripheral blood mononuclear cells (PBMCs) from adolescents born by CD were stimulated with TLR ligands (TLR2, peptidoglycan (Ppg); TLR3, poly(I:C); TLR4, LPS), and excretion of regulatory (IL-2, IL-10), inflammatory (IL-1β, TNF, IL-6), Th1 (IFN-γ, IL-12p70), and Th2 (IL-4, IL-13)-associated cytokines and a chemokine (CXCL8) was measured.

2 | METHODS

2.1 | Study population

The initial study on cesarean delivery and infections (KEISARI) consisted of 805 mother-child pairs who all underwent cesarean delivery after singleton gestations at Kuopio University Hospital, Finland, between 1990 and 1992 (for details, see 7, 12, 13, and supporting information). Data on obstetric factors were collected from patient data archives.

When the children reached the age of 15-17 years, clinical examinations including skin prick tests and blood sampling were conducted. From a subsample of children (n=111), another blood sample was collected for PBMC isolation and immunological tests. A random sample of children (n=79) was selected to the present study among children with sufficient number of PBMCs and full-term birth (>37 weeks). 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 | Definition of obstetric factors

Induction of labor was defined as induction by artificial rupture of amniotic sac or by prostaglandins or oxytocin (yes vs no). The status of amniotic membranes at the time of operation was categorized as intact or ruptured. Cervical dilation was defined as centimeter of cervical dilation at the time of operation (0-1 cm, 2-5 cm, over 6 cm). The type of CD was divided into three classes (elective, emergency, and urgent).

Neonatal antibiotic treatment and neonatal intensive care unit (NICU) treatment were defined as care received (yes vs no). Neonates who required special therapy after birth (immediately after birth within first postnatal week) were treated in separate NICU instead of normal postpartum ward. NICU treatment included, for example, intravenous therapy (antibiotics, fluids, or other medication), mechanical ventilation, phototherapy, or other supportive treatment. Neonate’s individual need for NICU treatment was based on the assessment of neonatologist or pediatrician after birth.

2.3 | Amniotic fluid and intrauterine swab bacterial cultures

Amniotic fluid or intrauterine swab samples were collected as described previously. The methods and initial results have been described in detail earlier. The results of microbial uterine or amniotic fluid culture were defined as negative, a-pathogenic, and pathogenic positive cultures (for details, see supporting information).

2.4 | PBMC isolation and stimulation

Blood samples were collected from the peripheral vein into lithium heparin tubes. PBMCs were isolated as described previously and cryopreserved by suspending in 2% HI-FCS in RPMI 1640 (Invitrogen, Grand Island, NY, USA) mixed 1:1 with 15% DMSO in HI-FCS (Sigma-Aldrich, St. Louis, USA).

For functional studies, PBMCs were thawed and resuspended at 1×10^6 viable cells/mL in RPMI 1640 supplemented with 10% human AB serum (Innovative Research, Novi, MI, USA), and 1% L-glutamine and 1% antibiotic/antimycotic (both from Invitrogen). The mean percentage of viable cells after thawing was 91.2% (SD: 5.3%). PBMCs were cultured in round-bottom 96-well plates at 2.5×10^5 cells/well in duplicate in medium alone (unstimulated control), with Ppg (10 μg/mL), poly(I:C) (50 μg/mL), and LPS (0.1 μg/mL; all from Sigma-Aldrich, St. Louis, MO, USA) for 5 hours. These concentrations were chosen on the basis of preliminary dose-response experiments. Cell-free supernatants were stored in −70°C until batch analysis of cytokines.

2.5 | Cytokine measurement

Cytokines were analyzed using Meso Scale Discovery (MSD) Sector Imager™ 2400A with Discovery Workbench® 3.0.18 software. Samples were analyzed with MSD Proinflammatory Panel 1 (human) V-PLEX kit (for CXCL8, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, and TNF; MSD, Rockville, MD, USA) according to manufacturer’s instructions, using reagents provided with the kit.

The detection limit (DL) was defined for each cytokine separately. Distributions of cytokines and detection ranges are shown in Table S1. Samples with concentrations below the DL were given value corresponding to DL of the respective cytokine assay.

