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RESEARCH

Mikko Lappalainen

Environmental Microbes and Immunological Development in Children

– The Role of Animal, Bacterial and Fungal Exposures

43

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Mikko Lappalainen

Environmental Microbes and Immunological Development in Children

-The Role of Animal, Bacterial and Fungal Exposures

ACADEMIC DISSERTATION

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ABSTRACT

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While the immune system adapts to respond to external antigens, it goes through a major maturation process during the first years of life, though the process actually starts before birth. During early infancy, also the most prevalent chronic diseases in childhood, asthma and allergies begin to develop. Even though the knowledge concerning these complex immunological phenomena has increased during recent decades, different steps and the causal components affecting these processes are still largely unidentified. Lately, increasing interest has been focused on environmental microbial exposures and animal contacts which appear to play one of the key roles not only in immunologic development but also in the manifestation of the allergic disorders.

The overall aim of the thesis was to elucidate the influence of environmental microbial and animal exposures on the development of immune functions during the first year of life. In addition, the contribution of birth-related factors on neonatal immune responses was assessed.

The research was based on two birth cohort studies (MAA and LUKAS). The MAA study consisted of 29 mother-child pairs (deliveries in 2000-2001). Participants (442 mother-child pairs) in the LUKAS cohort were recruited in two phases (LUKAS1 and LUKAS2) during three years (deliveries in 2002-2005). Blood samples were collected from both mothers and their children at birth, at 3 months (MAA) and at 1 year (LUKAS) after birth. Blood samples were stimulated with three different compounds and analyzed for immunological markers, called cytokines, as ways of evaluating the function of immune system. Indoor microbial exposure was characterized by measuring the levels of different bacterial and fungal species as well as chemical markers (describing the presence of microbes) from house dust samples (floor, bed and dust bag of the vacuum cleaner) using qPCR and GC-MS-MS -methods. Keeping animals and/or contacts and other potential immunomodulatory factors were surveyed by interviews and self-administered questionnaires.

Some perinatal determinants of cord blood cytokine responses were identified. The season of the birth had effects on neonatal cytokine production: the lowest levels of IL-5, IL-10 and IFN-γ were detected at the spring. Decreased levels of IL-10 and IFN-γ were measured after induction of labour with prostaglandins and in children with low birth weight (IL-10 only). Male gender of the child was associated with increased IL-5 responses, whereas maternal smoking during pregnancy

decreased IL-5 levels. In general, the cytokine -producing capacity of the newborns increased from birth to 1 year of age, though it still remained clearly weaker than maternal responses. Nevertheless, significant mother-to-child cytokine correlations were observed already at 3 months after birth.

During the first year of life, immune responses seemed to be affected by animal and indoor dust microbial exposures. Children of dog owners expressed decreased TNF-α responses at birth and at the age of 1 year compared to non-owners' children. No associations were detected between cat ownership and immune responses. At the age 3 months, a high concentration of the chemical marker for Gram-negative bacteria (3-hydroxy fatty acids, carbon chain $C_{10:0}$-$C_{14:0}$) in bed dust was associated with decreased production of one proinflammatory cytokine (TNF-α). In contrast, levels of the Gram-positive bacterial marker (muramic acid) in floor dust were associated with increased TNF-α and IL-6 responses. At the age of 1 year, Gram-positive bacterial exposure in general and the levels of the genus *Mycobacterium* in floor dust were associated with decreased Th1-type (IFN-γ) cytokine responses. Fungal exposure did not appear to have consistent influence on immune responses at birth, 3 months or 1 year after birth.

The results of this thesis indicate that even though neonatal cytokine responses strengthen during the first year of life, they still remain weaker than adult responses. Specific birth-related factors as well as maternal and neonatal characteristics seem to be associated with neonatal cytokine production. Continuous contact with dogs appears to be of importance in the development of the immune system. Also environmental microbial exposures have influence on immunologic maturation during the first year of life. In general, intensive exposure to microbes, especially to bacteria, appears to decrease cytokine responses in infancy. Microbes, however, may also have bidirectional immunomodulatory effects, i.e., the same exposure agent/s can either induce or inhibit immunological functions possibly depending on the developmental stage of the immune system.

In summary, the decreased immune responses in children after intensive dog and non-pathogenic microbial exposures may be related to the adaptation undergone by the immune system as it faces environmental antigens. The present search for the factors affecting the development of the neonatal immune system may offer new insights also for the future work aiming at prevention of allergies.

Key Words: Bacteria, birth, cat, chemical marker, cohort, cord blood, cytokine, dog, exposure, fungi, immune system, infant, microbe, stimulation, whole blood

TIIVISTELMÄ

Mikko Lappalainen. Environmental Microbes and Immunological Development in Children – The Role of Animal, Bacterial and Fungal Exposures [Elinympäristön eläin- ja mikrobialtisteiden merkitys lapsen immunologiseen kehitykseen]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 43. 156 sivua. Helsinki 2010.
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Ensimmäisien elinvuosien aikana, jopa jo ennen syntymää, lapsen immuunijärjestelmä käy läpi valtavan kypsymisprosessin, jolloin se oppii reagoimaan vierasaineita vastaan. Nykytietämyksen mukaan samoihin aikoihin saavat alkunsa myös astma ja allergiat, lapsuuden yleisimmät krooniset sairaudet. Vaikka tietämys näistä monimutkaisista immunologisista prosesseista on lisääntynyt viime vuosikymmeninä, eri vaiheet ja niihin vaikuttavat tekijät ovat kuitenkin edelleen laajalti tuntemattomat. Viimeaikoina kasvava mielenkiinto on kohdistunut erityisesti elinympäristöstä saatavaan mikrobi- ja eläinaltistukseen, jota pidetään merkittävänä tekijänä paitsi normaalin immunologisen kehityksen myös allergisten sairauksien puhkeamisen kannalta.

Tutkimuksen päätavoitteena oli selvittää elinympäristöstä peräisin olevan mikrobi- ja eläinaltistuksen vaikutusta lapsen immunologiseen kehitykseen ensimmäisen elinvuoden aikana. Lisäksi arvioitiin synnytykseen liittyvien tapahtumien vaikutusta vastasyntyneen immuunivasteisiin.

Väitöskirjatutkimus koostui osasta kahden syntymäkohorttitutkimuksen aineistoa (MAA ja LUKAS). MAA kohorttiin rekrytoitiin 29 äiti-lapsi paria (synnytykset 2000-2001). LUKAS tutkimukseen kutsuttiin kahdessa osassa (LUKAS1 ja LUKAS2) kolmen vuoden aikana (synnytykset 2002-2005) yhteensä 442 äiti-lapsi paria. Lapsista ja äideistä kerättiin verinäytteet syntymän yhteydessä sekä 3 kuukauden (MAA) ja 1 vuoden kuluttua (LUKAS) synnytyksestä. Verinäytteet stimuloitiin kolmella erityyppisellä yhdisteellä. Stimuloiduista verinäytteistä analysoitiin immuunijärjestelmän toimintaa kuvaavia merkkiaineita, sytokiineja. Sisätiloissa tapahtuva mikrobialtistuminen arvioitiin mittaamalla eri bakteeri- ja sienilajien sekä niiden läsnäoloa kuvaavien merkkiaineiden pitoisuuksia tutkimuskodeista kerätyistä pölynäytteistä (lattia, sänky ja imurin pölypussi) kahdella eri määritysmenetelmällä (qPCR ja GC-MS-MS). Haastatteluiden ja kyselylomakkeiden avulla selvitettiin altistuminen koti- ja tuotantoeläimille sekä muita immunologiseen kehitykseen mahdollisesti vaikuttavia tekijöitä.

Tietyt äidin ja lapsen ominaisuudet sekä syntymänläheiset tekijät olivat yhteydessä napaveren sytokiinivasteisiin. Syntymävuodenajalla oli vaikutusta vastasyntyneen sytokiinipitoisuuksiin: keväällä syntyneillä IL-5, IL-10 ja IFN-γ vasteet olivat matalimmat. Raskauden käynnistys prostaglandiineilla laski IL-10 ja IFN-γ tasoja. Myös lapsen matala syntymäpaino oli yhteydessä alentuneeseen IL-10

tuotantoon. Poikalapsilla IL-5 vasteet olivat korkeammat kuin tytöillä. Äidin raskausaikaisella tupakoinnilla oli laskeva vaikutus IL-5 tuotantoon. Vastasyntyneiden sytokiinituotantokyvyn havaittiin pääsääntöisesti voimistuvan syntymästä yhden vuoden ikään, mutta olevan edelleen selvästi heikompi kuin äideillä. Tästä huolimatta äidin ja lapsen sytokiinivasteet korreloivat jo 3 kuukautta synnytyksen jälkeen.

Ensimmäisen elinvuoden aikana lapsen verestä mitatuissa stimuloiduissa sytokiinivasteissa todettiin eroja eläin- ja mikrobialtistumisen suhteen. Koiran omistajien lapsilla havaittiin alentuneet TNF-α vasteet sekä syntymässä, että 1 vuoden iässä verrattuna lapsiin, joiden kodissa ei ollut koiraa. Kissa-altistumisella ei todettu olevan vaikutusta lasten immuunivasteisiin. Gram-negatiivisia bakteereja kuvaavan merkkiaineen (3-hydroksirasvahappo, hiiliketjut $C_{10:0}$-$C_{14:0}$) suuri määrä sänkypölyssä oli yhteydessä alentuneeseen tulehduksenvälittäjäaine (TNF-α) tuotantoon 3 kuukauden iässä. Sitä vastoin kasvavalla lattiapölyn Gram-positiivisen bakteerimerkkiaineen (muramiinihappo) määrällä oli TNF-α ja IL-6 tuotantoa nostava vaikutus. Lattiapölyn *Mycobacterium*-lajien pitoisuus sekä Gram-positiivinen bakteerialtistus ylipäänsä olivat yhteydessä alentuneeseen Th1-tyypin (IFN-γ) sytokiinituotantoon 1 vuoden iässä. Sienialtistus ei ollut johdonmukaisesti yhteydessä lapsen immuunivasteisiin syntymässä, 3 kuukauden tai 1 vuoden iässä.

Väitöskirjatutkimus osoitti, että vaikka lapsen immuunivasteet voimistuvat selvästi ensimmäisen elinvuoden aikana, ne jäävät silti vaimeammaksi kuin aikuisen vasteet. Tietyt synnytykseen liittyvät tekijät sekä äidin ja lapsen ominaisuudet ovat yhteydessä vastasyntyneen sytokiinituotantoon. Lisäksi tulokset viittaavat siihen, että toistuva koirakontakti ja elinympäristöstä saatava mikrobialtistus vaikuttavat lapsen kehittyviin immuunitoimintoihin ensimmäisen elinvuoden aikana. Pääsääntöisesti intensiivinen altistuminen mikrobeille, erityisesti bakteereille, näyttäisi laskevan varhaislapsuuden immuunivasteita. Mikrobeilla voi myös olla kaksisuuntaisia immuunijärjestelmän toimintaa muokkaavia vaikutuksia, koska sama altiste(et) saattaa joko tehostaa tai vaimentaa immuunitoimintoja, riippuen mahdollisesti immuunijärjestelmän kehittymisvaiheesta.

Runsaan ei-patogeenisen mikrobi- ja koira-altistuksen yhteydessä lapsilla havaitut alentuneet immuunivasteet saattavat viitata siihen, että immuunijärjestelmä on tottunut kohtaamaan ympäristön antigeeneja. Tämä immunologiseen kehitykseen vaikuttavia tekijöitä selvittänyt väitöskirjatutkimus voi osaltaan edistää myös allergioiden ehkäisyyn pyrkivää tutkimustyötä.

Asiasanat: Altistuminen, bakteeri, immuunijärjestelmä, kemiallinen merkkiaine, kissa, kohortti, koira, kokoveri, mikrobi, napaveri, sieni, stimulaatio, syntymä, sytokiinit, vastasyntynyt

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II Lappalainen M, Roponen M, Pekkanen J, Huttunen K, Hirvonen M-R. 2009. Maturation of cytokine –producing capacity from birth to 1 yr of age. Pediatric Allergy and Immunology 20: 714-25.

III Lappalainen MHJ, Huttunen K, Roponen M, Remes S, Hirvonen M-R, Pekkanen J. 2010. Exposure to dogs is associated with a decreased tumour necrosis factor-α –producing capacity in early life. Clinical and Experimental Allergy 40: 1498-506.

IV Lappalainen MHJ, Roponen M, Hyvärinen A, Nevalainen A, Laine O, Pekkanen J, Hirvonen M-R. 2008. Exposure to environmental bacteria may have differing effects on tumour necrosis factor -α and interleukin-6 –producing capacity in infancy. Clinical and Experimental Allergy 38:1483-92.

V Lappalainen MHJ, Hyvärinen A, Hirvonen M-R, Rintala H, Roivainen J, Renz H, Pfefferle PI, Nevalainen A, Roponen M, Pekkanen J. High indoor microbial levels are associated with reduced Th1 –cytokine secretion capacity in infancy. Submitted

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ABBREVIATIONS

APC	Antigen-presenting cell
CBMC	Cord blood mononuclear cell
CD	Cluster of differentiation
ConA	Concanavalin A
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPS	Extracellular polysaccharide
GC-MS-MS	Gas chromatography tandem mass spectrometry
FBS	Fetal bovine serum
GI	Gastrointestinal
CTL	Cytotoxic T-cell
HDL	High density lipoprotein
Ig	Immunoglobulin
IL	Interleukin
IFN-γ	Interferon gamma
LAL	*Limulus* Amebocyte Lysate assay
LPS	Lipopolysaccharide
LBP	LPS-binding protein
LUKAS	Lapsuuden kasvuympäristö ja allergiat -study
MAA	Mikrobialtistus ja allergiat -study
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PASTURE	Protection against Allergy -Study in Rural Environments
PBMC	Peripheral blood mononuclear cell
pg/ml	Picograms per millilitre
pg/10^6 WBC	Picograms per one million white blood cells
PMA	Phorbol 12-myristate 13-acetate

PRR	Pattern-recognition receptor
P/I	Combination of PMA and ionomycin
qPCR	Quantitative real time polymerase chain reaction
RPMI	Roswell Park Memorial Institute -medium
SEB	Staphylococcal enterotoxin B
TGF-β	Transforming Growth Factor-β
Th	T-helper lymphocyte
THL	National Institute for Health and Welfare
Treg	T-regulatory lymphocyte
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
WAO	World Allergy Organization
WBC	White blood cell
3-OH FAs	3-hydroxy fatty acids

1 INTRODUCTION

Our understanding of the development of human immune system has increased greatly during the past decades. Events occurring during pregnancy and at the time of birth have great significance, but especially the first years of life are the time of rapid maturation. During this period, the immune system encounters a wide range of different environmental challenges, many not faced during the fetal period. The response patterns against these antigens experienced in childhood may persist into adulthood. The search for causative factors and relevant mechanisms of the immune development have been under intensive investigations not only because they are still poorly understood, but also due to the fact that the most prevalent chronic diseases in childhood, i.e., asthma and allergies, are clearly immunological disorders originating also at the early stages of life. Their development is governed by the same kinds of exposure factors, which affect the immune maturation in general (Holt and Jones 2000; Jones et al. 2000; Warner et al. 2000, 2004).

The so-called hygiene hypothesis was created to offer an explanation for the increased prevalence of allergies in the Western world. It proposed that contact with an elder sibling and infections in childhood could offer protection against the development of allergic diseases (Strachan 1989). Later, the scope of the initial idea was expanded by speculating that the environmental microbial burden in general would play an important role, not the infections themselves (Martinez 2001; Martinez and Holt 1999).

The immunomodulatory capability of different microbial agents has been characterized extensively in many experimental studies. Nevertheless, these investigations have not been able to clarify totally the complex cascade between immune responses and different microbial exposures that humans encounter in their everyday lives. This dissertation aims to increase our understanding of these associations by adopting a multidisciplinary approach, using epidemiological birth cohorts along with microbiological and immunological methods. There is only a limited number of previous publications in this rather novel area of research. In addition, these reports have focused primarily on the exposure to a single microbial component, endotoxin. However, indoor environments include several other potential immunomodulatory microbial agents, and their effects on early immune development are investigated in this thesis.

2 REVIEW OF THE LITERATURE

2.1 Background-Hygiene hypothesis

Modern research in the field of allergic disorders encountered an important milestone about 20 years ago, when it was noticed that children with elder siblings experienced less hay fever in adulthood than firstborn children (Strachan 1989). The concept of the hygiene hypothesis, which included urbanization with improved hygienic conditions, smaller family size and the decrease in childhood infections, was offered as an explanation for the dramatic increase in the prevalence of allergies in Western societies. However, this hypothesis has also faced opposition, especially about the putative protective effect of childhood infections, because infections can also be harmful to health (Björkstén 2009; Platts-Mills et al. 2005). Nonetheless, the original finding concerning the protective effect of the elder siblings has still remained as one of the major explanatory factors (Strachan 2000). The hygiene hypothesis received support from studies showing that children growing up in rural environments, especially on farms, experience less atopic diseases than their counterparts living in urban environments (von Mutius 2007). These studies also highlighted the importance of the early exposure to non-pathogenic microbes in conferring protection against childhood allergies (Braun-Fahrländer et al. 2002; von Mutius et al. 2000a). From the immunological point of view, the initial interpretation of the hygiene hypothesis included the balance between two subpopulations of T-helper cells (Th1 vs Th2). It was speculated that increased microbial exposures could drive the development of immune system towards non-allergic Th1-type responses (Martinez and Holt 1999). Briefly, in view of the impressive progress in immunologic research in tandem with the findings in the field of microbiology and epidemiology, it now appears that the original idea of hygiene hypothesis relying primarily to the major importance of infections may have been oversimplified, possibly even misleading (Björkstén 2009; Platts-Mills et al. 2005). Nowadays the “modified” hygiene hypothesis includes three main tenets: 1) it is not only viral, bacterial and helminthic infections, but also 2) environmental microbial exposures and 3) their effects on both innate and adaptive immune responses (Schaub et al. 2006; von Mutius 2007). Thus modified hypothesis emphasizes the significant role of the non-pathogenic microbial exposures in modulating the functions of the immature immune system (Martinez 2001). However, the causal microbial species / groups, their quantity, as well as the importance of the timing and duration of the exposure are not well known.

2.2 Immune system

The immune system has two primary functions: 1) defense reactions targeted against foreign substances including microbes, toxins and allergens as well as different proteins and polysaccharides regardless of their pathogenicity 2) ability to distinguish self (=cells, tissues, organs of the host) from non-self. In order to achieve these goals, the immune system possesses several regulatory and detection mechanisms and responses, which can be divided into two co-operationally working parts: innate immunity (called also natural or native immunity) and adaptive immunity (called also acquired immunity). The immune system includes many lymphoid organs, which are classified as primary or secondary organs / tissues depending on the phase of lymphocyte development within them. Primary (or central) organs include bone marrow and thymus whereas secondary (or peripheral) organs are spleen, lymph nodes, lymphatic vessels and mucosa-associated lymphoid tissue (MALT) found in various sites of the body.

2.2.1 Innate immunity

The innate immune system is the first line of defense against invading microorganisms. Innate immunity elicits rapid responses, but the mechanisms are non-specific to different microbes, which means that microbial structures are recognized in a generic way, without any long-lasting memory functions.

The primary components of the innate immunity include physical and chemical barriers (e.g. epithelial cells, secreted mucus layer, epithelial cilia, defensins, ficolins), phagocytes (magrophages, neutrophils and dendritic cells), Natural-killer cells (NK-cells), eosinophils, basophils and mast cells, the complement system and finally, the wide range of different cytokines, which are discussed later in 2.2.4 (Abbas et al. 2007; Chaplin 2010). Neutrophils are the most common leucocytes. They mediate the earliest phases of innate immune responses by recognizing, ingesting and killing microbes. Macrophages are derived from blood monocytes. These cells possess several important properties and play a key role not only in later innate responses against microbes but also in adaptive immunity. Macrophages are important in the recognition, phagocytocis and destruction of microbes, as well as in the secretion of cytokines and antigen presentation to other cells. Dendritic cells are the most potent professional antigen presenting cells (APCs), initiating adaptive T-cell dependent immune responses. These cells are capable of producing cytokines and they have a critical role in linking innate and adaptive immunity. NK-cells are lymphocytes (distinct from T or B lymphocytes) in the first line of defense against virus infected cells and tumour cells. Cytokine production by the NK-cells can activate macrophages to kill phagocytosed microbes. Eosinophils, basophils and mast cells are able to release the mediators involved in the immediate hypersensitivity reactions, which are important in allergic diseases (Abbas et al. 2007). The complement system has several essential functions in both innate and

adaptive immunity, but due to the complex nature of these biochemical cascades, they will not be discussed here in detail.

2.2.2 Pattern recognition receptors for the detection of microbes

Antigen presenting cells (APCs) of the innate immunity, such as macrophages and dendritic cells, have cell surface proteins called pattern recognition receptors (PRRs), which are able to detect highly conserved microbial structures called pathogen associated molecular patterns (PAMPs). Despite the name, PAMPs are found not only on pathogenic but also on non-pathogenic microbes. Recognition of the PAMPs by PRRs increases the expression of the co-stimulatory molecules and major histocompatibility complex class II (MHC II) molecules on the cell-surface of the APCs, which enables antigen presentation to the T-cells. At the same time, the production of cytokines is induced. The quality and quantity of the cytokine secretion guides the type of the T-cell responses. Toll-like receptors (TLRs) are a class of PRRs with important functions in orchestrating the innate and adaptive immune responses to infection, inflammation and tissue injury. So far, at least 11 TLRs have been described in humans (Garantziotis et al. 2008; Medzhitov 2001; Pasare and Medzhitov 2004). However, TLRs are not the only recognition receptors, also other PRR families have been characterized, namely C-type lectin receptors, nucleotide binding and oligomerization domain (NOD) -like receptors and retinoic acid-inducible gene 1 -like receptors (Pålsson-McDermott and O´Neill 2007).

The molecular basis for Gram-negative bacterial LPS recognition and signaling is quite well understood (Heumann and Roger 2002). According to the current knowledge, the system for LPS recognition is rather complex involving several components of the innate immune system. The death or division of the Gram-negative bacteria leads to the release of LPS. At first, LPS aggregates with LPS-binding protein (LBP), which acts as a carrier of LPS. This complex becomes attached with the soluble or membrane bound cluster of differentiation 14 (CD14) molecules, which enables recognition of LPS by the Toll-like receptor 4 (TLR4) along with accessory molecule MD-2 on the surface of macrophages or monocytes, leading to cell activation and finally to secretion of the proinflammatory cytokines (Heumann and Roger 2002). The LPS-LBD complex may also bind to high density lipoprotein (HDL), which results in neutralization of the LPS. TLR4 is probably the main signalling pathways for LPS, but it is possible that also other receptors are involved in recognition (Triantafilou and Triantafilou 2005).

Another bacterial cell wall component, peptidoglycan, has a broader spectrum of recognition molecules than LPS including TLR2, CD14, NOD1, NOD2 and peptidoglycan recognition proteins (PGRPs) (Dziarski 2003; Guan and Mariuzza 2007). TLR2-mediated responses of peptidoglycan do not necessarily require CD14 but their effects are usually enhanced by the presence of CD14 (Dziarski 2003).

Many receptors associated with innate immunity have been reported also to recognize fungal components such as β-glucans (Goodridge et al. 2009).

2.2.3 Adaptive immunity

Compared to innate immunity, in evolutionary terms adaptive immunity is younger and more sophisticated. Adaptive immune responses are not as fast as innate responses, but their greater specificity ensures better targeted responses against distinct antigens. In addition, the adaptive immune system has a greater propensity to recognize different antigens, but the most significant difference is in the immunological memory, which is lacking in innate immunity. Adaptive memory functions provide the basis to trigger an enhanced response against the same antigen after repeated exposure. Both innate and adaptive immunity have the ability to discriminate between self and non-self.

Adaptive immunity expresses two types of responses: 1) humoral responses, which are mediated by B-lymphocytes (B-cells) and their secreted antibody proteins and 2) cell-mediated responses orchestrated by T-lymphocytes (T-cells) and their products, cytokines. Both B and T-lymphocytes can differentiate into long-lasting memory cells.

The main function of the activated B-cells is antibody production, but they can also act as APCs. Human B-cells can secrete five different antibody isotypes namely IgA, IgD, IgE, IgG and IgM, which have several differing biological properties, though their main function is antigen binding. All T-cells are able to produce cytokines and can be categorized into different subpopulations: helper T-cells (Th-cells), cytotoxic T-cells (CTLs) and regulatory T-cells (Treg-cells) (Abbas et al. 2007). There are several subsets of Th cells including Th1, Th2, Th17 and Th9. So far, the best described subtypes are Th1 (multiple function, e.g. contribution to cell-mediated immunity by activating macrophages) and Th2 (multiple functions, e.g. take part in humoral immunity by activating B-cell antibody production). CTLs are able to destroy tumor cells and cells infected with intracellular microbes (Abbas et al. 2007). Tregs have several subgroups which can suppress the activation of immunologic cells either by producing anti-inflammatory mediators (cytokines) or by acting as contact -dependent manner and in that way help to maintain homeostasis and self-tolerance in immune system (Bluestone and Abbas 2003; Romagnani 2004a).

2.2.4 Cytokine network

Cytokines are the key signal mediators in the immune system. These compounds are produced principally by leukocytes but also by other cells such as endo- and epithelial cells. They are small soluble proteins, secreted rapidly after synthesis with both local and systemic effects. Cytokines have multiple properties on immune system: pleiotropism (one cytokine having effects on different cell types),

redundancy (different cytokines having the same or overlapping effects), synergy (two or more cytokines having greater than additive effects, 1+1>2) and antagonism (one cytokine having inhibitory effect on the response induced by another cytokine) (Abbas et al. 2007).

Cytokines cannot be classified strictly to the innate or adaptive immunity, because the same cytokine can be produced by the cells of both innate and adaptive system and it may have overlapping effects on both sides. The nomenclature of the cytokines is somewhat misleading. For example, interleukins (ILs) were originally regarded as cytokines, which were produced only by leukocytes and affecting only on leukocytes but today it is known that there are interleukins, which are secreted and act on other cells than leukocytes. In addition, there are chemokines, interferons (IFNs) and tumor necrosis factors (TNFs). Chemokines are chemotactic cytokines, which are able to enhance leukocyte movement and regulate the migration of the leukocytes from blood to tissue. Interferons were named according to their ability to interfere viral replication. Especially type I interferons, such as IFN-α and IFN-β, protect against viral infections. IFN-γ (type II interferon) does not have very strong antiviral activity but it serves important functions in innate immunity and in adaptive cell-mediated immunity against intracellular microbes. The name of the TNFs refers to their properties to cause necrosis to tumors. TNFs have key role in mediating inflammatory responses (Abbas et al. 2007). Sometimes cytokines are classified according to their inflammatory capability. Proinflammatory cytokines (e.g. TNF-α and IL-1) can promote inflammation causing fever, tissue damage, septic shock or even death, whereas anti-inflammatory cytokines (e.g. IL-10 and TGF-β) are able suppress the proinflammatory responses. Moreover, there are hematopoietic cytokines, which stimulate the growth and differentiation of the leukocyte precursors in bone marrow. An overview of the complex cytokine network is presented in Table 1, which includes only some of all the identified cytokines.

Table 1. Central cytokines in immune system

Cytokine	Main sources	Principal actions
IFN-γ	Th1-cells, CTLs, NK-cells	Major Th1 cytokine. Macrophage activation, promotes Th1 and inhibits Th2 differentiation, stimulation of the expression of cell surface receptors in several cell types, promotes B-cell functions
IL-1	Macrophages, endothelial and epithelial cells	Proinflammatory responses, local inflammation, systemic effects (fever, thrombosis, production of acute phase proteins)
IL-2	Th-cells	Increases cell proliferation (T-cells, NK-cells and B-cells) and cytokine (T-cells) and antibody (B-cells) production, promotes Treg-cell development
IL-4	Th2-cells, mast cells	Major Th2 cytokine. Promotes Th2 differentiation, key mediator in immediate hypersensitivity (increases B-cell isotype switching to IgE), increases cell proliferation (mast cells, Th2-cells)
IL-5	Th2-cells, mast cells	Eosinophil activation and increased production, increases B-cell proliferation and the production of IgA
IL-6	Macrophages, endothelial cells, T-cells	Proinflammatory responses (production of acute phase proteins), increases proliferation of antibody producing B-cells
IL-8	Macrophages, epithelial and endothelial cells	Chemokine (known also as CXCL8), the only chemokine named originally as interleukin, neutrophil chemotaxis
IL-10	Macrophages, Th2-cells, Treg-cells	Anti-inflammatory cytokine, inhibition of macrophage and dendritic cell functions (IL-12 ↓, MHC II ↓)
IL-12	Macrophages, dendritic cells	Inducer of cell-mediated immunity, promotes Th1 differentiation, increases IFN-γ secretion (CTLs, Th1-cells, NK-cells) and cytotoxic activity (NK-cells, CTLs)
IL-13	Th2-cells, CTLs, mast cells	Mediator in immediate hypersensitivity (increases B-cell isotype switching to IgE), promotes mucus production in lung epithelial cells, promotes tissue repair by increasing collagen synthesis in macrophages and fibroblasts
IL-15	Macrophages, many cell types	Promotes the proliferation of NK-cells and T-cells, similar functions than IL-2
IL-18	Macrophages	Promotion of IFN-γ production (NK-cells and T-cells), synergic functions with IL-12
TGF-β	T-cells, macrophages, many cell types	Anti-inflammatory cytokine. Inhibits cell proliferation (T and B cells) and activation (macrophages, T-cells), promotes tissue repair
TNF-α	Macrophages, T-cells	Main proinflammatory cytokine, local inflammation, systemic effects (production of acute phase proteins, fever, hypoglycemia, cachexia, thrombosis, apoptosis, septic shock), activates neutrophils, monocytes and endothelial cells
TNF-β	Th1-cells, CTLs	Known also as lymphotoxin. Mediator in acute inflammatory response (activates endothelial cells and neutrophils)

Modified from Abbas et al. 2007

2.3 Early phases of the immune system

The common conception that the immune system of human newborn is fully immature or deficient has been proven to be wrong (Holt and Jones 2000). There is evidence that the fetal immune system is capable of recognizing antigen-like stimuli already during the second trimester of pregnancy as reviewed earlier by Holt (2005). In addition, some functions in fetal spleen are considered to be completely immunocompetent by 18 weeks of pregnancy (Holt and Jones 2000). Langerhans cells (dendritic cells in the epidermis) also seem to resemble adult phenotype by the second trimester (Holt and Jones 2000). It is not only the genes in the infant that control the immune development. Also exposure to microbial and animal proteins and other potential maternal exposure factors from daily life during pregnancy may contribute to the development of newborn's immune functions e.g. maternal dietary habits such as fish oil consumption (Dunstan et al. 2003; Prescott et al. 2007), use of probiotics (Prescott et al. 2008), use of farm dairy products (Pfefferle et al. 2010), maternal smoking (Noakes et al.2003, 2006; Prescott 2008) and maternal stress (Wright et al. 2010).

At birth, the infant has to confront the immunological challenges of living in an antigen-rich environment outside the uterus. It has been shown that events during the time of birth have the potential to modify the neonatal immune system: the birth process itself launches the secretion of acute phase proteins and proinflammatory cytokines and is clearly a stressful event to the newborn especially those born by vaginal delivery (Malamitsi-Puchner et al. 2005; Marchini et al. 2000). Moreover, many maternal and neonatal characteristics at the time of birth and other labour-related immunomodulatory factors have been described e.g. microbial colonization in the vagina (Keski-Nisula et al. 2009; Ly et al. 2006; Stencel-Gabriel et al. 2009), maternal age (Keski-Nisula et al. 2004; Omori et al. 2008), intrauterine infections (Matsuoka et al. 2001), season of birth (Gold et al. 2009; Sullivan Dillie et al. 2008), birth weight (Gold et al. 2009), birth height (Keski-Nisula et al. 2003), duration of gestation (Gold et al. 2009; Keski-Nisula et al. 2003; Rogers et al. 2002). Breastfeeding has been rather widely recognized as an important factor supporting immunological maturation during the neonatal period and providing protection against environmental antigens (Paramasivam et al. 2006; Piirainen et al. 2009).

Practically all newborns express Th2-biased immune responses after birth (Prescott et al. 1998). This is probably due to maternal Th2-balanced cytokine environment needed for successful pregnancy. An increase in maternal proinflammatory (TNF-α) and Th1 (IFN-γ) responses seems to have harmful effects during pregnancy, causing pre-term delivery and even abortion (Daher et al. 2004; Raghupathy 1997; Vitoratos et al. 2006). It is well known that during the neonatal period, most of the functions and the cells of both innate (Levy 2007) and adaptive (Adkins et al. 2004) immunity are still deficient as compared to the situation in adults. Since neonatal adaptive immunity does not have any significant memory

functions due to lack of previous antigen contacts, the defence mechanism depends largely on innate immunity. Impairment of the adaptive Th1 responses combined with the poor APC activity in innate immunity means that neonates are vulnerable to microbial infections. However, immediately after birth the immune system begins to mature in an age-dependent manner and during this period there is a clear consolidation of the several cytokine responses (Härtel et al. 2005). Indeed, it has been shown that under specific experimental conditions neonatal monocytes and cord blood mononuclear cells (CBMCs) are able to produce even more intense stimulated cytokine responses than adults (Angelone et al. 2006; Yerkovich et al. 2007). Thus, it should be noted that although neonates lack many of the complete mature immune functions, it is incorrect to state that neonates are fully immunodeficient. It is more likely that neonates express several immune responses, ranging from deficient or deviant to fully mature, depending on the conditions of the antigen exposure. Consequently, the more relevant term describing the neonatal immunity would be "*immunodeviant*" (Adkins et al. 2004).

2.3.1 Linkage between immune development and allergy

The terminology to describe allergic and allergy-like reactions is confusing. World Allergy Organization (WAO) defines allergy as "*a hypersensitivity reaction initiated by specific immunologic mechanism. Allergy can be antibody-mediated or cell-mediated"* (Johansson et al. 2004). Atopy is "*a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema.*" (Johansson et al. 2004) Atopy is commonly determined as allergen specific IgE antibody levels $\geq$ 0.35 kU/L in serum (sIgE) or as positive skin prick test results.

It is important to note that the development of the immune system and the allergy are distinct from each other. It is true that they have a close relationship because allergies are disorders of the immune system. Furthermore, due to the neonatal antigen specific reactivity at birth, the priming of allergies occurs at a very early age, probably already *in utero* (Jones et al. 2000; Warner et al. 2000), during the normal immunologic maturation process. Thus the search for the causal components affecting the development of the neonatal immune system in general may offer new insights also into the complex mechanism of allergy development.

It is well documented that the balance between adaptive T-helper cell type 1 (Th1) and 2 (Th2) responses plays a central role in the development of atopy. Increased Th2 responses together with the complex cytokine network including numerous adhesion molecules are characteristic in atopic diseases. For example, Th2-cells secrete cytokine IL-4, which regulates the release of the IgE (one of the key mediators in immediate hypersensitivity) from the B-cells. In contrast, non-

atopic individuals manifest mainly cell-mediated Th1 responses, e.g. characterized by the production of IFN-γ (inhibition of Th2 responses) (Romagnani 2000). As mentioned earlier (see 2.3) the infant's cytokine milieu is skewed towards Th2 cytokine responses after birth, so newborns' have atopic cytokine responses as the default. It was proposed that Th2 responses are dampened quickly after birth in a non-atopic immunological maturation process (Figure 1, line A), whereas in individuals with an atopic phenotype, the Th2 responses continue to consolidate (Figure 1, line C). Lack of attenuation of Th2 responses in children who become atopic may be a result of the delayed maturation of cytokine responses, especially in Th1 cytokine (IFN-γ) secretion, as shown in children with atopic heredity (Prescott et al. 1999). Delayed immune development is also one possible explanation for the fact that some atopic infants become non-atopic later in childhood (Figure 1, line B).