More than 30% of stimulated IL-4 and IFN-γ were below the lower DL and more than 30% of unstimulated CXCL8 values were above the higher DL (despite of sample dilution) and therefore these cytokine variables were dichotomized using DLs as cutoff points for further analyses. Unstimulated IL-12p70, IL-4 and IFN-γ and stimulated IL-12p70 were excluded from the analyses due to high prevalence of values below the lower DL (>80%). Stimulated CXCL8 was excluded due to high prevalence of values above the higher DL despite of sample dilution (Table S1).

2.6 | Statistics

As distributions of continuous cytokine variables (IL-1β, IL-2, IL-6, IL-10, IL-13, and TNF) deviated from normal distributions, they were log-transformed to fulfill assumptions of parametric testing. Associations between continuous cytokine variables and obstetric factors were analyzed with linear regression and reported as geometric mean ratios (GMR) and 95% confidence intervals (CIs). Associations between dichotomized cytokine variables (CXCL8, IL-4, IFN-γ) and obstetric factors were analyzed with logistic regression and reported as odds ratios (OR) and 95% CI. Other confounders, such as BMI at teenage, mother’s parity, maternal allergic diseases, paternal allergic diseases, maternal education, maternal education, maternal smoking during
TABLE 1  The prevalence of basic characteristics, obstetric variables, microbial exposure, and neonatal exposures

<table>
<thead>
<tr>
<th>Basic characteristics</th>
<th>N (%)</th>
<th>Obstetric variables</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male)</td>
<td>47 (60)</td>
<td>Section type</td>
<td></td>
</tr>
<tr>
<td>Maternal allergic diseases (yes)</td>
<td>43 (56)</td>
<td>Emergency</td>
<td>18 (23)</td>
</tr>
<tr>
<td>Maternal education</td>
<td>Urgent</td>
<td>33 (42)</td>
<td></td>
</tr>
<tr>
<td>&lt;12 years</td>
<td>29 (37)</td>
<td>Elective</td>
<td>28 (35)</td>
</tr>
<tr>
<td>&gt;12 years</td>
<td>49 (63)</td>
<td>Cervical dilatation in section</td>
<td></td>
</tr>
<tr>
<td>Previous births (yes)</td>
<td>40 (51)</td>
<td>0-1 cm</td>
<td>27 (34)</td>
</tr>
<tr>
<td>Maternal age at birth</td>
<td>2-5 cm</td>
<td>29 (37)</td>
<td></td>
</tr>
<tr>
<td>&lt;28 years</td>
<td>24 (34)</td>
<td>Over 6 cm</td>
<td>23 (29)</td>
</tr>
<tr>
<td>28-33 years</td>
<td>24 (34)</td>
<td>Induction of labor (yes)</td>
<td>17 (22)</td>
</tr>
<tr>
<td>&gt;33 years</td>
<td>22 (31)</td>
<td>Amniotic membranes (ruptured)</td>
<td>49 (62)</td>
</tr>
<tr>
<td>Antibiotics during pregnancy (yes)</td>
<td>28 (38)</td>
<td>Microbial exposure</td>
<td></td>
</tr>
<tr>
<td>Pregnancy week</td>
<td>Uterine/amniotic fluid culture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37-39</td>
<td>32 (40)</td>
<td>Negative</td>
<td>39 (65)</td>
</tr>
<tr>
<td>40-43</td>
<td>47 (60)</td>
<td>A-pathogenic</td>
<td>13 (22)</td>
</tr>
<tr>
<td>Child’s birth weight</td>
<td>Pathogenic</td>
<td>8 (13)</td>
<td></td>
</tr>
<tr>
<td>Up to 3200 g</td>
<td>23 (29)</td>
<td>Neonatal exposures</td>
<td></td>
</tr>
<tr>
<td>3200-4000 g</td>
<td>36 (46)</td>
<td>Neonatal antibiotic treatment (yes)</td>
<td>13 (17)</td>
</tr>
<tr>
<td>over 4000 g</td>
<td>20 (25)</td>
<td>Neonatal intensive care treatment (yes)</td>
<td>22 (28)</td>
</tr>
<tr>
<td>Paternal allergic diseases (yes)</td>
<td>37 (47)</td>
<td>Breastfeeding</td>
<td></td>
</tr>
<tr>
<td>Paternal education</td>
<td>&lt;3 months</td>
<td>22 (30)</td>
<td></td>
</tr>
<tr>
<td>&lt;12 years</td>
<td>42 (55)</td>
<td>4-9 months</td>
<td>31 (43)</td>
</tr>
<tr>
<td>&gt;12 years</td>
<td>34 (45)</td>
<td>&gt;9 months</td>
<td>20 (27)</td>
</tr>
</tbody>
</table>