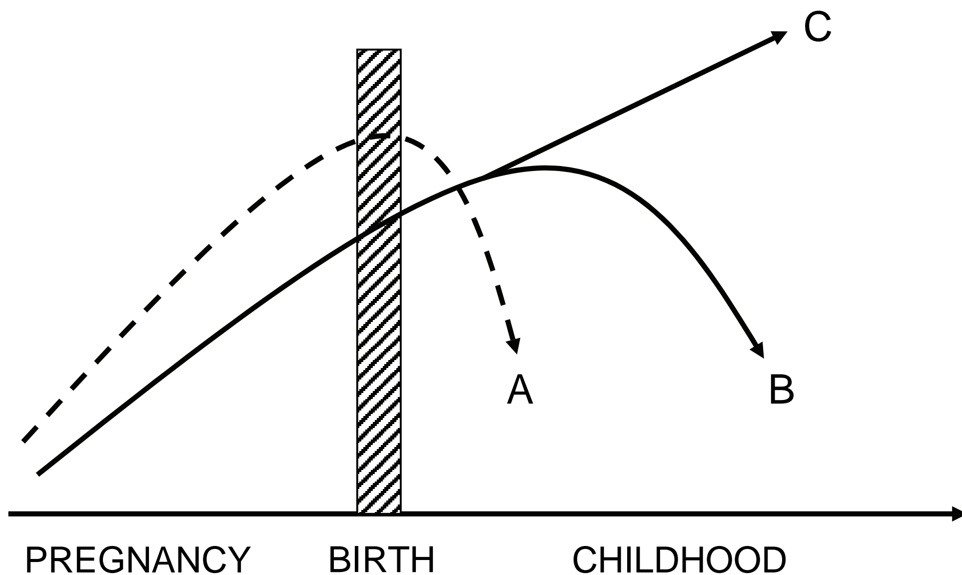

Figure 1. **The development of Th2 responses in children who do not become atopic (A), who are atopic in infancy but not later in childhood (B) and who become persistently atopic (C).** (Modified from Jones et al. 2000)

Ever since this initial interpretation for atopy development was proposed, failure to shift from the default allergic Th2 cytokine environment to the non-allergic Th1 - balanced cytokine profile (called also "*missing immune deviation*") has received considerable support (Romagnani 2004a, 2004b). Due to the major progress in the field of microbiology and immunology, also new aspects for immunologic mechanisms have emerged. A subpopulation of T-lymphocytes called T -regulatory cells (Tregs) has been under intensive investigation lately, i.e., it has been postulated that reduced suppressive activity of the Tregs may maintain Th2-mediated immunity in atopic individuals (Akdis et al. 2004; Foley et al. 2007; Ling et al. 2004; Seroogy

and Gern 2005). However, many leading immunologists have also criticized this so called "*reduced immune suppression*" as the main explanatory mechanism for the hygiene hypothesis (Romagnani 2007). The hypothesis of "*missing immune deviation*" took a step further when the focus was expanded from the adaptive T-cell responses to the innate immunity including APCs such as dendritic cells and their cell surface proteins called pattern recognition receptors (e.g. toll-like receptors, TLRs). Dendritic cells appear to have a central role in Th1 / Th2 differentiation and possibly in the previously mentioned delayed maturation of Th1 responses (Upham and Stumbles 2003). These cells may also take part in mediating the tolerogenic immune process against inhaled antigens (Upham and Stumbles 2003). A diminished environmental microbial stimulus was regarded as the key factor in "*missing immune deviation*" (Martinez 2001; Martinez and Holt 1999). With respect to this theory, it appears that the reduction in microbial burden leads to poor stimulation of TLRs resulting in weak Th1 responses and skewing of the immune system towards Th2-type reactions (Bauer et al. 2007; Horner 2006).

In summary, immediately after birth, the neonatal immune system maturates in a stepwise manner towards Th1 responses. The risk of atopy may depend on the quantity and the speed of this individual developmental process of immune system. In the light of the current knowledge, in addition to the Th1 and Th2 responses, it also seems likely that the functions of the innate immunity play a crucial role in driving the immune system either towards an allergic or to a non-allergic phenotype. However, this immunological maturation process is governed by several different key factors including genetics and site, dose, type and timing of the antigen exposure (Belderbos et al. 2009).

2.4 Indoor microbial exposures

Microbes are simple, usually single-celled organisms, which are too small to be seen by the naked eye. Soon after birth, the respiratory tract, GI-tract, skin and mucus membranes all over the body are colonized by the surrounding microbial flora. All of these routes are relevant in terms of microbial exposure. Microbes include a wide range of different micro-organisms, but only those bacterial and fungal species, which are in the focus of this thesis, are described.

2.4.1 Gram-positive and Gram-negative bacteria

Gram-staining is an old method, which classifies bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. The Gram-positive bacterial cell wall contains a thick layer of cross-liked polysaccharide-peptide matrix called peptidoglycan, whereas in Gram-negative bacterial cell, this layer is much thinner. In addition, the Gram-negative cell wall is composed of polysaccharide-lipid molecules called

lipopolysaccharides (LPS) (Salyers and Dixie 2000). LPS is commonly called endotoxin. Endotoxin is a toxin that is released when the cell is lysed or during cell division. Both peptidoglycan and LPS are known to be potent inducers of the immune responses.

Lipid A is the immunostimulatory part of the LPS. Lipid A contains 3-hydroxy fatty acids (3-OH FAs,) and thus 3-OH FAs are regarded as a chemical marker for Gram-negative bacteria (Binding et al. 2004; Saraf et al. 1997). Due to good correlation with the common endotoxin test, *Limulus* amebocyte lysate assay (LAL), 3-OH FAs especially with a carbon chain length from 10 to 14, seems to be appropriate markers for bioactive endotoxin (Saraf et al. 1997). In contrast to LAL-method, 3-OH FAs measurements detect LPS irrespective of its bioactivity.

N-acetyl-muramic acid is the major component of the peptidoglycan. Since the amount of peptidoglycan is higher in Gram-positive bacteria (30-70% of cell wall) than in Gram-negative bacteria (<10% of cell wall) (Starr et al. 1981), muramic acid is regarded as a chemical marker for Gram-positive bacteria (van Strien et al. 2004).

Mycobacteria are ubiquitous in all kinds of environments e.g. water, soil and dust (Falkinham 1996). Although genus *Mycobacterium* includes some highly pathogenic species, the most common indoor *Mycobacterium* species seem to be non-pathogenic (Torvinen et al. 2010). However, both pathogenic and non-pathogenic species can elicit immune responses, at least under experimental conditions (Huttunen et al. 2001; Jussila et al. 2002a). There is an on-going debate about the importance of childhood mycobacterial infections on atopy development (Obihara et al. 2007).

Streptomycetes are very common Gram-positive bacteria in soil and also in other environments such as fodder, compost and aquatic habitats (Kutzner 1986). These bacteria are frequently found in indoor environments, especially in moisture damaged buildings (Rintala et al. 2004). Genus *Streptomyces* produces a wide spectrum of secondary metabolites and some of these compounds can be toxic (Andersson et al. 1998) but on the other hand, about half of the known antibiotics with therapeutic properties are produced by streptomycetes (Demain 1999). Similarly to mycobacteria, also streptomycetes are capable of inducing immunologic responses *in vitro* (Huttunen et al. 2003) and *in vivo* (Jussila et al. 2001, 2003).

2.4.2 Fungi

Fungi are classified as a kingdom of eukaryotic organisms, separate from plants, animals or prokaryotic bacteria. Ergosterol is a component of fungal cell membranes, equivalent to cholesterol in animal cells (Salyers and Dixie 2000). This membrane lipid is used commonly as a chemical marker for describing in quantitative terms the fungal biomass (Saraf et al. 1997)

Lately, the special interest has been focused on the certain fungal species, *Penicillium* spp., *Aspergillus* spp., *Paecilomyces variotii*, *Trichoderma viride*,

Trichoderma atroviride, *Trichoderma koningii* and *Wallemia sebi*, because they are found frequently in indoor environments and their concentrations are believed to be associated with the severity of moisture damage (Lignell et al. 2008). Although many of the analyzed fungal species are potential toxin producers (Degenkolb et al. 2008; Reverberi et al. 2010), the role of the multiple indoor fungal exposures on human health or especially on immunologic development in early childhood is not clear.

2.5 Double-edged health effects of animal exposure

It has been postulated that exposure and contacts to different animal species can have very different outcomes, i.e., promote, confer protection against or have no consistent effect on the development of asthma and allergic conditions in childhood. Similar factors which govern the effects of microbial exposures (see 2.7), i.e., genetics and the exposure event, are likely affecting also in the case of animal exposures. Contradictory findings concerning the effects of animal exposure may also be partly explained by the complex mixture of allergens and different microbes that animals carry and spread indoors (see 2.6.1). It is obvious that the levels of major dog and cat allergens are increased in homes with these respective animals (Arbes et al. 2004; Raunio et al. 1998) and that these allergens can cause symptoms in sensitive subjects. The problem in assessing the true contribution of the dog and cat exposures is that dog and cat allergens are found virtually in every household, even inside homes without these pets (Arbes et al. 2004). Thus there is no truly unexposed control group available. Not only the levels of allergens but also the concentrations of microbial compounds may be increased by the presence of furry pets. For example, elevated endotoxin levels have been detected from the house dust (Gereda et al. 2001; Heinrich et al. 2001) and indoor air (Park et al. 2001a) of the dog owners' households. Also avoidance and/or removal of pets may lead to the difficulties in the interpretation of the results. It is possible that most of the pet owners are those who do not experience symptoms from pets (so-called "healthy pet-keeping effect"). In addition, some parents may avoid pets because they believe that pet exposure is a risk factor for childhood asthma and allergies (Bornehag et al. 2003; Brunekreef et al. 1992).

Epidemiological studies concerning the possible protective effect of farm environment have enabled the assessment of farm animal exposures. Exposure to stables, especially during the first year of life, has been linked to a lower frequency for asthma, hay fever and atopic sensitization (Riedler et al. 2001). Even prenatal stable and barn exposures (maternal exposure) seem to decrease the risk for suffering atopy in childhood (Ege et al. 2006, 2008). In addition, pig keeping and animal barn exposure of the child have been associated with a decreased risk for childhood asthma (Ege et al. 2007). Nonetheless, there are also contradictory

findings reporting that exposure to farm animals in general may increase the risk for asthma in children (Hugg et al. 2008).

It is very common to keep domestic animals not only in rural areas but also in urban environments and the interest has been focused primarily on the furry pets such as cats and dogs. Dog keeping has been reported to decrease the risk for asthma, wheezing, atopy, atopic dermatitis and hay fever in children (Bufford et al. 2008; Chen et al. 2008; Gern et al. 2004; Hugg et al. 2008; Litonjua et al. 2002; Ownby et al. 2002; Remes et al. 2001, 2003; Waser et al. 2005), but also increased risk of atopic sensitization has been reported (Al-Mousawi et al. 2004). In some studies, the presence of a cat in the household appeared to decrease the risk for atopy and wheezing in childhood (Ownby et al. 2002; Remes et al. 2003; Waser et al. 2005), but in some other settings, it increased the risk for allergic asthma and also for atopic sensitization (Al-Mousawi et al. 2004; Hugg et al. 2008; Lindfors et al. 1999; Melen et al. 2001). It is not clear whether having multiple dogs or cats in the household has a stronger impact on atopic and asthmatic outcomes than having only one pet (Ownby et al. 2002; Remes et al. 2001). On the other hand, some findings have suggested that dog or cat ownership does not have any influence on allergic outcomes. Dog ownership was not associated with atopy (Lindfors et al. 1999; Melen et al. 2001; Remes et al. 2001) nor has cat keeping been linked with wheezing, atopy and atopic dermatitis (Bufford et al. 2008; Gern et al. 2004; Remes et al. 2001).

2.6 Exposure to animals and neonatal immune functions

Although the contribution of animal exposure to childhood allergy and asthma has been investigated broadly, the literature concerning the effects of animal exposures on immune responses during early life is still quite limited, covering mainly dog and cat exposure.

A prospective birth cohort study of 285 families detected associations between dog ownership and elevated mitogen-induced IL-10 and IL-13 secretion at the age of 1 year (Gern et al. 2004). Moreover, dog keeping appeared to be protective against the development of atopic dermatitis in infants with the specific genotype in the gene encoding for a co-recognition receptor for endotoxin (CD14-159TT) (Gern et al. 2004). The presence of dog and/or cat in the household may increase also the mitogen-stimulated IFN-γ -producing capacity 3 months after birth (Roponen et al. 2005). Mononuclear cell yield and IFN-γ levels were increased in cord blood of dog owners' newborn but the differences in IFN-γ production were small and not consistent between different stimuli (Sullivan Dillie et al. 2008). It should be noted that also exposure to pet-derived allergens has the potential to modify immune responses during childhood. The levels of Can f 1 (the major dog allergen) in house

dust were associated with the increased concentrations of IL-10, IL-5 and IL-13 at the age of 1 year and IL-5 and IL-13 at 3 years of age (Bufford et al. 2008).

There is only minor evidence that cat exposure can modify cytokine responses in infants. It has been shown that cat keeping is inversely associated with cord blood IL-5 responses (Sullivan Dillie et al. 2008). However, the difference in the IL-5 response was small and seen only in one of the three stimuli. Some other studies have failed to detect any associations between cytokine responses during the first years of life and levels of either of the major cat allergen (Fel d 1) (Bufford et al. 2008) or cat keeping (Gern et al. 2004). Despite the lack of associations in cytokines, exposure to cats (Hesselmar et al. 2003) and cat allergens (Platts-Mills et al. 2001) may be linked to the increased immunoglobulin G4 (IgG4) levels in childhood.

Farm studies have shown that maternal contact with an increasing number of farm animal species during pregnancy is associated with enhanced cord blood TNF-α and IFN-γ responses (Pfefferle et al. 2010; Schaub et al. 2009) and the expression of Treg-cell markers in children (Schaub et al. 2009). Furthermore, farm children expressed higher levels of those cytokines than their non-farming counterparts (Pfefferle et al. 2010).

2.6.1 Possible underlying immunologic mechanisms of animal exposures

The postulated relationships between the presence of a dog, the levels of allergens and microbial antigens, immune responses and development of allergy are rather complex (Figure 2). Increased allergen exposures could lead to an increment in Th2 cytokine responses, which would promote the development of allergies. Elevated endotoxin exposure may be protective via enhanced Th1 responses, but on the other hand, it may increase the risk of respiratory irritation and symptoms (see 2.7). Finally, it is not known which types of microbial exposures are affected by the presence of dog and whether other microbial exposures have influence on the immune responses.

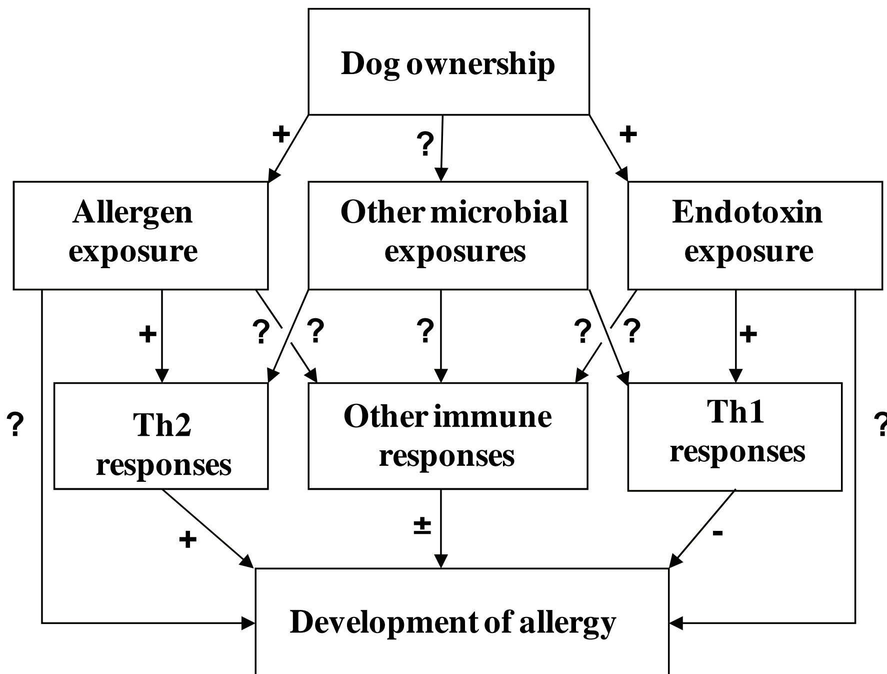

Figure 2. **Hypothesized model for the associations between dog keeping, allergen and microbial exposures, immune responses and the development of allergy.** (Modified from Wright et al. 2008)

The role of different microbial components in mediating the effects of dog exposure is also under debate and largely unclear. There is evidence that a high endotoxin load together with the presence of several dogs, but neither alone, confers protection against wheezing in childhood (Campo et al. 2006). On the other hand, many studies have failed to demonstrate that the effects of dog exposure on health outcomes or immune responses are due to the elevated exposure to endotoxin or other microbial antigens (Bufford et al. 2008; Chen et al. 2008; Litonjua et al. 2002; Waser et al. 2005). Even if microbes were to be one of the most important causal components in dog exposure, it is likely that also allergens and possibly so far uncharacterized lifestyle factors relating to the dog keeping have a role in this complex chain of immunological events.

It needs to be considered that different animal species, such as cat and dogs, may have divergent effects on immune system. It is a generally accepted theory that atopic disorders are dominated by Th2 responses and that levels of IgEs are often increased in atopic individuals. However, investigators found that children exposed to cats and high levels of cat allergen produced increased amounts of IgG and IgG4 antibodies without any increase in the secretion of IgE (Hesselmar et al. 2003;

Platts-Mills et al. 2001, 2005). In other words, highly cat exposed children were less likely to be sensitized to cats. Since also IgG4 is a Th2-dependent antibody, these findings raised the concept of “modified Th2 responses”. This phenomenon could be related to the adaptation of the immune system to face cat allergens and challenges further the initial Th1/ Th2 -concept in the development of allergic diseases.

The current understanding of the development of the human immune system and allergic disorders emphasizes the role of animal and microbial exposures. However, it is becoming clear that the bidirectional nature (adverse or protective) of these exposures is complicated by the mixture of genetics and type, timing, duration and intensity not only of animal and microbial exposures themselves but also exposure to numerous other pre- and postnatal environmental factors as well as the physiological state of the child.

2.7 Double-edged health effects of microbial exposure

There is growing body of epidemiological evidence which suggests that early life microbial exposures and contacts are associated with the development of asthma and allergic diseases in childhood. It is becoming clear that microbial exposures can be either beneficial or adverse, even both. Type, dose (low vs high), duration (short-term vs continuous) site of antigen encounter (gut vs respiratory tract vs skin), age when the exposure occurs and other individual characteristics and environmental factors relating to the events of the exposure all contribute to the bidirectional effects evoked by microbes (Renz and Herz 2002). Moreover, the complex interactions between environmental exposures and the genetic constitution of the child make it difficult to assess the influence of one particular environmental stimuli, since the same exposure may either increase or decrease (or have no influence on) the risk of asthma or allergic sensitization, depending on the genetic susceptibility of the child (Simpson et al. 2006; Upham and Holt 2005; Vercelli 2006).

The Gram-negative bacterial cell wall component, LPS (endotoxin), is the most widely evaluated single microbial agent in the field of allergy research. Several studies have shown that the exposure to house dust endotoxin can offer protection against hay fever, eczema, asthma, atopic sensitization and atopic asthma in childhood (Böttcher et al. 2003; Braun-Fahrländer et al. 2002; Douwes et al. 2006; Gehring et al. 2001, 2002; Gereda et al. 2000; Phipatanakul et al. 2004; Simpson et al. 2006; von Mutius et al. 2000a). On the other hand, there is an almost equivalent amount of literature indicating that endotoxin is a potent respiratory irritant linked with the increased risk for wheezing, respiratory infections and bronchitis (Bolte et al. 2003; Gehring et al. 2001; Litonjua et al. 2002; Park et al. 2001b; Simpson et al. 2006) and the incidence of childhood respiratory diseases in general (Dales et al. 2006). Extensive exposure to endotoxin may be a risk factor for non-atopic wheeze, particularly in children with certain genetic characteristics (Simpson et al. 2006).

Especially in occupational settings in adulthood, endotoxin exposure has been shown to be harmful (Douwes et al. 2002).

Another bacterial cell wall constituent, peptidoglycan (muramic acid) has quite recently been promoted as a promising bacterial agent for allergy research. The knowledge concerning the health effects of muramic acid is still limited but high levels of muramic acid in mattress dust from the children's bed have been inversely associated with the prevalence of wheezing (van Strien et al. 2004). There is evidence that infections caused by Gram-positive mycobacteria may confer protection against childhood asthma (von Mutius et al. 2000b). However, the influence of mycobacterial infections on atopy development is rather controversial (Obihara et al. 2007).

Moisture damage and the presence of mold in the buildings are associated with a wide range of adverse respiratory health effects including worsening of respiratory symptoms and the development of asthma (Bornehag et al. 2001, 2004; World Health Organization 2009). However, the causal components mediating dampness-related health problems remain still to be discovered. Some studies have observed relationships between the levels of specific indoor fungal components / species and the health outcomes of the inhabitants. Increased exposure to spores of *Penicillium / Aspergillus* and *Alternaria* -species has been associated with the prevalence (Osborne et al. 2006) and the risk for allergic sensitization (Garrett et al. 1998; Jacob et al. 2002), asthma (Garrett et al. 1998), wheezing and persistent cough (Gent et al. 2002) in children. The relationship between atopy and *Cladosporium* exposure is rather controversial (Garrett et al. 1998; Jacob et al. 2002; Osborne et al. 2006). On one hand, some studies have concluded that exposure to some fungal components such as extracellular polysaccharides (EPS) and β-glucans may be beneficial by offering protection against childhood asthma, wheezing, atopic wheezing and atopic sensitization (Douwes et al. 2006; Iossifova et al. 2007; Schram-Bijkerk et al. 2005). On the other hand, the health effects related to ergosterol exposure (marker for fungal biomass) are poorly recognized. In adults, an increased risk for atopic sensitization and wheezing (Dharmage et al. 2001) has been reported but the effect in children is almost completely unknown (Hyvärinen et al. 2006a).

2.8 Exposure to microbes and neonatal immune functions

Experimental studies have characterized extensively the immunomodulatory capability of different microbial derived products e.g. LPS and peptidoglycan, by stimulating the specific cell lines or exposing animals to these compounds. Nevertheless, results from *in vitro* and *in vivo* studies have not been able to unravel the complex interplay between immune responses and different microbial exposures that humans encounter in their everyday lives. Thus quite recently, the influence of environmental microbial exposures on neonatal immunity has been investigated in a

multidisciplinary manner by including an epidemiological study design along with microbiological and immunological methods. The single microbial component, which has received the greatest interest in this rather novel area of research, is endotoxin and thus at present, the associations between other microbial agents and immune responses at early age are poorly understood.

About 10 years ago, it was proposed that reduced contact with environmental antigens might impair the deviation of infant's immune system from the naturally occurring Th2-skewed humoral immune responses into the non-allergic Th1-balanced cell-mediated immune functions (Martinez and Holt 1999). In support of this hypothesis, it was shown that increased concentrations of house dust endotoxin decrease the levels of allergen-induced Th2 cytokine IL-13 in early childhood (Abraham et al. 2005). However, no associations were found with respect to proinflammatory (TNF-α), Treg (IL-10) or Th1 (IFN-γ) cytokine responses. A subsequent study offered further support by concluding that the change in functional capacity of whole blood leukocytes to produce IFN-γ following mitogen stimulation was greater in the period from birth to 3 months of age in those children with high levels of house dust endotoxin (Roponen et al. 2005). In addition, IL-6 responses at birth were associated with house dust endotoxin levels. Similarly, a recent publication described a significant correlation between the levels of settled dust endotoxin and IFN-γ responses at the age 3 years, but not at the age of 1 year (Bufford et al. 2008). However, also the levels of IL-13 were increased with respect to endotoxin concentrations. Associations with another bacterial cell wall component, muramic acid, were similar to endotoxin (Bufford et al. 2008). In addition, significant correlation between the concentrations of house dust endotoxin and the proportions of IFN-γ -producing $CD4^+$ T cells has been observed in children younger than 2 years (Gereda et al. 2000). Levels of endotoxin in different types of dust samples may be related to increased IFN-γ and TNF-α responses also in cord blood (Pfefferle et al. 2010). Although there are several supporting findings for the above mentioned hypothesis, it has become clear that the effects of microbial exposures on neonatal immunity are more complex than previously thought and thus the basic concept of a Th1/Th2 -balance may be oversimplified and cannot explain entirely the immunologic spectrum relating to the development of immune system or allergy.

Important results from the cross-sectional study with over 800 children from rural areas across the Europe offered a new point of view for the potential causal mechanism. Levels of mattress dust endotoxin were inversely associated with clear down-regulation of LPS-induced immune responses (TNF-α, IFN-γ, IL-10 an IL-12 cytokines) in school-aged children (Braun-Fahrländer et al. 2002). The authors concluded that continuous, high-level exposure to environmental endotoxin could have promoted the adaptation of the immune system against external antigens, which may have prevented the development of allergy. Other microbial agents were not measured, but the authors speculated that these associations were probably due

to exposure to a much wider spectrum of microbes than Gram-negative bacteria alone. In another context, endotoxin tolerance, which is reflected as an impaired cytokine release after LPS stimulation of the leukocytes or whole blood *ex vivo*, has been previously characterized in patients with sepsis (severe inflammatory state of the whole body usually caused by bacterial infection) (Cavaillon 1995; West and Heagy 2002).

The influence of fungal exposures on neonatal immunity is almost completely unknown. Immune responses of children at the age of 1 and 3 years were not affected by their exposure to indoor dust ergosterol (Bufford et al. 2008). However, levels of fungal extracellular polysaccharides (EPS) in house dust were related to the increase in TNF-α secretion from cord blood (Pfefferle et al. 2010). When exposure to other fungal components was studied in adults, it was found that high levels of airborne β-glucans increased the production of TNF-α and reduced the proportion of certain T-cells (Beijer et al. 2003).

According to current knowledge, the functions of the recognition receptors for microbes in innate immunity may play a key role in the early immune modulation (Manicassamy and Pulendran 2009). Relating to the expression of Toll-like receptors, it has been shown that maternal exposure to environments rich in microbes (maternal stable exposure) and contacts with an increasing number of different farm animal species during pregnancy may increase the expression of the TLR2, TLR4 and CD14 genes in offspring (Ege et al. 2006). In addition, certain farm characteristics and the farming status of the children themselves seem to be associated with increased expression of the genes of those recognition receptors (Ege et al. 2006, 2007; Lauener et al. 2002).

3 AIMS OF THE STUDY

The overall aim of the thesis was to study the effect of environmental microbial and animal exposures on the development of immune functions during the first year of life.

The specific aims were:

1. To examine the contribution of labor-related factors and maternal and neonatal characteristics on the cord blood cytokine responses. (I)
2. To assess the development of immune responses from birth to 1 year of age and to compare responses between mothers and neonates. (II, IV)
3. To elucidate whether animal exposure during pregnancy and during the first year of life affect the cytokine -producing capacity at birth and 1 year after birth. (III)
4. To clarify the effect of indoor microbial exposure on cytokine responses at birth (IV), at the age of 3 months (IV) and at 1 year. (V)

4 MATERIALS AND METHODS

4.1 Study design

This thesis is based on the prospective birth cohort studies (MAA, LUKAS1 and LUKAS2), which consisted of an epidemiological study design combined with immunological and microbiological measurements. Most of the work was conducted on the LUKAS2 data. The data obtained from different follow-ups of each cohort are presented in Table 2.

Table 2. **Data included into the thesis.**

Cohort	Data	Follow-up when data was collected				
		Pregnancy	Birth	2 months	3 months	12 months
MAA	Cytokines, child		X		X	
	Cytokines, mother				X	
	IgE analysis					
	Microbial analyses				X	
	Questionnaires	X	X		X	
LUKAS1	Cytokines, child		X			
	Cytokines, mother					
	IgE analysis					
	Microbial analyses					
	Questionnaires	X	X			
LUKAS2	Cytokines, child		X			X
	Cytokines, mother					X
	IgE analysis*		X			X
	Microbial analyses			X		
	Questionnaires	X	X	X		X

*At birth mothers only, 12 months children only.

4.1.1 Study population -MAA (IV)

The focus in Mikrobialtistus ja Allergiat (MAA) birth cohort was to assess the possible associations between indoor microbial exposures and the development of allergy.

Patient records from maternal outpatient clinics at Kuopio University Hospital was used for screening of the background information and an invitation letter was sent to healthy pregnant women living in actively operating farms. Thus mothers who gave birth at the Department of Obstetrics at Kuopio University Hospital after a

clinically normal pregnancy, were recruited to the study during a 5-month period (11/2000 – 4/2001) (n=12). Non-farming controls (n=17) were randomly selected (1/2001 – 3/2001) from rural and suburban areas near the city of Kuopio. Criteria for exclusion were as follows: gestational diabetes, pre-eclampsia, acute severe maternal chronic infection during pregnancy, congenital abnormalities in neonate, vaginal delivery <35 weeks of gestation, uterine infection during delivery and breaking of amniotic fluid >24 hours before delivery. A written informed consent was obtained from all mothers. Consent and the study protocol were approved by the Research Ethics Committee, Hospital District of Northern Savo.

4.1.2 Study population -LUKAS1 (I) and LUKAS2 (I–III, V)

Similarly to the MAA study, this ongoing prospective birth cohort study, Lapsuuden Kasvuympäristö ja Allergiat (LUKAS), aims to clarify the role of indoor exposure to various microbial products on the development of childhood asthma and allergic diseases in rural environments. Moreover, immunological and genetic pathways mediating the effect of these exposures are being investigated. Publication I is made up of the data from the LUKAS1 and LUKAS2 cohorts, whereas II, III and V consist only of the LUKAS2 cohort.

Participants in LUKAS study were enrolled in two phases (Figure 3). The first half of the study (LUKAS1) is the Finnish part of the European birth cohort study PASTURE (Protection against Allergy -Study in Rural Environments; includes mothers and their children also from Austria, Switzerland, France and Germany) (von Mutius and Schmid 2006). Home addresses, farming-status and expected date of delivery of mothers were obtained from questionnaires distributed by maternal outpatient clinics. The subjects were mothers living in farms with livestock and an equally-sized group of non-farming controls from rural areas in eastern and middle Finland and who had given birth between 09/2002-05/2004 in one of the local major hospitals (Kuopio, Iisalmi, Jyväskylä and Joensuu). Inclusion criteria (maternal age of ≥18 years, singleton pregnancy, native language Finnish, no plans of moving from the study area, expected place of delivery in one of the study hospitals, siblings of the study child not participating in the study) were fulfilled by 340 of the screened mothers (Figure 3). After delivery, 214 participants fulfilled further inclusion criteria (parturition at ≥37 weeks of gestation, no congenital abnormalities in newborn, successful cord blood sampling).

LUKAS2 is an extension to the LUKAS1 cohort. Initially it was established to serve a larger study population for investigating childhood asthma and additional studies started only in LUKAS1 study, such as more detailed assessment of dietary habits and broader determinations of moisture problems and microbial environment. Most of the recruited 228 mothers in LUKAS2 were non-farmers (95.2%) living in rural and suburban households near the city of Kuopio. Inclusion criteria were otherwise similar to LUKAS1, but only mothers who gave birth in Kuopio

University Hospital were invited (deliveries between 05/2004 – 05/2005). In order to keep housing conditions comparable between LUKAS2 and LUKAS1, only mothers from non-apartment households were enrolled into the LUKAS2. Due to laboratory logistic reasons, only children born between Sunday afternoons and Thursday mornings were included. Written informed consent was acquired from all LUKAS mothers. Ethical permission was granted by the Research Ethics Committee, Hospital District of Northern Savo.

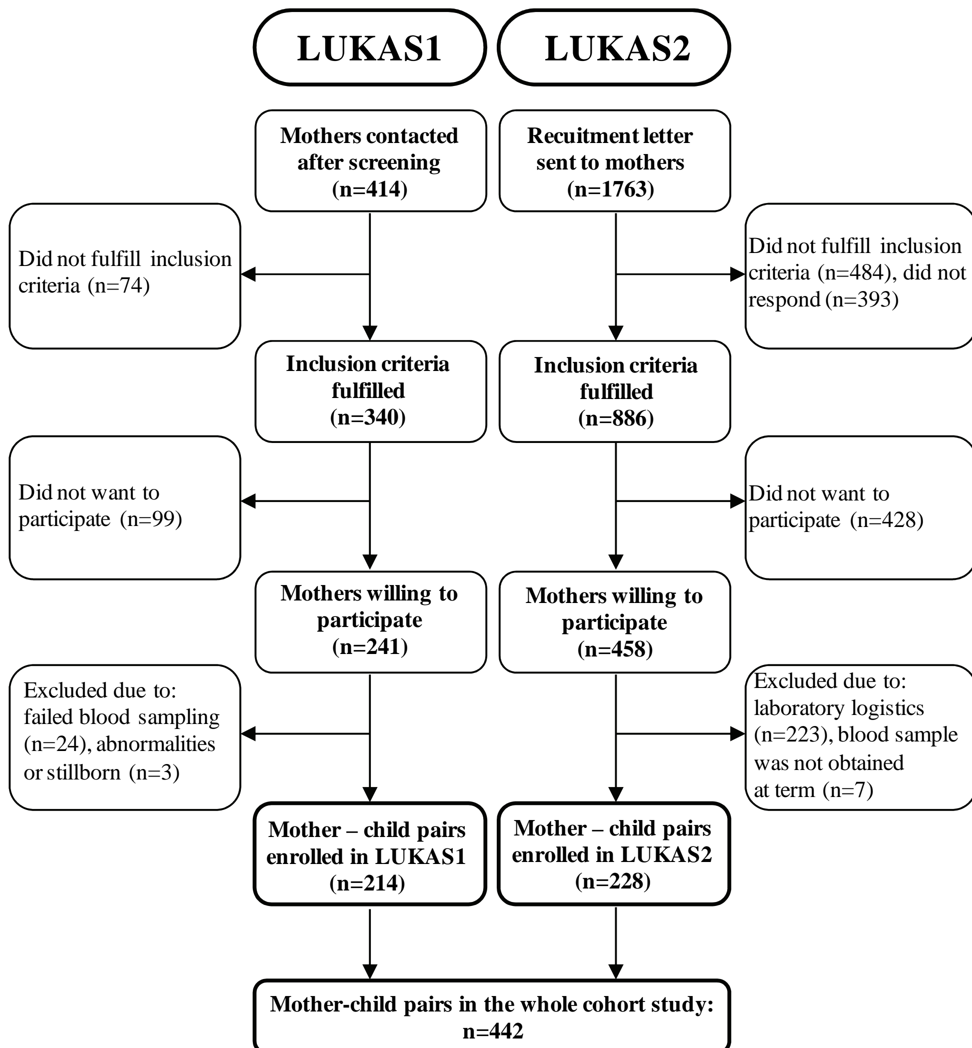

***Figure 3*. Flowchart of the recruitment process in LUKAS birth cohort.** (Modified from Karvonen et al. 2009)

4.2 Questionnaires (I–V)

In MAA cohort, self-administered questionnaires were submitted to participating mothers during the last trimester of pregnancy and also at 3 months after birth.

Pregnancy questionnaire concerned maternal and paternal health status (e.g. allergies, respiratory symptoms and diseases), pregnancy-related questions (e.g. maternal illnesses and use of antibiotics during this pregnancy, number of previous pregnancies and deliveries), general information about housing (e.g. number of inhabitants, cleaning habits, living environment, the presence of visible mold, activities undertaken for allergy avoidance or due to allergy), educational and occupational information, parental smoking, animal ownership / contacts and dietary habits including use of vitamins and probiotics.

In order to characterize possible changes in background information, questionnaire submitted 3 months after delivery contained some of the same topics as the previous form (maternal health status, housing information, animal ownership and contacts, smoking, farming status). In addition, obstetric factors (e.g. time and mode of birth, child's weight, height, gender), maternal illnesses and use of antibiotics during the remaining pregnancy time, infant's diseases, diet and use of antibiotics, breast feeding and day-care arrangements were queried.

In the LUKAS study, mothers filled in questionnaires during the third trimester of pregnancy, and at 1 year after birth of the child. In addition, mothers were interviewed 2 months after delivery. Questionnaires were formulated on the basis of the previous national and international studies. LUKAS questionnaires were more detailed compared to MAA questionnaires, but the topics were same. In particular, maternal participation in farm work (included also mothers' own farm exposures during her childhood) and animal contacts, child's and mother's respiratory disorders and symptoms as well as atopic sensitization were enquired more accurately compared to MAA subjects. In addition, allergic and asthmatic disorders of the siblings and grandparents of child were clarified in LUKAS. Obstetric data were derived from a separate form filled in at the time of delivery.

4.3 Blood sampling (I–V)

In the MAA cohort, neonatal cord blood samples (n=29) and peripheral venous blood samples of mothers at birth (n=29) were collected in lithium-heparin -tubes by an aspiration technique (Vacutainer, Becton&Dickinson, Plymouth, United Kingdom) at the Laboratory of Kuopio University Hospital. Sampling was repeated at 3 months after birth (n=29 for both mothers and children). Samples were stored and transferred at 4°C and processed for further analyses within 24 hours.