pregnancy, number of younger and older siblings, duration of breastfeeding, and child’s birth weight, were tested in all models. However, they did not change estimates more than 10% and thus were not included in the analysis. Since obstetric factors may be highly dependent on each other, the associations between advanced cervical dilatation and neonatal intensive care treatment were adjusted with each other and with the induction of labor and neonatal antibiotic treatment in final analysis. Statistical analyses were performed using SPSS Statistics 21-software (IBM Corporation, Armonk, NY, USA). Values of P<.05 were considered statistically significant.

3 | RESULTS

3.1 | Study population

Characteristics of the study population are shown in Table 1. As inclusion criteria of KEISARI study, all studied children were born by CD (elective [35%], urgent [42%], and emergency [23%]). Twenty-eight percent of the children had NICU treatment immediately after birth, while 17% had antibiotic treatment. Data on microbial cultures at birth were available from 60 of the 79 subjects. Of them, 65% had negative cultures, whereas a-pathogenic microorganisms were detected in 22% and pathogenic microorganisms in 13% of the cultures. Mean age of studied teenagers was 15.8 (SD: 0.6). When comparing the whole population (N=111) with study population (N=79), we did not see significant differences in the prevalence of basic characteristics, obstetric variables, microbial exposure, or neonatal exposures (data not shown).

3.2 | Indicators of natural progress of childbirth associate with spontaneous and TLR-stimulated cytokine production

Advanced cervical dilatation associated with higher cytokine secretion. If the mother had entered the active phase (cervical dilatation of 6 cm or more) of labor before the time of operation, increased spontaneous production of IL-2, inflammatory, and TH2 cytokines (Figure 1A) and a CXCL8 (OR: 3.75, 95% CI: 1.16-12.12) was observed at teenage. Similar associations were seen between cervical dilatation and TLR4-stimulated inflammatory and TH2 (Figure 1A)- and TH1-related cytokines (IFN-γ, TNF, IL-12, IL-6, IL-10).

![Figure 1](attachment:image.png)
OR: 4.58, 95% CI: 1.09–19.27). Similarly, albeit not always as significant, associations were also seen in TLR2 and TLR3 stimulations (Fig. S1A).

Induction of labor was associated with the lower spontaneous production of inflammatory cytokines but not with TLR4-stimulated (Figure 1B) or TLR2- and TLR3-stimulated production of cytokines (Fig. S1B).

The rupture of amniotic membranes before operation associated with higher unstimulated production of CXCL8 (OR: 3.11, 95% CI: 1.19–8.16) and as a trend with IL-1β (Table 2), higher TLR4-induced TNF (Table 2), higher TLR2-induced IFN-γ (OR: 2.88 95% CI: 1.12–7.41), and lower TLR3-induced IL-4 cytokine production (OR: 0.43 95% CI: 0.15–1.18).

The type of CD (elective, emergency, and urgent) did not associate with spontaneous or stimulated cytokine production at teenage (data not shown).

3.3 | Neonatal intensive care treatment associates with lower cytokine response

Adolescents who had received NICU treatment at birth had lower spontaneous production of all studied cytokines (Figure 2A) and CXCL8 (OR: 0.32, 95% CI: 0.11–0.92). Similar associations were observed between NICU treatment and TLR4-stimulated cytokine production (Figure 2A) as well as following TLR2 and TLR3 stimulation (Fig. S2A). Neonatal antibiotic treatment did not associate with spontaneous or TLR4-induced cytokine production (Figures 2B and S2B).

3.4 | Association of microbial exposure and cytokine secretion

The intrauterine microbial growth did not associate significantly with spontaneous or TLR4-stimulated cytokine secretion (Figure 3). In TLR2 stimulation, elevated IL-13 (TH2-related cytokine) was associated with the presence of pathogenic microbes in intrauterine cultures at birth (Fig. S3B).