Blood was sampled in LUKAS1 and LUKAS2 studies similarly to MAA study with minor differences: In addition to Kuopio University Hospital, central hospitals also in the cities of Joensuu, Jyväskylä and Iisalmi performed sampling in LUKAS1.

Blood was collected at birth (LUKAS1, 195 children; LUKAS2, 228 children; immunochemical analysis were not done in the maternal samples collected at delivery) and at 1 year after birth (LUKAS2, 200 children, 208 mothers; LUKAS1 1 year samples were not included into this thesis). Blood was processed < 27 hours after sampling. Serum samples (mothers at delivery and children at the age of 1 year) were collected for IgE analyses and EDTA-blood (Vacutainer) for the white blood cell analysis (see 4.4.2).

4.4 Whole blood culture and cytokine stimulation (I–V)

In MAA study, whole blood cell cultures were performed to determine stimulated cytokine production in leukocytes of the studied children and their mothers (cord blood and peripheral blood collected 3 months after birth). Briefly, heparinized blood was diluted 1:4 with cell culture medium (RPMI 1640 supplemented with 1% penicillin-streptomycin mixture, 10% FBS and 1% L-glutamine, all from Gibco, Paisley, United Kingdom) and stimulated with the combination of phorbol 12-myristate 13-acetate (PMA) (final concentration in the well 15 ng/ml) and concanavalin A (ConA) (10 µg/ml) for 8 and 24 hours (5 % CO_2, 37 ^oC). After incubation, cultures were centrifuged at 380*g* for 10 minutes and the supernatants were collected and stored at -70 °C for later cytokine analysis.

In LUKAS studies, preparation of whole blood cell culture and the stimulation protocol were similar to MAA study with following exceptions: heparinized blood (cord blood and 1 year after birth) was diluted 1:8 with RPMI 1640 with Glutamax I (Gibco, Paisley, United Kingdom) cell culture medium (supplemented with 1% Antibiotic-Antimycotic from Gibco, Paisley, United Kingdom and 10% heat inactivated FBS Gold from PAA Laboratories GmbH, Pasching, Austria). To induce the production of cytokines, the whole blood culture was stimulated with three different stimuli (+control) for 24 and 48 hours: Staphylococcal enterotoxin B (SEB) (final concentration in the well 100 ng/ml), LPS (100 ng/ml) and the combination of PMA (5 ng/ml) and ionomycin (1 µg/ml) (all from Sigma Chemicals, St. Louis, MO, USA, except LPS from Research Center Borstel, Borstel, Germany). After incubation, blood culture was transferred to Eppendorf tubes, centrifuged for 10 minutes at 800*g* and cell free -supernatant was collected and stored at -70 °C for later cytokine measurements.

In LUKAS study, following compounds were used in cytokine stimulation: Staphylococcal enterotoxin B (SEB) (Gram-positive bacterial origin, superantigen, activates cells in both innate and adaptive immunity), Gram-negative bacterial LPS (cell wall component of the Gram-negative bacteria, stimulates innate immunity) and the combination of the mitogenic protein kinase C -activator phorbol 12-myristate 13-acetate (PMA) and calcium ionophore ionomycin (induces adaptive responses). Superantigens, such as SEB, are able to bind at the same time to MHC II

molecules on the surface of APCs as well as T-cell receptors (TCRs) in T-cells (Müller-Alouf et al. 2001). As mentioned earlier, LPS is recognized by the TLRs in APCs. In MAA study, the combination of two mitogens, PMA/ConcanavalinA, was used to stimulate the production of cytokines.

4.4.1 Cytokine assay

In the MAA study, concentrations (pg/ml) of interleukin-6 (IL-6), tumor necrosis factor -alpha (TNF-α), interferon-gamma (IFN-γ) and IL-4 were measured from supernatants by using enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). The samples were run in duplicate and the analysis was performed according to the manufacturer's instructions. In-house validated sensitivity limits were as follows: 10 pg/ml (TNF-α), 4 pg/ml (IL-6), 8 pg/ml (IL-4) and 5 pg/ml (IFN-γ). The values of stimulated cytokine responses were more optimal following 8h stimulation and thus the values from 24h stimulation were excluded from further analyses.

In LUKAS studies, concentrations (pg/ml) of TNF-α, IFN-γ, IL-5, IL-8 and IL-10 were measured similarly to MAA by using ELISA (OptEIA™ Human ELISA set, BD Biosciences, San Diego, CA, USA). Determination of cytokines was performed according to the manufacturer's instructions with minor modifications (optimal antibody concentrations were selected according to the in-house titration experiments and the number of washes was increased). The ranges of valid cytokine measurements were as follows: IFN-γ (12.5 – 800 pg/ml), IL-5 (4.7 – 300 pg/ml), IL-8 (15.6 – 1000 pg/ml), IL-10 (6.3 – 400 pg/ml) and TNF-α (15.6 – 1000 pg/ml). Only the linear part of the standard curve was utilized. Cytokine concentrations below the detection limit were given a value 0 pg/ml. If the concentration of the measured cytokine exceeded the upper limit of the standard curve, the sample was diluted as many times as needed to obtain a concentration within the range. Cytokine results from both 24h and 48h stimulation were used in publication II. In publications I, III and V, the values for each cytokine were selected at the time point (24h or 48h) with higher percentage of samples exceeded the detection limits and when the measured concentrations were at their highest. LUKAS2 and MAA cytokine measurements were conducted in Environmental Toxicology Unit, THL, Kuopio. LUKAS1 cord blood cytokines were measured in PASTURE central laboratory (Philipps University of Marburg, Germany).

4.4.2 White blood cell count

Cytokine responses of LUKAS children were analyzed as absolute levels (pg/ml, publication I) and as the capacity of 10^6 white blood cells (WBC) to produce the measured cytokine (pg / 10^6 WBC, publications I-III, V). For the latter analysis, individual leukocyte counts were calculated from EDTA blood using Sysmex KX-21N blood cell analyzer (Sysmex Corporation, Kobe, Japan) (Environmental

Toxicology Unit, THL, Kuopio). In the MAA cohort, white blood cell counts were not preformed.

4.5 Analysis of immunoglobulin E (I–III, V)

In the LUKAS studies, allergen-specific immunoglobulin E (sIgE) for 13 inhalant and 6 food allergens was determined from serum samples of both mothers (at delivery) and their children (1 year after birth) by using the Allergy Screen Test Panel for Atopy (Mediwiss Analytic, Moers, Germany) (Herzum et al. 2005). IgE analyses were done in PASTURE central laboratory (Philipps University of Marburg, Germany). Test panel of aeroallergens included house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*), pollens (alder, birch, European hazel, grass pollen mixture, rye, mugwort and plantain) danders (cat, horse and dog), and the mold (*Alternaria alternata*) and a panel of food allergens contained hen's egg, cow's milk, peanut, hazelnut, carrot and wheat. Atopic sensitization was defined as sIgE -value $\geq$ 0.35kU/L for any of the measured allergens. Atopic sensitization was assessed separately for inhaled allergens (excluding food allergen sensitization) and food allergens (excluding inhalation allergen sensitization) and also for the combined group (sensitization against at least one inhalation and one food allergen). The prevalence of atopy and medium levels of sIgEs for LUKAS2 population are found in publication II, otherwise atopy was used as a confounding factor in the statistical analysis (III, V).

4.6 Microbial determinations (IV, V)

4.6.1 Collection of house dust

To determine house dust microbial composition in MAA homes, three different indoor dust sample types were collected 3 months after birth: bed dust, floor dust and dust from the bag of the home's vacuum cleaner. The collection method has been described earlier (Hyvärinen et al. 2006b). In brief, bed dust was sampled by vacuuming the mother's mattress for 2 minutes (duvets and blankets were removed but not undersheets). Floor dust was vacuumed from the rug of mother's bedroom (2 minutes, area of 1m^2) or directly from the floor in the absence of rug. A cumulative dust bag dust sample from the vacuum cleaner of each home was obtained for a known period (2 to 4 months). At that time a new dust bag had been placed in home's vacuum cleaner or in the case of central vacuum cleaner, the dust container had been cleaned.

In LUKAS2 homes, microbial analyses used in this thesis were done only from floor dust samples, which were collected from the living room floor 2 months after delivery. The living room was defined as the room where family spent most of the

time in the evenings after dinner. Dust was sampled from the rug by vacuuming the area of $1m^2$ for 2 minutes or in the absence of rug, area of $4m^2$ from the bare floor.

4.6.2 Determination of chemical markers (IV, V)

Both MAA and LUKAS2 house dust samples were analyzed for same chemical markers using gas chromatography tandem mass spectrometry (GC-MS-MS): ergosterol (ng/mg) as a marker of fungal biomass, 3-hydroxy fatty acids with carbon chain lengths from 10 to 14 (3-OH FAs, $C_{10:0}$-$C_{14:0}$) indicating the presence of Gram-negative bacteria (µmol/mg) (marker for endotoxin) and muramic acid (ng/mg) as a Gram-positive bacterial marker (marker for peptidoglycan). Chemical markers were measured in the Environmental Microbiology Unit, THL, Kuopio. For a detailed description of the GC-MS-MS assay see publication IV.

4.6.3 Quantification of microbial species (V)

Concentrations for specific bacterial and fungal species were determined only from LUKAS2 floor dust samples using quantitative real time polymerase chain reaction (qPCR) as described earlier (Haugland et al. 2004; Rintala and Nevalainen 2006; Torvinen et al. 2010). Cell amounts (cells per milligram of dust, cells/mg) of two Gram-positive bacterial genera were quantified: *Mycobacterium* spp. and *Streptomyces* spp. In addition, concentrations of the following fungi were measured: *Trichoderma viride* / *atroviride* / *koningii* (=*Trichoderma viride* group), *Wallemia sebi* and the combined assay group for *Penicillium* spp., *Aspergillus* spp. and *Paecilomyces variotii* (=*PenAsp* group). All qPCR-analyses were performed in the Environmental Microbiology Unit, THL, Kuopio.

4.7 Statistical analysis

In statistical tests, cytokine levels were always treated as continuous variables. Children in the MAA cohort were classified into two exposure groups according to the median concentration of each chemical marker: “low exposure group” as below the median and “high exposure group” as above the median. In LUKAS2, children were classified into three categories of low, moderate and high microbial exposure using upper and lower quartiles of indoor dust microbial concentration as cutoff points (low = <25%, medium = 25-75%, high >75%). In addition, in order to make a better estimation about the overall exposure to microbes, categorized microbial variables were numbered (Low = 1, Medium = 2 and High = 3), summed and further divided into tertiles. The following combined microbial variables were created: combined Gram-positive bacteria (including muramic acid, *Mycobacterium* spp. and *Streptomyces* spp.), combined indoor fungi (PenAsp group, *T.viride* group, *W. sebi*), combined fungi (PenAsp group, *T.viride* group, *W. sebi* and ergosterol) and combined chemical markers (muramic acid, 3-OH FAs and ergosterol). Statistical

methods used in thesis are listed in Table 3. All analyses were made using SPSS software versions 15.0 – 17.0.

Table 3. **Statistical methods used in studies I - V**

Study	Analysis	Method
I	- Differences in maternal and neonatal factors between two parts of the cohort	Chi-square test / Mann-Whitney U-Test
	- Univariate associations between cytokine responses and maternal, neonatal and birth -related factors	Mann-Whitney U-Test / Kruskal-Wallis Test
	- Adjusted associations between cytokine responses and selected maternal, neonatal and birth -related factors	Linear Regression
II	-Correlation between cytokine responses	Spearman's rank correlation
	-Differences in cytokine responses between mothers vs children and birth vs 1 year of age.	Wilcoxon signed rank test
	-Associations between cytokine responses and atopy	Mann-Whitney U-Test
III	-Univariate associations between cytokine responses and animal exposures	Mann-Whitney U-Test
	-Adjusted associations between cytokine responses and animal exposures	Linear Regression
IV	-Cytokine production in relation to chemical marker exposure	Mann-Whitney U-Test
	-Correlation between maternal and neonatal cytokine responses	Spearman's rank correlation
	-Correlation between chemical marker concentrations	Pearson correlation
	-Correlation between different dust sampling methods	Pearson correlation
	-Chemical marker concentrations in relation to farming	Independent-Samples T-test
V	-Screened associations between cytokine responses and microbial exposures	Mann-Whitney U-Test / Kruskal-Wallis Test
	-Adjusted associations between cytokine responses and microbial exposures	Linear Regression
	-Correlation between microbial concentrations	Spearman's rank correlation
	-Differences between determinants and microbial concentrations	Mann-Whitney U-Test / Kruskal-Wallis Test

4.7.1 Adjustment for covariates (I, III, V)

In publications III and V, linear regression models were used to adjust for the possible confounding effect of several variables. The following variables were selected for further analysis since they were considered to have a biologically plausible effect and since the preliminary results obtained in multivariate linear regression tests, which included wide variety of different potential covariates suggested associations on the stimulated cytokine responses: mode of birth, number of earlier deliveries, use of antibiotics during pregnancy, maternal atopy (sIgE $\geq$ 0.35 kU/L), maternal doctor-diagnosed asthma, maternal education, number of 0 to 12 year old siblings in the household, maternal smoking and gender of the child.

In publication I, the covariates included partly the same variables than in publications III and V (mode of birth, number of earlier deliveries, maternal education, maternal smoking and gender of the child). In addition, many potential obstetric factors and maternal characteristics were considered (see publication I for details). The findings were always adjusted for the cohort (LUKAS1 or LUKAS2), paternal farming status and the presence of maternal hay fever.

5 RESULTS

5.1 Effects of perinatal factors on cord blood immune responses (I)

The influence of different maternal, neonatal and obstetric factors on cord blood cytokine responses were analyzed for P/I-stimulated IL-5, IL-10 and IFN-γ levels from the LUKAS1 and LUKAS2 study population.

In univariate tests, children born in the spring (March-May) had lower levels of IL-5, IL-10 and IFN-γ than those born in the fall (September-November). Even when cytokine production was corrected with the WBC counts (unit as pg/10^6 WBC), associations remained similar (Figure 4). Multivariate covariance analysis did not change the results. An increasing duration of labour appeared to result in a linear increment in the levels of cord blood IL-5 and IFN-γ and WBC counts. However, after WBC correction, the associations between cytokines and duration of labour were no longer statistically significant. In addition, mode of delivery had an influence on the IL-5 responses and the number of leukocytes at birth: the highest levels were measured after assisted vaginal and lowest after elective caesarean section. Statistical significance was not reached after correction of IL-5 levels with the WBC count. Induction of the labour with prostaglandin decreased IFN-γ levels, also after WBC-correction, and IL-10 levels only after WBC-correction. The results concerning prostaglandin induction were also similar after covariance analysis.

In the univariate tests, the production of IL-5, IL-10 and IFN-γ in cord blood was influenced by certain parental and neonatal characteristics. Compared to girls, boys produced more IL-5 at birth, also after WBC-correction. The lowest cord blood IL-5 responses, also WBC-corrected levels, were measured in those children whose mothers smoked during pregnancy. Neonates with the lowest birth weight had also the lowest leukocyte counts and IL-10 responses. Apgar scores were inversely associated with the levels of IFN-γ. Firstborn children seemed to have higher levels of IL-5 and IFN-γ than children with elder siblings. However, WBC-corrected cytokine responses were not associated with birth weight, apgar scores or with maternal parity. The presence of maternal hay fewer was associated with higher WBC-corrected production of IL-5. High concentration of cord blood IL-10 seemed to be associated with academic schooling of mothers. Paternal farming status was associated with a decrement of the all measured cytokine responses and IL-5 and IFN-γ also after WBC-correction. In the multivariate models, associations remained statistically significant for gender of the child, birth weight, maternal education and maternal smoking.

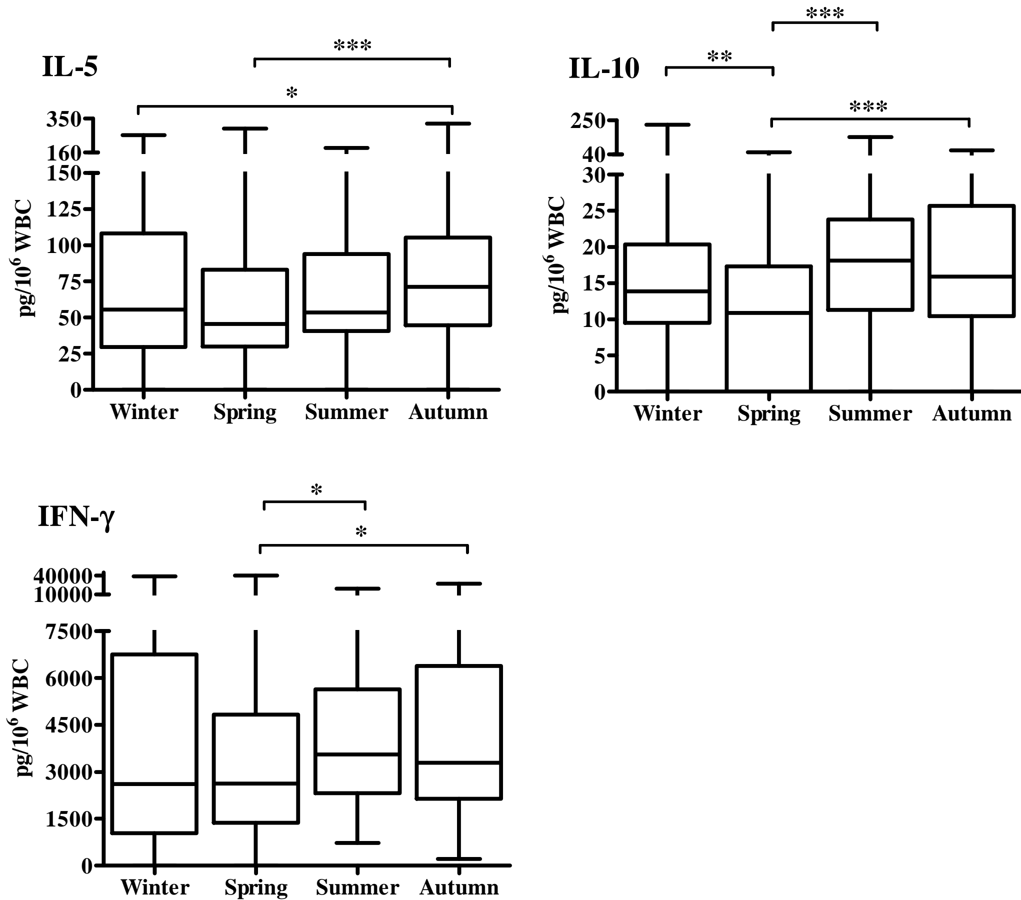

Figure 4. Cord blood cytokine responses in relation to the season of the birth. **Associations between birth season and the white blood cell corrected levels (pg/10^6WBC) of IL-5, IL-10 and IFN-γ in cord blood following stimulation with phorbol ester and ionomycin (P/I). Box plots represent the interquartile range, median (horizontal line) and extremes (whiskers) of individual delta values. Mann-Whitney U-Test, *p < 0.05; **p < 0.005; ***p < 0.001.**

5.2 Development of immune responses from birth to 1 year

In MAA cohort (IV), 8-hour PMA/ConA-stimulated cytokine responses were measured at birth (children) and 3 months after delivery (children and mothers) (Figure 5). Median concentrations (pg/ml) of TNF-α and IL-6 were higher at birth than at the age of 3 months and also higher than in the maternal samples. Secretion of IFN-γ was the lowest at birth and the highest in maternal samples. Levels of IL-4 were mostly below the detection limits and thus could not be analyzed further.