3.5 | Cervical dilatation and neonatal intensive care treatment were independent predictors of cytokine production

The most consistent associations were found between cervical dilatation and NICU treatment and cytokines. After adjustment with other obstetric factors, advanced cervical dilatation associated with higher unstimulated production of inflammatory and TH2-related cytokines, and CXCL8. NICU treatment associated with lower production of regulatory, inflammatory, and TH2-related cytokines (Table 3), similar, albeit not always as strong associations were seen after TLR stimulations (Tables S3 and S4).

4 | DISCUSSION

Although many studies have shown that the birth by cesarean section is associated with several diseases later in life,14-15 studies observing the effect of CD and related factors on the immune system are rare and often focus on neonates or first years of life.16-18 It is also often ignored that not all CDs are similar: The progression of birth (e.g., release of hormones driving the birth process, cervical dilatation, rupture of amniotic membranes) and early microbial exposure may vary in different CDs. As emergency or urgent cesarean deliveries may occur after the onset of labor, factors affecting neonatal immune system may be

**TABLE 2** Associations between ruptured amniotic membranes at the time of cesarean delivery and spontaneous and TLR4-induced cytokine secretion at teenage

<table>
<thead>
<tr>
<th>Ruptured membranes</th>
<th>Regulatory, GMR (95% CI)</th>
<th>Inflammatory, GMR (95% CI)</th>
<th>Th2, GMR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-10</td>
<td>IL-2</td>
<td>IL-1β</td>
</tr>
<tr>
<td>Control</td>
<td>49</td>
<td>0.78 (0.48–1.26)</td>
<td>1.20 (0.81–1.79)</td>
</tr>
<tr>
<td>TLR4</td>
<td>49</td>
<td>1.24 (0.74–2.08)</td>
<td>1.17 (0.76–1.81)</td>
</tr>
</tbody>
</table>

GMR, geometric mean ratio with 95% confidence interval (CI), calculated using linear regression.

Reference group: amniotic membranes intact at the time of cesarean delivery (N=30), P-values <.1 bolded.

**FIGURE 2** The effect of (A) neonatal intensive care treatment (N=22) and (B) neonatal antibiotic treatment (N=13) on spontaneous and TLR4-induced cytokine secretion at teenage. Figures show geometric mean ratios (GMR) with 95% confidence interval (CI), calculated using linear regression. Reference group: (A) no neonatal intensive care treatment (N=57), (B) no neonatal antibiotic treatment (N=66)
similar to those operating during vaginal birth. Our study is the first one showing that obstetric factors, intrauterine microbial exposure, and neonatal treatment may have long-term effects on the function of the immune system in children born by CD. We observed that spontaneous and TLR-stimulated cytokine release was increased among teenagers whose mothers had entered the active phase of the labor before CD. In contrast, neonatal intensive care treatment associated with lower immune responses later in life. Some indications on the associations between microbial exposure in utero and innate immune responses were also discovered.

Cervical dilatation of ≥6 cm indicates a transition of the labor to the active phase. It is thought that at this active phase mother's body is preparing the child for the outside world by releasing several molecules, aiming to wake the child's own immune system. Interestingly, we observed more pronounced spontaneous and also TLR-stimulated cytokine release in teenagers whose mothers had entered the active phase of labor before CD. In our previous study, increased spontaneous production of cytokines observed in 4.5-year-old children living in farm environment was considered as a marker of a healthy immune development, as farm children are less likely to suffer from atopic diseases than non-farmers. Cervical dilatation together with the rupture of the membranes allows the colonization of the amniotic fluid with microbes, especially in the case of extended labor. In our study, however, associations between cytokines and cervical dilatation were independent from the intrauterine microbial findings (data not shown), suggesting that cervical dilatation per se plays a role in induction of child's immune maturation. Microbial cultures were, however, performed over 20-25 years ago, thus limiting the reliability of the negative culture results. Maternal use of antibiotics during pregnancy may also alter the microbiota of the fetus and influence the immune development of the child. In our study, it did not, however, associate with cytokine responses at adolescence (data not shown).

Induction of birth could be considered as "artificial" in vaginal birth. In CD, however, induction of birth may introduce some "normal" components to the birth process, as compared to pre-planned, elective CD where labor has not started at the time of CD. Although cervical dilatation and induction of birth can be considered to be correlated among CDs, in our study, they did not associate similarly with cytokines. Unfortunately, we did not separate the type of induction more specifically. It would be interesting to evaluate whether there were detectable differences in immunity between those whose mothers were treated by oxytocin or prostaglandin products, which are strong inflammatory mediators, and those who underwent only amniotomy.

In contrast to elevated cytokine levels, we observed lower spontaneous and TLR-stimulated production of cytokines in teenagers who had been treated in NICU after birth. This may be due to already-impaired immune defense at birth or, alternatively, factors related to NICU treatment, for example, the lack of exposures during the first days, which could have led to the attenuated immunity later in life. NICU treatment could be related to lower extent of maternal skin contact, delayed start of breastfeeding, and the exposure to the hospital environment. Interestingly, similar associations were not observed in children treated with antibiotics after birth.

When interpreting our results, it is important to take into account possible correlations between studied obstetric factors. That is, advanced cervical dilatation by the time of section may indicate that there has been a need for emergency cesarean delivery. This may have led to the treatment of an infant in NICU, potentially with antibiotics. It is noteworthy that advanced cervical dilatation and neonatal intensive care treatment were independent predictors of cytokine secretion in adolescence. This suggests that they may have an important role in early life immunomodulation, potentially with lasting effects up to early adulthood.

All studied teenagers were born by CD; that is, we could not contrast observed immune responses to those of vaginally delivered children. Our results, however, indicate that the lack of natural processes of delivery and neonatal treatments may lead to impaired cytokine responses. Children with decreased cytokine response to TLR stimuli may, furthermore, have altogether compromised response to external pathogenic stimuli and are thus in greater risk of infectious diseases. Since several studies have reported that children born by CD have increased risk of health problems, such as asthma, juvenile arthritis, immune deficiencies, and leukaemia, and as the prevalence of CD exceeds the recommendations by World Health Organization, we should take action to decrease the number of CDs performed upon request without a medical reason. Our results suggest that the long-term effect of not only CD but also other obstetric factors on children's health and immune system should be researched further.

Our study is the first one investigating associations between intrauterine microbial growth at birth and immune responses at adolescence. Although pathogenic bacteria cultures associated with higher risk of

**FIGURE 3** Associations between detected (A) a-pathogenic (N=13) and (B) pathogenic (N=8) microbes in intrauterine samples at the time of cesarean delivery and spontaneous and TLR4-induced cytokine secretion at teenage. Figures show geometric mean ratios (GMR) with 95% confidence interval (CI), calculated using linear regression. Reference group: (A and B), no detected microbes in intrauterine samples (N=39).
<table>
<thead>
<tr>
<th>Table 3: Unadjusted and adjusted associations between obstetric factors and spontaneous cytokine secretion at term.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Obstetric Factors</strong></td>
</tr>
<tr>
<td>Advanced cervical dilation (cm)</td>
</tr>
<tr>
<td>Unadjusted</td>
</tr>
<tr>
<td>Adjusted</td>
</tr>
<tr>
<td>Neonatal ICU treatment</td>
</tr>
<tr>
<td>Unadjusted</td>
</tr>
<tr>
<td>Adjusted</td>
</tr>
</tbody>
</table>

**Note:** GMR = geometric mean ratio with 95% confidence interval. aGMR = adjusted GMR. BMI = body mass index. CD = cytokine density. TLR = toll-like receptor. UTI = urinary tract infection. IQR = interquartile range. ECM = extracellular matrix. OR = odds ratio. 95% CI = 95% confidence interval. P-value = statistical significance level. " = P-value < .05. * = P-value < .01. ** = P-value < .001. "*" = P-value < .0001.

Unadjusted and adjusted associations between obstetric factors and spontaneous cytokine secretion at term. Values are geometric mean ratios with 95% confidence intervals. aGMR = adjusted GMR.

CD-related factors did not associate with specific TLRs, but with innate immune system and cytokine secretion more generally. It is noteworthy that the levels of cytokines correlated strongly, especially following TLR stimulation (Table S5), which needs to be considered when interpreting results. Comparison of cytokine responses measured in different studies is also challenging as cytokine responses and TLR signaling are age dependent.24,25 We tested a number of potential confounders (eg, maternal use of antibiotics and smoking during the pregnancy, number of siblings, BMI, and exercise at teen age, pets, and living conditions), but they did not interfere with our results. One should keep in mind, however, the long period of time between the incident of interest (birth) and the measurement of the outcome (cytokine responses at adolescence), meaning that there might be a number of unknown factors that could have affected the obtained results.