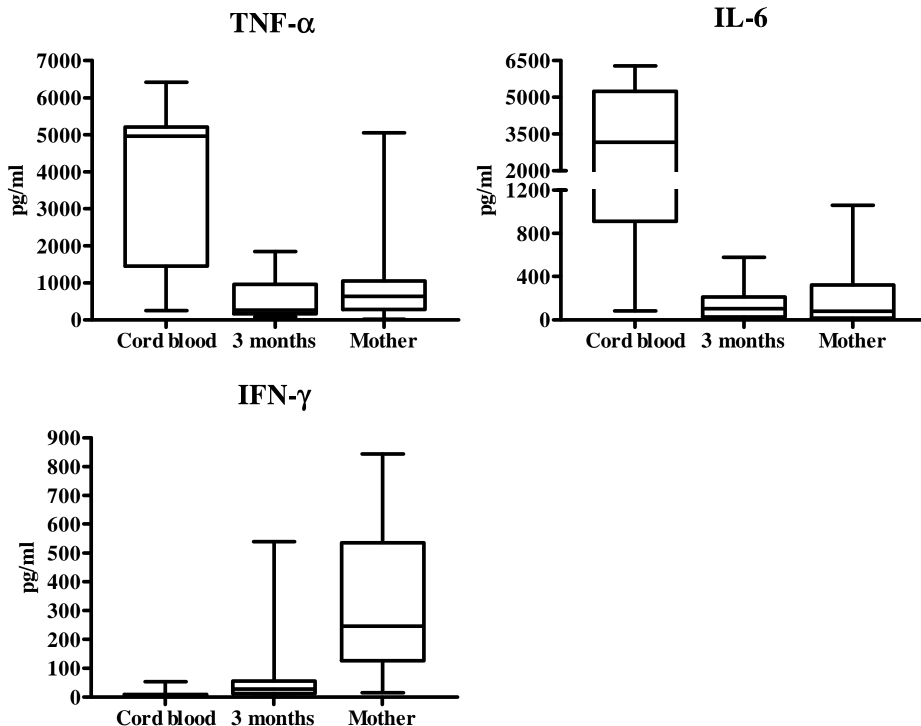

Figure 5. Development of cytokine responses from birth to 3 months of age and comparison to mothers. **Production (pg/ml) of TNF-α, IL-6 and IFN-γ following 8h stimulation with PMA/ConA in cord blood and in peripheral blood of children and their mothers 3 months after birth. Box plots represent the interquartile range, median (horizontal line) and extremes (whiskers).**

Immune development in LUKAS2 (II) was evaluated as changes in cytokine - producing capacity of 10^6 white blood cells (pg/10^6 WBC) at birth (children) and at 1 year after birth (mothers and children). In general, the children's cytokine - producing capacity increased from birth to 1 year of age independently of the stimuli, but was lower compared to the maternal responses (Figure 6). However, P/I and LPS-stimulated IL-8 responses appeared to be an exception, being at their highest in cord blood.

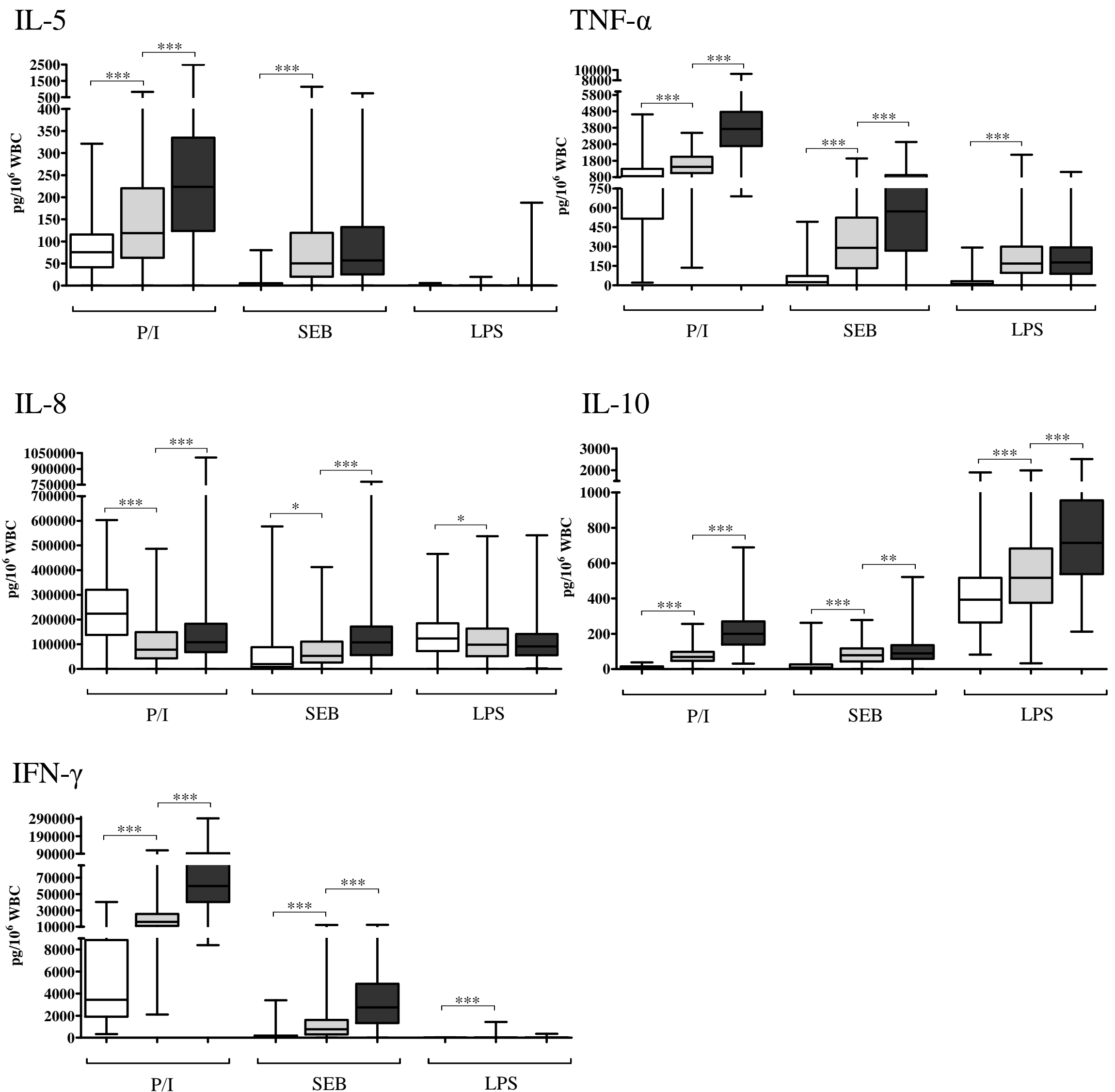

Figure 6. Development of cytokine -producing capacity from birth to 1 year of age and comparison to mothers. **White blood cell corrected levels (pg/10^6 WBC) of IL-5, TNF-α, IL-8, IL-10 and IFN-γ following 48h stimulation with P/I, SEB and LPS in cord blood (empty boxes) and in peripheral blood of children (light grey) and their mothers (dark grey) 1 year after birth. Box plots represent the interquartile range, median (horizontal line) and extremes (whiskers) of individual delta values. Wilcoxon signed rank test, *:p≤0.05; **:p<0.01; ***:p<0.001.**

5.2.1 Comparison of maternal and neonatal cytokine responses (II,IV)

Among the MAA participants, statistically significant mother-to-child cytokine correlations were detected for the proinflammatory cytokines (Spearman's rho: TNF-α: r=0.659, $p<0.01$; IL-6: r=0.480, $p<0.05$), but not for the Th1 cytokine (IFN-γ: r=0.308, p=0.142) at 3 months after birth.

In LUKAS2, all of the stimulated cytokine responses showed significant mother-to-child correlations 1 year after birth (Table 4) whereas intraindividual correlations between cytokine levels at birth and at 1 year of age were weaker: Statistically significant correlations were seen between IL-10 (LPS stimulation), TNF-α (P/I and LPS), IFN-γ (P/I and SEB) and IL-8 (P/I) responses.

Table 4. **Spearman's correlation coefficients between cytokine responses (birth-to-1 year and mother-to-child, 1 year) following 48h stimulation with PI, SEB and LPS.**

	P/I	SEB	LPS	P/I	SEB	LPS
	Birth vs 1 year of age			Child vs Mother, 1 year after birth		
IL-5	0.14	0.08	n.a.	0.30***	0.42***	n.a.
N	140	142		191	193	
IL-10	0.04	-0.01	0.20*	0.30***	0.26***	0.26***
N	140	142	143	191	193	194
TNF-α	0.31***	-0.06	0.31***	0.31***	0.48***	0.40***
N	140	142	142	191	193	193
IFN-γ	0.20*	0.18*	n.a.	0.28***	0.43***	n.a.
N	140	142		191	193	
IL-8	0.24**	0.02	0.11	0.25***	0.15*	0.25***
N	140	142	143	190	189	193

n.a. = not applicable. Of the measured cytokine values > 80% were below the detection limits.

*p<0.05, **p<0.01, ***p<0.001

5.2.2 Neonatal cytokine -producing capacity in relation to atopy (II)

In this thesis, associations between atopic sensitization and cytokine responses at age 1 year were assessed only among the LUKAS2 participants. In addition, the influence of atopic heredity on infants' immune responses was analyzed. Atopic sensitization was defined as any allergen specific IgE-value ≥ 0.35kU/L. Atopic heredity was defined as maternal sensitization against any of measured allergens. In the children, the prevalence of sensitization against inhaled allergens (21%) was close to that of food allergens (23%). In the mothers, sensitization against inhalant allergens was more prevalent than against food allergens (52% vs 21%, respectively).

The capacity to secrete cytokines did not differ consistently between children sensitized against inhalation allergens and non-sensitized children either at birth or at the age of 1 year. Similarly, only a few associations were found between cytokine -producing capacity and food allergen sensitization in children. Interestingly, children sensitized against both inhalation and food allergens had lower 48h P/I-induced IL-5 responses and higher IFN-γ/IL-5 -ratio (24h and 48h P/I; 48h SEB stimulation) at the age of 1 year than their non-sensitized counterparts.

The influence of atopic heredity (maternal sensitization) on neonatal 24h and 48h P/I-stimulated cytokine responses is presented in Table 5. Maternal sensitization against inhalation allergens was associated with increased cord blood IL-10 and IL-5 production. No differences were found in cytokine -producing capacity at 1 year of age in relation to maternal sensitization against inhalation allergens.

Food allergen sensitization of the mothers was associated with decreased P/I-stimulated IFN-γ/IL-5 -ratio and increased IL-10 responses at birth (Table 5). At the age of 1 year, P/I-induced ratio of IFN-γ to IL-5 and IFN-γ levels were decreased in children of food allergen sensitized mothers. Maternal sensitization against both inhaled and food allergens was not consistently associated with their children's immune responses. Only 48h LPS-induced IL-10 secretion in cord blood was associated with maternal atopy (atopic median 516 pg/10^6 WBC vs non-atopic 396, p=0.04).

Table 5. **The effects of atopic heredity (maternal atopic sensitization) on 24h and 48h P/I-stimulated cytokine responses (median, pg/10^6 WBC) of children at birth and at the age 1 year.**

		Maternal inhalation atopy*			**Maternal food atopy****		
		No	**Yes**	**P-value**	**No**	**Yes**	**P-value**
	Cytokine	N=85	N=80		N=85	N=14	
Cord blood							
P/I, 24h	IL-5	27.7	39.9	**<0.01**	27.6	33.6	0.22
	IL-10	13.2	14.8	0.08	13.2	17.3	0.14
	TNF-α	978	1080	0.45	978	1230	0.45
	IFN-γ	2770	3050	0.82	2770	1380	0.16
	IL-8	110000	101000	0.36	110000	108000	0.50
	IFN-γ/IL5	94.3	87.7	0.10	94.3	43.7	**0.01**
P/I, 48h	IL-5	63.6	88.2	**<0.01**	63.6	79.8	0.36
	IL-10	9.87	12.6	**<0.01**	9.87	13.9	**<0.01**
	TNF-α	823	898	0.36	823	753	0.98
	IFN-γ	2980	3770	0.31	2980	2800	0.79
	IL-8	251000	209000	0.33	251000	293000	0.23
	IFN-γ/IL5	57.6	47.2	0.32	57.6	39.2	0.29
Children, 1 year after birth							
P/I, 24h	IL-5	58.5	58.4	0.76	58.5	93.6	0.27
	IL-10	78.7	86.7	0.29	78.7	86.4	0.46
	TNF-α	1330	1280	0.86	1330	1270	0.90
	IFN-γ	9050	7380	0.21	9050	4690	**<0.01**
	IL-8	37300	38000	0.55	37300	43600	0.16
	IFN-γ/IL5	160	138	0.48	160	76.3	**<0.01**
P/I, 48h	IL-5	117	117	0.61	117	159	0.54
	IL-10	68.6	72.5	0.23	68.6	71.2	0.55
	TNF-α	1440	1420	1.00	1440	1800	0.70
	IFN-γ	16400	17100	0.82	16400	11000	**0.02**
	IL-8	84200	68900	0.50	84200	79400	0.88
	IFN-γ/IL5	168	146	0.39	168	61.7	**<0.01**

IFN-γ/IL5 is the ratio of IFN-γ to IL-5. *Maternal sensitization against inhalation allergens only, mothers sensitized against food allergens excluded. **Maternal sensitization against food allergens only, mothers sensitized against inhalation allergens excluded. Statistical test: Mann-Whitney U-Test.

5.3 The effects of animal exposures on immune development (III)

Associations between animal exposures and immune responses were assessed only from the LUKAS2 cohort. Keeping animals and/or contacts were surveyed by self-administered questionnaires collected during the last trimester of pregnancy.

During the last trimester of pregnancy, it was more common to have a dog (36%) than a cat (17%) in the household. Pets were typically kept indoors. Only 7% of the dogs and 10% of the cats were exclusively kept outdoors. Maternal contacts with farm animals were uncommon, but 11% of the mothers were regarded as being horse exposed. Only 4.8% of the mothers reported living in an actively operating farm.

The main finding was that having a dog in the household during pregnancy and during the first year of life was associated with decreased TNF-α -producing capacity of the children both at birth (P/I stimulation) and 1 year after birth (P/I and LPS stimulation). Associations remained statistically significant also after adjustment for confounding factors and exclusion of children from farming families (Figure 7). Moreover, maternal horse exposure during pregnancy was associated with lowered cord blood TNF-α responses (SEB and LPS stimulation) in univariate tests, but not after controlling for confounders. Cat keeping did not have any consistent influence on the measured cytokine responses.

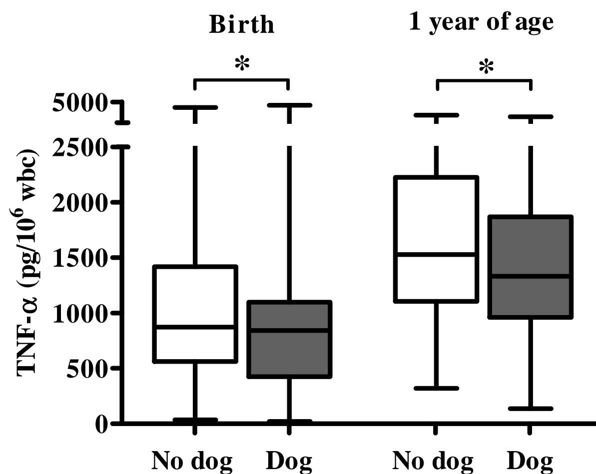

Figure 7. Associations between dog ownership and TNF-α responses. **P/I-stimulated (48h) TNF-α -producing capacity (pg/10^6 wbc) at birth and 1 year after birth in children from non-farming households with (filled boxes) or without (empty boxes) a dog. Box plots represent the interquartile range, median (horizontal line) and extremes (whiskers) of individual cytokine Δ-values. *p≤0.05 (after multiple linear regression analysis of ln-transformed data).**

5.4 The effects of microbial exposures on immune development (IV, V)

5.4.1 Exposure to bacteria

Both MAA and LUKAS2 house dust samples were analyzed for the concentrations of 3-hydroxy fatty acids with a carbon chain length from 10 to 14 (3-OH FAs, $C_{10:0}$-$C_{14:0}$) indicative of the presence of Gram-negative bacteria and muramic acid assessing the presence of Gram-positive bacteria. In addition, two Gram-positive bacterial genera (*Mycobacterium* spp. and *Streptomyces* spp.) were analyzed from LUKAS2 house dust.

In the MAA cohort (IV), markers were determined from three different types of house dust samples: floor dust, bed dust and dust from a vacuum cleaner (see 4.6.1). Inverse associations were found between the levels of PMA/ConA-induced proinflammatory cytokine TNF-α at the age 3 months and concentrations of 3-OH FAs ($C_{10:0}$-$C_{14:0}$) in bed dust (Figure 8). High exposure to muramic acid (determined from floor dust) was associated with elevated TNF-α responses (Figure 8). In addition, the production of another proinflammatory cytokine, IL-6, was increased in children both at birth and at the age 3 months if the concentrations of floor dust muramic acid were high (IL-6 at birth, high exposure median 5200 pg/ml vs. low exposure median 912 pg/ml, $p<0.01$; at 3 months, see publication IV Figure 3.). With the exception of the above mentioned association between muramic acid and the production of IL-6 at birth, cord blood cytokine responses were not associated with the levels of chemical markers. The chemical markers analyzed from the dust bag dust were not consistently associated with any of the measured cytokines.