The strength of this study is a unique study design, which includes data on intrauterine growth of microorganisms by the time of birth and a long-term follow-up at adolescence. Analyses of an extensive cytokine pattern were made in one batch, using a highly sensitive and validated method. PBMCs could only be obtained from a small number of participants as compared to the original sample size of KEISARI study. Our data, however, suggest that the effect of obstetric factors on immune responses later in life should be studied further in larger populations. It is also essential to investigate which obstetric factors determine immune responses in vaginally delivered children and how they compare to our results.

In summary, we observed increased immune responses in teenagers whose mothers had entered the active phase of the delivery by the time of the CD, whereas ICU treatment at birth associated with lower responses later in life. Our novel data suggest that the lack of natural processes of delivery and neonatal intensive care treatment leads to impaired cytokine responses later in life. Some indications on the associations between microbial exposure in utero and innate immune responses were also discovered. The effect of obstetric factors on children’s health and immune system should be researched further among children born by CD but also in vaginally delivered children. Obtained data from these studies could be used as the risk assessments for the obstetric decisions made during the delivery and in neonatal phase.

**Acknowledgments**

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CONFLICT OF INTERESTS
Authors do not have any potential, perceived, or real conflict of interests that could inappropriately influence their work. The sponsors of this study had no involvement in study design; the collection, analysis, and interpretation of data; the writing of the report; and the decision to submit the manuscript for publication.

AUTHOR CONTRIBUTIONS
All authors approved the submitted version. Martikainen Maria-Viola contributed to data collection, statistical analyses, manuscript preparation, and drafting. Jakupović Hermina contributed to data collection and quality control. Karvonen Anne and Hirvonen Maija-Riitta contributed to manuscript preparation. Pekkanen Juha, Keski-Nisula Lea, and Roponen Marjut contributed to study concept and design, and manuscript preparation.

REFERENCES

SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

Study III


“Integrating farm dust and urban air PM studies in search for human immunoregulatory mechanisms operating in protective and high-risk environments.”

[Epub ahead of print]

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

**Running title:** Environment and immunoregulation

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\textbf{e} School of the Environment, Nanjing University, Nanjing, China \\
\textbf{f} Department of Paediatrics, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong, China. \\
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Integrating farm and air pollution studies in search for immunoregulatory mechanisms operating in protective and high-risk environments

Pediatr Allergy Immunol

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

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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.

CONFLICT OF INTEREST STATEMENT
Authors do not have any actual or potential conflicts of interest including any financial, personal or other relationships that could inappropriately influence, or be perceived to influence, their work.

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 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 were analysed in detail.

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 6/18 were atopic (specific IgE against any studied allergen >3.5IU/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

PBMCs 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

PBMCs 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, ThermoFisher) to the concentration of 1 x10^6 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% CO₂ 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 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 (0.01mg/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 was analysed using Meso Scale Discovery (MSD) Sector Imager™ 2400A with Discovery Workbench® 3.0.18 software. Detailed information of analyses are in Supplementary (See Supplementary 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 are in Supplementary (See Supplementary p. 3) and in Jalava et al. (13).

PM 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 using16S rRNA gene amplicon sequencing. The detailed information of analyses are in Supplementary (See Supplementary p. 4).

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2.6 Statistical analyses

The data from immunophenotyping (cell variables (DC and monocyte)) was expressed as percentages of cells positive for specific markers, and data from cytokine measurements as concentrations of cytokines (pg/ml). Data on PAH contents was 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 non-parametric 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 (Fig. 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$_{2.5}$ 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 comparison. 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 (See Supplementary; Fig. 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 comparison (Fig 2.). Urban PM$_{1-0.2}$ 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 (See Supplementary; Fig. 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$_{1-0.2}$ stimulation and weakest after farm dust stimulation (See Supplementary; 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$_{1-0.2}$ and PM$_{0.2}$ induced significantly different expression of receptors and production of cytokines compared to PM$_{2.5-1}$ while effects induced by PM$_{0.2}$ did not differ very much from those induced by PM$_{1-0.2}$ (Table 1).