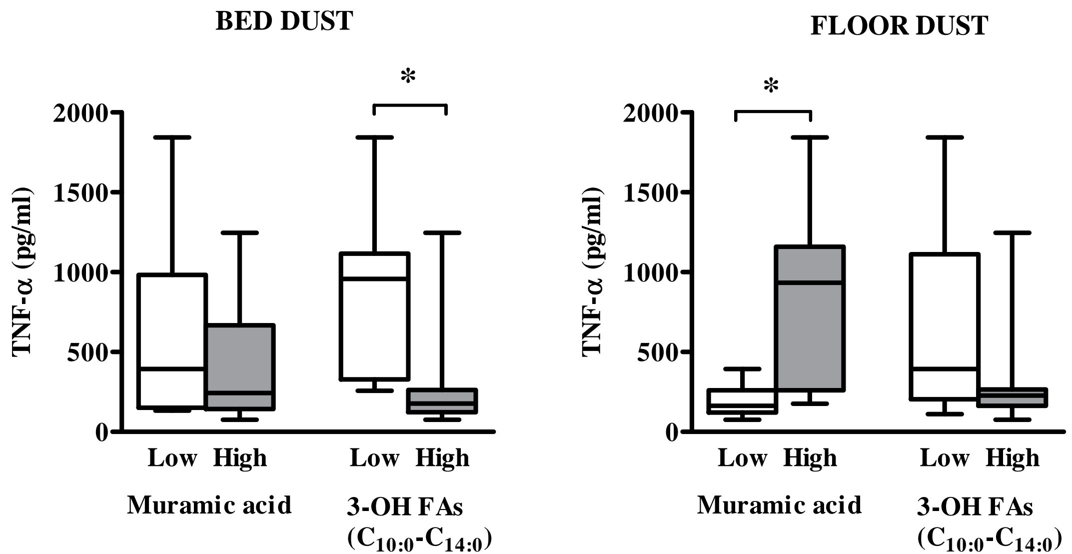

Figure 8. Proinflammatory cytokine responses at the age 3 months in relation to bacterial marker exposure. **PMA/ConA-induced production of TNF-α in whole blood cultures of 3-months old children with low and high concentrations of Gram-negative (3-OH fatty acids) and Gram-positive (muramic acid) bacterial markers in bed and in floor dust. Box plots represent the interquartile range, median (horizontal line) and extreme values (whiskers). Mann-Whitney U-Test, *p<0.01.**

In LUKAS2 (V), only floor dust microbial data were included in this thesis. An increase in the house dust levels of *Mycobacterium spp.* and in Gram-positive bacterial exposure in general (combined Gram-positive bacterial -variable, see 4.7) were associated with the reduced IFN-γ -producing capacity following SEB stimulation, especially at the age of 1 year (Figure 9). Adjustment for covariates had only a minor influence on the findings at this age. Similar associations were found also for *Streptomyces* spp., especially at birth, but after controlling for confounders, the relationships became weaker. No associations were observed between cytokine responses and the concentrations of 3-OH FAs ($C_{10:0}$-$C_{14:0}$).

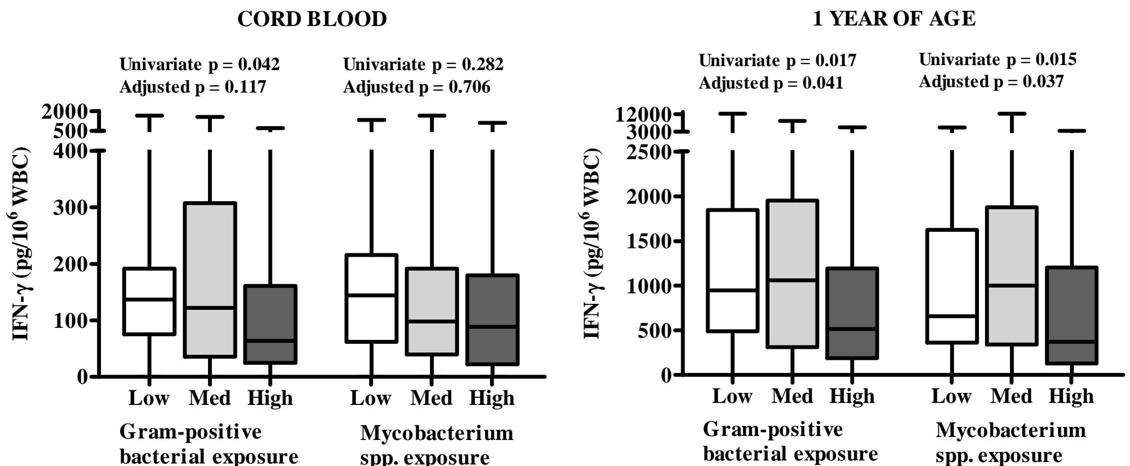

Figure 9. Th1 cytokine responses at birth and 1 year after birth in relation to Gram-positive bacterial exposures. **SEB-induced (48h) IFN-γ -producing capacity (pg/10^6 WBC) in relation to indoor dust Gram-positive bacterial exposure (low, medium high). Box plots represent the interquartile range, median (horizontal line) and extremes (whiskers) of individual cytokine Δ-values. Univariate p-value from Kruskal-Wallis test, adjusted p-value from linear regression.**

5.4.2 Exposure to fungi

Ergosterol, as a marker for fungal biomass, was measured from both LUKAS2 and MAA dust samples. Fungal species specific analyses (qPCR) were conducted only from the LUKAS2 floor dust samples, which included *Trichoderma viride* group, *Wallemia sebi* and *PenAsp* group (for the group definitions see 4.6.3).

Ergosterol exposure was not consistently associated on measured immune responses either in MAA or in LUKAS2 study. In LUKAS2 study, fungal exposure in general (combined fungal variables) was associated with decreased production of SEB-stimulated IFN-γ in univariate tests, but not after adjustment for confounders.

6 DISCUSSION

The findings from these prospective birth cohort studies demonstrate that most of the immune responses in the newborns are clearly strengthened during the first year of life, though they are still weaker as compared to adults. Some birth-related factors along with certain maternal and neonatal characteristics were associated with neonatal cytokine production. There were also indications that early life immune functions are likely to be affected by indoor microbial exposures and also by exposure to dogs.

6.1 Perinatal determinants of cord blood immune responses

Associations were assessed from the LUKAS participants for P/I-stimulated cord blood IL-5, IL-10 and IFN-γ. Several potential neonatal, maternal and obstetric factors were detected, which appeared to have an influence on the secretion of these cytokines.

One interesting finding was that the season of the birth had very consistent effect on neonatal cytokine responses. Levels of IL-5, IL-10 and IFN-γ were lower in children who were born in the spring compared to those who were born in the fall. Correction of the cytokine production by taking into account the white blood cell count (WBC) or the adjustment for confounders did not change these results. Since the associations were similar also with WBC-corrected cytokine values, it appears that the birth season affects the functional capacity of the cord blood leukocytes to produce these cytokines. These results are in agreement with the recent findings showing that stimulated production of IL-10 and IFN-γ in cord blood mononuclear cells (CBMCs) is greatest in the fall and winter (Gold et al. 2009) or in the summer and fall (Sullivan Dillie et al. 2008). However, it appears that the true influence of the season of the birth on neonatal cytokine responses is not straightforward, since highest secretion of IL-5 and IL-13 in CBMCs has been measured in children born during spring and summer time (Sullivan Dillie et al. 2008). On the other hand, no consistent associations were found between cytokine production (IL-5 and IFN-γ) in CBMCs and winter birth in another study (Lendor et al. 2008). The higher cord blood cytokine levels in children born in the fall may be due to maternal allergen exposures during spring and summer. High-level pollen exposures during pregnancy may increase the risk of sensitization and symptoms of childhood atopy (Kihlstrom et al. 2003). However, the influence of month of birth on atopy development is far from clear (Knudsen et al. 2007; Saitoh et al. 2001; Yoo et al. 2005).

In addition to allergen exposures, the effect of the birth season could be explained by the different amount of sunlight exposure that the mothers experience during pregnancy. Serum levels of biologically active D-vitamin depend greatly on the skin exposure to ultraviolet light B, which in Finland is highest in summer time. Seasonal changes in maternal D vitamin status are reflected in the neonatal serum levels of vitamin D metabolites (Kuoppala et al. 1986; Nehama et al. 1987). An increase in cutaneous vitamin D may promote the proliferation of immunosuppressive T-cell population, T-regulatory cells (Tregs) (Loser and Beissert 2009), which are able to produce IL-10. Furthermore, in an epidermal cell line, vitamin D metabolite (1alpha,25-dihydroxyvitamin D3, known as calcitriol) is able to induce the expression of IL-10 receptors (Michel et al. 1997). Indeed, low neonatal levels of calcitriol have been linked to the reduced cord blood IL-10 concentrations (Zittermann et al. 2004). Calcitriol-treatment may increase the secretion IL-5 and IL-10 in peripheral blood mononuclear cells (PBMCs), but on the other hand they may decrease the production of IFN-γ (Jirapongsananuruk et al. 2000; Rausch-Fan et al. 2002). The production of IL-10 is poor in dendritic cells which have matured in the absence of the vitamin D metabolite (Canning et al. 2001).

Increased duration of labour and the assisted vaginal delivery appeared to be associated with an increase in the levels of IFN-γ, IL-5 and WBC counts in cord blood. The normal vaginal birth process is a stressful event for neonates, initiating the production of acute phase proteins and proinflammatory cytokines (Marchini et al. 2000). As expected, no statistically significant associations were observed after WBC-correction of the cytokine levels, which indicates that elevated cytokine responses are mainly attributable to the birth related leukocytosis. The production of IL-10 was not associated with the above mentioned birth related factors, which is in line with an earlier report (Power et al. 2002).

In this thesis, inverse associations were found between the levels of cord blood IL-10 and IFN-γ and prostaglandin induced labour. Prostaglandins are commonly used to hasten the onset of labor. The effects of prostaglandin induction on cord blood cytokines have not been evaluated previously. The importance of the observed associations on neonatal immune development is not clear and needs to be studied in more detail.

With respect to the neonatal and maternal characteristics, male gender of the child was associated with increased cord blood IL-5 responses and maternal smoking during pregnancy was linked with decreased IL-5 levels. In another study, no differences were found in cord blood IL-10, IL-5 and IFN-γ responses between boys and girls with atopic heredity (Uekert et al. 2006). However, at the age 3 years, boys had higher IL-5 levels than girls (Uekert et al. 2006). Although smoking during pregnancy seems to be an important risk factor for childhood respiratory disorders (Prescott 2008), the effect on cord blood cytokine responses is not clear (Macaubas et al. 2003; Noakes et al. 2003, 2006). An inverse association was found between

birth weight and the levels of IL-10 in this study. In the recent study, levels of IL-10 in CBMCs were not affected but IFN-γ responses were reduced in children with low birth weight (Gold et al. 2009).

In summary, these findings indicate that there are several neonatal and maternal characteristics and birth-related factors, which may alter the functions of sensitive immature immune system of the child.

6.2 Neonatal immunologic maturation

6.2.1 Development of cytokine responses during the first year of life

Among the LUKAS2 children, independently of the stimulation, white blood cell corrected production of IL-5, IL-10 TNF-α and IFN-γ was lower at birth than at the age 1 year or in their mothers. However, IL-8 -producing capacity was highest at birth (even higher than maternal production) following P/I and LPS stimulation. There is an extensive literature to support the present findings that most of the adaptive and innate immune responses following antigen stimulation are deficient during the neonatal period (Adkins et al. 2004; Holt and Jones 2000; Levy 2007; Levy et al. 2004; Marodi 2006; Schultz et al. 2007) but they increase rapidly with age (Buck et al. 2002; Halonen et al. 2009; Härtel et al. 2005; Smart and Kemp 2001). The observed attenuation of several neonatal cytokine responses may be one reason for the fact that newborns are susceptible to pathogenic microbial infections.

It is important to consider that the presentation of the cytokine data is crucial in how one interprets the final conclusions. For example, MAA children had higher stimulated proinflammatory cytokine concentrations (both TNF-α and IL-6) in cord blood than at the age of 3 months or in the maternal samples. Indeed, under certain experimental circumstances, neonatal immune responses may exceed that those found in adults (Angelone et al. 2006; Levy et al. 2006; Yerkovich et al. 2007). The cytokine responses in MAA study were presented only as absolute levels without the WBC-correction. Cord blood has higher leukocyte counts than blood samples of older children or mothers and this seems to be the major explanatory factor for the high cord blood cytokine responses in MAA study design (see 6.5. for more details).

It was interesting to note that already at 3 months of age, proinflammatory cytokine responses correlated with those of mothers. IFN-γ responses did not correlate, probably because the production of IFN-γ is poor during the first year of life (Holt and Jones 2000; Rowe et al. 2001). One year after birth, all of the measured cytokines, independently of the stimuli, expressed statistically significant mother-to-child correlations. However, the correlation coefficients were not very high, which suggests that maternal cytokine environment explains only a part of the variation of cytokine responses in children. There appears to be a correlation also between the numbers of cytokine -producing cells in mothers and their 2-year old

children (Larsson et al. 2006). Presumably, genetic factors are an explanation for observed mother-to-child cytokine correlations but it is likely that the correlations are strengthened by the same living environment and lifestyle habits between mothers and their children.

In summary, these findings postulate that in general terms the neonatal capacity to respond to different innate and adaptive stimuli is deficient. Although the secretion capacity of the whole blood leukocytes is enhanced for most of the cytokines from birth to 1 year of age, certain cytokine responses in specific situations may be at their highest at birth, which further indicates that not all neonatal immune responses are immature.

6.2.2 Differences in neonatal cytokine response in relation to atopy

The risk of neonates to manifest the atopic diseases depends strongly upon the family history of atopy (Moore et al. 2004; Tamay et al. 2007; Tariq et al. 1998). All newborns have naturally a humoral, "allergic" Th2 -emphasized cytokine environment (Prescott et al. 1998). It has been postulated that soon after birth, secretion of Th2 cytokines is augmented in those children who will develop atopy later in childhood whereas in non-atopic children, the corresponding responses will be down-regulated (Jones et al. 2000). Some studies have implied that neonates with an atopic heredity have a stronger bias towards Th2-balanced responses after antigen stimulation (Gabrielsson et al. 2001; Kopp et al. 2001; van der Velden et al. 2001). Also in this thesis, maternal history of atopy (defined as any allergen specific IgE $\geq$ 0.35 kU/L) was associated with an increased capacity to secrete Th2 cytokines (IL-10 and IL-5) and decreased ratio of IFN-γ to IL-5 in both neonatal cord blood and at the age 1 year. This phenomenon may be related to the delayed maturation of IFN-γ secretion of the T-cells among children with atopic heredity (Holt et al. 1992).

Although in the present study maternal atopy appeared to have some influence on neonatal immune functions, atopy of the children at 1 year of age was not consistently associated with stimulated cytokine responses at birth. In fact, there was a tendency towards a higher SEB and P/I-induced cell-mediated Th1-type response at the age of 1 year in the children sensitized against both food and inhalation allergens compared to their non-atopic counterparts. This is interesting because some findings with allergen-specific IFN-γ responses have been rather similar (Kimura et al. 2002; Ng et al. 2002; Prescott 2003; Smart and Kemp 2002), but not with SEB stimulation (Smart and Kemp 2002). Since early childhood is the time of rapid and extensive immunologic development, it is possible that this natural maturation process evokes a distortion to the sensitive cytokine network during the first year of life, and thus children, who became atopic, express a mixed Th1/Th2-model (=Th0) rather than clear cut Th2 responses (Heaton et al. 2005; Holt et al. 2000; Ng et al. 2002). On the other hand, when the definition of atopy is based merely on allergen specific IgE measurements in early life, there are some aspects

which should be taken into account. Early sensitization to inhaled allergens may not predict very well later atopy development, whereas early life sensitization against food allergens may be a better predictor (Kjaer et al. 2009). The lack of consistent associations in this study could also be due to possible atopy misclassification caused by rather extensive IgE sensitization measurements including 19 different allergens. In the classification used here, children with only one positive IgE result (sIgE ≥ 0.35 kU/L) were regarded as being atopic, probably the definition of atopy was not strict enough and this may have increased the number false positive cases.

In summary, a positive family history of allergy could be a better predictor for the later development of atopy than the levels of early life allergen specific IgEs or cytokines (Prescott et al. 2003). It also seems likely that the immunologic mechanism for the development of atopy cannot be explained simply by the balance between Th1 and Th2 responses. It is more likely that the complex interplay between different cells in both innate and adaptive immunity and the early immunological maturation process itself determines who becomes atopic later in life.

6.3 Animal exposures and immune development in early life

This study revealed that proinflammatory cytokine (TNF-α) responses were decreased in children of dog owners at birth and at the age of 1 year. Cat ownership did not have any influence on measured cytokine responses, which may be partly explained by the differences in social behavioral factors and species specific characteristics between dogs and cats. For example, direct contact with cats may be less intense than that with dogs. Because cats are usually smaller than dogs, they may carry and spread less microbial particles and allergens indoors. The current literature concerning the effects of early life animal exposures on cytokine responses is very limited. Gern et al (2004) observed that postnatal exposure to dogs may influence immune development by increasing IL-10 and IL-13 levels in dog-exposed children at the age 1 year with a family history of atopy and asthma. However, these workers did not measure the production of TNF-α. Moreover, cat and/or dog ownership has been linked to increased IFN-γ responses at the age 3 months but no associations were detected in TNF-α responses (Roponen et al. 2005).

Avoidance and/or removal of pets can cause a serious bias in the observed associations between pets and measures of immune response (Bornehag et al. 2003; Brunekreef et al. 1992). In this study, about 25% of the households reported avoidance of pets or removing pets at some phase of life (before the birth of the study child) because of allergies. The potential confounding effect of pet avoidance was assessed in regression models, but the observed associations between dog exposure and cytokine responses were not affected. At least dog allergy of the mothers (maternal serum sIgE ≥ 0.35 kU/L against dog dander) did not seem to be a major reason to avoid dogs, because dog keeping among dog dander -sensitized

mothers (38%) was similar to those of non-sensitized (35%). In addition, cat keeping was similar between cat-sensitized mothers (19%) and their non-sensitized counterparts (16%). Removal or acquiring of pets during the study period did not cause distortion to the associations because pet keeping remained virtually constant from birth to 1 year.