3.4 Composition of the samples

There were some variations in the ionic and elemental compositions of the environmental samples (See Supplementary; 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$_{0.2}$ (See Supplementary; 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. (See Supplementary; Fig. 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 i.e. 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 co-operating 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 e.g. 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 homeostasis 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 non-farming children (12), suggesting that conclusions about cell function cannot be based solely on markers as immune responses are multi-layered.

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 non-farm 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 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$_{2.5}$. 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$_{1-0.2}$ 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 of 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.

5. ACKNOWLEDGEMENTS
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REFERENCES

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FIGURE LEGENDS

Fig. 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, horizontal line indicates the median. Significances were calculated using non-parametric Wilcoxon Signed rank test. *= P-value<0.05, compared to control, **= P-value < 0.01, compared to control. Ψ p-value < 0.05 after adjustment for multiple comparisons with Bonferroni correction.

Fig. 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, horizontal line indicates the median. Significances were calculated using non-parametric Wilcoxon Signed rank test. *= P-value <0.05, compared to control, **= P value < 0.01, compared to control. Ψ p-value < 0.05 after adjustment for multiple comparisons with Bonferroni correction.
Table 1. Cross-associations between immune responses induced by different PM size-fractions

<table>
<thead>
<tr>
<th>Cross-associations</th>
<th>PM&lt;sub&gt;0.2&lt;/sub&gt; vs PM&lt;sub&gt;1-0.2&lt;/sub&gt;</th>
<th>PM&lt;sub&gt;0.2&lt;/sub&gt; vs PM&lt;sub&gt;2.5-1&lt;/sub&gt;</th>
<th>PM&lt;sub&gt;1-0.2&lt;/sub&gt; vs PM&lt;sub&gt;2.5-1&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
</tr>
<tr>
<td>Cell subsets</td>
<td></td>
<td></td>
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<tr>
<td>CD80+ monocytes (%)</td>
<td>0.756</td>
<td>0.002†</td>
<td>0.001†</td>
</tr>
<tr>
<td>CD80+ pDCs (%)</td>
<td>0.346</td>
<td>0.224</td>
<td>0.087</td>
</tr>
<tr>
<td>CD80+ mDCs (%)</td>
<td>0.433</td>
<td>0.015</td>
<td>0.009</td>
</tr>
<tr>
<td>ILT4+ monocytes (%)</td>
<td>0.005</td>
<td>0.041</td>
<td>0.001†</td>
</tr>
<tr>
<td>ILT4+ pDCs (%)</td>
<td>0.53</td>
<td>0.48</td>
<td>0.033</td>
</tr>
<tr>
<td>ILT4+ mDCs (%)</td>
<td>0.814</td>
<td>0.433</td>
<td>0.221</td>
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<td>Cytokines</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IL-1β</td>
<td>0.069</td>
<td>0.001†</td>
<td>0.000†</td>
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<td>IL-2</td>
<td>0.019</td>
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<td>0.001†</td>
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<td>0.003†</td>
<td>0.001†</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.004</td>
<td>0.001†</td>
<td>0.000†</td>
</tr>
<tr>
<td>IL-12/IL-23p40</td>
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<td>0.001†</td>
<td>0.000†</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.009</td>
<td>0.002†</td>
<td>0.001†</td>
</tr>
<tr>
<td>IL-17A</td>
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<td>0.005</td>
<td>0.000†</td>
</tr>
<tr>
<td>IFN-γ</td>
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<td>0.008</td>
<td>0.000†</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.001†</td>
<td>0.006</td>
<td>0.000†</td>
</tr>
</tbody>
</table>

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
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, horizontal line indicates the median. Significances were calculated using non-parametric Wilcoxon Signed rank test. *= P-value<0.05, compared to control, **= P-value < 0.01, compared to control. Ψ p-value < 0.05 after adjustment for multiple comparisons with Bonferroni correction.
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Although there is strong evidence that different exposures early in life can alter the risk of allergic diseases, the underlying mechanisms are unclear and the development of preventive strategies has been delayed. The present thesis demonstrates the impact of protective and harmful exposures on children’s immune status ex vivo and in vitro. Some changes in immune responses were observable up to teenage. This dissertation advances the knowledge of the relationship between early life exposures, immune development and the risk of allergic diseases.