The presence of dogs may increase the levels of endotoxin in house dust (Campo et al. 2006; Heinrich et al. 2001) and in indoor air (Park et al. 2001a). It has been proposed that endotoxin, may be a crucial causative agent mediating the effect of dog exposure, because the protection against wheezing was found only when there was a high endotoxin load together with multiple dogs (Campo et al. 2006). The role of endotoxin was highlighted also in another study where dog ownership was inversely associated with the risk of eczema during the first year of life, but after adjustment for the levels of endotoxin, the relationship was no longer statistically significant (Phipatanakul et al. 2004). There is also some evidence for important gene-environment interactions: dog exposure may be protective against allergic diseases, especially in children with a critical polymorphism in the gene encoding for the endotoxin receptor (Gern et al. 2004). On the other hand, several studies have not managed to reveal any influence of dog exposure on health outcomes and immune responses as a consequence of the increased exposure to endotoxin. Dog ownership has been linked to a reduced risk of asthma and wheezing (Litonjua et al. 2002; Waser et al. 2005) as well as common allergic outcomes such as hay fever and inhalant sensitization in childhood (Chen et al. 2008; Waser et al. 2005), independently of house dust endotoxin levels. Moreover, the levels of major dog allergen (Can f 1) in house dust have been associated with the increased cytokine responses at the age of 1 year, also after adjustment of the associations for the concentrations of endotoxin and two chemical markers (muramic acid and ergosterol) (Bufford et al. 2008).

It should be noted that dog exposure may have double-edged effects on the immune system (see Figure 2 and Wright et al. 2008). Dog -derived allergen and microbial exposures may activate different cytokine pathways: allergens may promote allergic Th2 responses, whereas the appropriate endotoxin load could enhance cell-mediated Th1 responses. Although dog exposure remained as the primary explanatory factor for observed associations even after adjustment for the levels of chemical markers (3-OH FAs, muramic acid and ergosterol), it is likely that endotoxin and allergens may play a role in mediating the effect of dog exposure. Possibly also other microbial exposures, for which dog exposure only serves as a surrogate, participate in this complex spectrum of immunological events.

In summary, in addition to this thesis, so far only few studies have investigated the effects of dog exposure on immune responses at early life (Gern et al. 2004, Roponen et al 2005) and the results seem to be confusing. Thus, more studies are needed to evaluate the true influence of dog exposure on neonatal immunity. However, the current knowledge suggest that early life exposure to dogs may

contribute to the immune maturation by offering immunological challenges, observed as modulation of cytokine responses during the first year of life.

6.4 Microbial exposures and immune development in early life

At the age of 3 months, high exposure to Gram-negative bacterial 3-OH FAs ($C_{10:0}$-$C_{14:0}$) seemed to reduce the production of proinflammatory cytokines, whereas exposure to Gram-positive bacteria appeared to have opposite effects. At the age of 1 year, the most pronounced finding was that increased exposure to indoor Gram-positive bacteria in general and also separately for Gram-positive bacterial genus *Mycobacterium* resulted in decreased Th1 cytokine -producing capacity. Factors which may account for the divergent results between MAA and LUKAS studies will be discussed in more detailed in "*Methodological consideration*" (see 6.5.). What makes it difficult to interpret these findings is the fact that there are only limited amount of literature investigating the associations between bacterial exposures (other than endotoxin) and neonatal immune responses.

Endotoxin is the most widely studied single microbial component in allergy research, whose effects on health are very complex and not straightforward (see 2.7). Some epidemiological studies have investigated the associations between endotoxin exposure and immune responses in early life (Abraham et al. 2005, Braun-Fahrländer et al. 2002, Bufford et al. 2008, Gereda et al. 2000, Pfefferle et al. 2010, Roponen et al. 2005). Relationships between the exposure to endotoxin and cytokines / immune responses seem to be not consistent, because some of the above mentioned publications have reported elevated IFN-γ levels after intensive exposure to endotoxin, but also generally depressed cytokine responses have been measured (Braun-Fahrländer et al. 2002). In this thesis, Gram-negative bacterial exposures were not characterized extensively (endotoxin was not measured, only 3-OH FAs represented Gram-negative bacteria) and only single univariate associations between cytokine levels and 3-OH FAs at 3 months of age were observed.

The observed inverse associations between bacterial exposures and cytokine responses at the age of 3 months (associations on 3-OH FAs) and 1 year may be related to the immunologic adaptation against microbial agents (Braun-Fahrländer et al. 2002). Immunologic adaptation could be a beneficial phenomenon for the maturation of the immune system and could also be related for the decreased risk to develop atopy in childhood. On the other hand, if down-regulation occurs only in Th1 responses, it may lead to the augmentation of the atopy promoting Th2-type cytokine responses. Thus, the present results do not clarify whether these microbial exposures are beneficial or detrimental for immune development and human health but the immunologic adaptation against invading non-pathogenic microbes is one plausible explanation for the observed associations.

Very little is known about the effects of fungal exposures on the functions of immune system. Recent study did not find any associations between the levels of ergosterol and mitogen-stimulated cytokine responses (IFN-γ, IL-5, IL-10, IL-13) at the age 1 year and 3 years (Bufford et al. 2008). However, the PASTURE core study reported some evidence that maternal exposure to fungal extracellular polysaccharides (EPS) could be associated with increased cord blood TNF-α responses, but the results were not very consistent (Pfefferle et al. 2010). In this study, fungal exposure seemed have similar trend on Th1 cytokine responses at the age 1 year, as was also observed with Gram-positive bacteria. Thus it seems likely that the observed effects on Th1 responses are a consequence of an exposure to a broader spectrum of microbes.

Only few associations were found between cord blood cytokine responses and microbial levels in house dust. However, these few associations indicate that microbial exposures already during pregnancy may have the potential to modify neonatal immune functions. This concept is supported by the previous study concluding that maternal exposure to environments rich in microbes during pregnancy increases the expression of the recognition receptors of the innate immunity in children (Ege et al. 2006).

In summary, microbial exposures, especially bacterial, may be able to influence the development of immune system. It also appears that microbes may have bidirectional immunomodulatory effects, because the same exposure agent/s may stimulate or inhibit immune functions, possibly depending on the level of exposure and the maturation stage of the immune system. However, due to the limited number of previous reports available and the inconsistent nature of the findings, the effects of different microbial exposures on immune development should be studied further.

6.5 Methodological considerations

In this thesis, protocol for immunology included immunostimulatory compounds and stimulation timepoints, which are widely used in experimental studies. Cytokines, which were selected for this study, represented different functions of the immune system including chemokine, proinflammatory, Th1 and Th2 responses. The volume of the sample available for ELISA analysis restricted the number of measured cytokines. However, today there are new technologies available to measure a wide range of different cytokines and other immunological parameters from a small-volume sample. Microbial analyses were extensive, but the inclusion of endotoxin measurements and the characterization of specific Gram-negative species in indoor dust and the assessment of individual microbial colonization of GI and respiratory tracts would have strengthened the study.

In vivo cytokine levels are commonly determined from plasma and serum, whereas both basal and stimulated concentrations can be measured from specific

leukocytes such as polymorphonuclear leukocytes, PBMCs, lymphocytes and also from whole blood culture. The cytokine responses in this thesis were assessed using whole blood samples. Whole blood culture contains circulating plasma components not present in specific leukocyte culture, which may either inhibit or intensify the effects of specific stimulants. In addition, smaller blood volumes and less time for blood processing (followed by reduced costs) are needed for whole blood culture as compared to specific cell cultures. However, when using whole blood culture the exact number of leukocytes in each stimulation is not the same between individuals and it is not known which cell types are the major sources of the cytokines. Individual variation in leukocyte counts can be to some extent controlled by determining the white blood cell count of the blood sample and by standardizing the measured cytokine concentration against this value.

There were differences in the results between the two distinct cohorts (MAA and LUKAS2), e.g. in the concentrations of the measured cytokines and associations between children and mothers. However, some potential explanations for the discrepant results can be found. Cytokine production was expressed in two different ways. 1) By measuring the concentrations of cytokines in 1 milliliter of supernatant (pg/ml) (done in both MAA and LUKAS) 2) By standardizing the concentrations of cytokines with the individual white blood cell count (pg/10^6 WBC) (done only in LUKAS). Moreover, in order to avoid the effect of individual variation in baseline cytokine production, LUKAS cytokine data was expressed as Δ -values (stimulated cytokine values minus unstimulated values). The differing cytokine results between MAA and LUKAS studies are largely due to the white blood cell (WBC) transformation. In the LUKAS study, cytokine production was assessed as the capacity of 10^6 white blood cells to secrete cytokines, i.e., this is data about the functional capability of blood leukocytes to produce cytokines. In the MAA study, cytokine responses were expressed only as the absolute levels because the WBC count was not as a part of that study protocol. In the LUKAS2 study, the number of leukocytes was higher in cord blood than at 1 year of age or mothers. If the WBC-correction was not performed for LUKAS2 cytokines, for example TNF-α production (pg/ml, P/I, 24h stimulation, data not shown) would have been highest in cord blood and lowest in mothers, which is similar to MAA findings. Correspondingly, correction of cytokine production with the leukocyte count would have decreased the levels of cord blood cytokine responses in MAA children.

There were also some other methodological differences between these two cohorts e.g. in terms of stimuli (type, concentration), duration of cytokine stimulation, dilution of blood samples in stimulation (MAA 1:4 vs LUKAS 1:8), different classification of pet ownership and microbial exposure and different study population (the focus on MAA was farm vs. non-farm setting, whereas in LUKAS2 95% of the households were non-farming).

In the MAA cohort, microbial concentrations were determined from three different types of house dust samples: bed dust, floor dust and the dust bag of the

vacuum cleaner. In the LUKAS2 study, microbial analyses only from floor dust were included in the thesis. In publication IV, there were significant differences in the associations between microbial exposure variables and cytokine responses according to the dust sample type. The different findings between bed dust and floor dust samples are probably partly due to different sources of bacteria: the majority of the Gram-negative bacteria in bed dust originate from humans themselves (86%), whereas floor dust Gram-negative bacterial species are mostly from environmental sources (79%) (Täubel et al. 2009). Furthermore, Gram-negative bacteria account for only 21% of the floor dust and 8% of the bed dust bacteria (Täubel et al. 2009), whereas the respective numbers for the occurrence of Gram-positive bacteria are 74% (Rintala et al. 2008) to 79% (Täubel et al. 2009) for floor dust and 92% for bed dust (Täubel et al. 2009). This clearly indicates that the house dust bacterial flora is dominated by bacteria of Gram-positive origin. Although Gram-negative bacteria contain potent immunomodulatory substances, due to the predominance of the Gram-positive bacterial species in the indoor dust and observed associations with cytokine responses in this thesis, more attention should be paid to the possible health effects (protective or adverse) evoked by exposure to Gram-positive bacteria.

When assessing individual exposure to microbes from the house dust samples, there is a lot of room for exposure misclassification. While airborne levels of microbes are known to vary considerably in the different seasons, floor and bed dust samples, which exhibit higher reproducibility, have been preferred in epidemiological studies, including the present study. However, since the exposure to environmental microbes occurs to a large degree via the air, better estimate of exposure could be achieved with long time personal air sampling. In the case of young children, also skin and GI-tract are important exposure routes, because neonates spend considerable time on the bed or floor and therefore have more direct contact with the dust on these locations. Assessing prenatal exposure to microbes by using house dust may include a potential source of error because it is not known how much mothers are exposed to different microbes outside the home. However, this problem is not so significant in the case of young children because they spend most of their time indoors in close contact with house dust covered surfaces.

7 CONCLUSIONS

1. Stimulated neonatal cytokine production was influenced by several birth-related factors and maternal and neonatal characteristics including season of birth, birth weight, gender of the child, maternal smoking during pregnancy and induction of labour with prostaglandin.

2. Stimulated cytokine responses increased from birth to 1 year of age, but still remained weaker than maternal responses. Cytokine responses of the children began to correlate with those of their mothers during the first year of life.

3. Continuous intensive contacts with dogs early in life and already during pregnancy seemed to have immunomodulatory effects during the first year of life.

4. Also microbial exposures may modify the immature immune system. In general, high-level exposure to indoor microbes, especially to bacteria, appeared to decrease immune responses in infancy. Present findings also highlight the bidirectional nature of the microbial exposures: the same exposure agent/s may have stimulatory or inhibitory effects on immune functions, possibly depending on the maturation stage of the immune system.

In summary, this thesis has extended the current understanding of the immunologic development during the first year of life and the different immunomodulatory factors, especially the role of microbial and animal exposures. These new data will hopefully provide new insights for the future studies attempting to elucidate factors and mechanism modulating the immunological development in a favourable direction, with the ultimate aim of preventing major chronic disorders e.g. asthma and allergies.

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9 REFERENCES

Abbas, A. K., Lichtman, A. H. and Pillai, S. 2007. Cellular and Molecular Immunology. 6th ed. Philadelphia, Pennsylvania, USA, Saunders Elsevier.

Abraham, J. H., Finn, P. W., Milton, D. K., Ryan, L. M., Perkins, D. L. and Gold, D. R. 2005. Infant home endotoxin is associated with reduced allergen-stimulated lymphocyte proliferation and IL-13 production in childhood. J Allergy Clin Immunol 116(2): 431-7.

Adkins, B., Leclerc, C. and Marshall-Clarke, S. 2004. Neonatal adaptive immunity comes of age. Nat Rev Immunol 4(7): 553-64.

Akdis, M., Verhagen, J., Taylor, A., Karamloo, F., Karagiannidis, C., Crameri, R., Thunberg, S., Deniz, G., Valenta, R., Fiebig, H., Kegel, C., Disch, R., Schmidt-Weber, C. B., Blaser, K. and Akdis, C. A. 2004. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 199(11): 1567-75.

Al-Mousawi, M. S., Lovel, H., Behbehani, N., Arifhodzic, N., Woodcock, A. and Custovic, A. 2004. Asthma and sensitization in a community with low indoor allergen levels and low pet-keeping frequency. J Allergy Clin Immunol 114(6): 1389-94.

Andersson, M. A., Mikkola, R., Kroppenstedt, R. M., Rainey, F. A., Peltola, J., Helin, J., Sivonen, K. and Salkinoja-Salonen, M. S. 1998. The mitochondrial toxin produced by *Streptomyces griseus* strains isolated from an indoor environment is valinomycin. Appl Environ Microbiol 64(12): 4767-73.

Angelone, D. F., Wessels, M. R., Coughlin, M., Suter, E. E., Valentini, P., Kalish, L. A. and Levy, O. 2006. Innate immunity of the human newborn is polarized toward a high ratio of IL-6/TNF-alpha production *in vitro* and *in vivo*. Pediatr Res 60(2): 205-9.

Arbes, S. J., Jr., Cohn, R. D., Yin, M., Muilenberg, M. L., Friedman, W. and Zeldin, D. C. 2004. Dog allergen (Can f 1) and cat allergen (Fel d 1) in US homes: results from the National Survey of Lead and Allergens in Housing. J Allergy Clin Immunol 114(1): 111-7.

Bauer, S., Hangel, D. and Yu, P. 2007. Immunobiology of toll-like receptors in allergic disease. Immunobiology 212(6): 521-33.

Beijer, L., Thorn, J. and Rylander, R. 2003. Mould exposure at home relates to inflammatory markers in blood. Eur Respir J 21(2): 317-22.

Belderbos, M., Levy, O. and Bont, L. 2009. Neonatal innate immunity in allergy development. Curr Opin Pediatr 21(6): 762-9.

Binding, N., Jaschinski, S., Werlich, S., Bletz, S. and Witting, U. 2004. Quantification of bacterial lipopolysaccharides (endotoxin) by GC-MS determination of 3-hydroxy fatty acids. J Environ Monit 6(1): 65-70.

Björkstén, B. 2009. The hygiene hypothesis: do we still believe in it? Nestle Nutr Workshop Ser Pediatr Program 64: 11-8; discussion 18-22, 251-7.

Bluestone, J. A. and Abbas, A. K. 2003. Natural versus adaptive regulatory T cells. Nature Reviews Immunology 3(3): 253-257.

Bolte, G., Bischof, W., Borte, M., Lehmann, I., Wichmann, H. E. and Heinrich, J. 2003. Early endotoxin exposure and atopy development in infants: results of a birth cohort study. Clin Exp Allergy 33(6): 770-6.

Bornehag, C. G., Blomquist, G., Gyntelberg, F., Jarvholm, B., Malmberg, P., Nordvall, L., Nielsen, A., Pershagen, G. and Sundell, J. 2001. Dampness in buildings and health. Nordic interdisciplinary review of the scientific evidence on associations between exposure to "dampness" in buildings and health effects (NORDDAMP). Indoor Air 11(2): 72-86.

Bornehag, C. G., Sundell, J., Bonini, S., Custovic, A., Malmberg, P., Skerfving, S., Sigsgaard, T. and Verhoeff, A. 2004. Dampness in buildings as a risk factor for health effects, EUROEXPO: a multidisciplinary review of the literature (1998-2000) on dampness and mite exposure in buildings and health effects. Indoor Air 14(4): 243-57.

Bornehag, C. G., Sundell, J., Hagerhed, L. and Janson, S. 2003. Pet-keeping in early childhood and airway, nose and skin symptoms later in life. Allergy 58(9): 939-44.

Braun-Fahrländer, C., Riedler, J., Herz, U., Eder, W., Waser, M., Grize, L., Maisch, S., Carr, D., Gerlach, F., Bufe, A., Lauener, R. P., Schierl, R., Renz, H., Nowak, D. and von Mutius, E. 2002. Environmental exposure to endotoxin and its relation to asthma in

school-age children. N Engl J Med 347(12): 869-77.

Brunekreef, B., Groot, B. and Hoek, G. 1992. Pets, allergy and respiratory symptoms in children. Int J Epidemiol 21(2): 338-42.

Buck, R. H., Cordle, C. T., Thomas, D. J., Winship, T. R., Schaller, J. P. and Dugle, J. E. 2002. Longitudinal study of intracellular T cell cytokine production in infants compared to adults. Clin Exp Immunol 128(3): 490-7.

Bufford, J. D., Reardon, C. L., Li, Z., Roberg, K. A., DaSilva, D., Eggleston, P. A., Liu, A. H., Milton, D., Alwis, U., Gangnon, R., Lemanske, R. F., Jr. and Gern, J. E. 2008. Effects of dog ownership in early childhood on immune development and atopic diseases. Clin Exp Allergy 38(10): 1635-43.

Böttcher, M. F., Björkstén, B., Gustafson, S., Voor, T. and Jenmalm, M. C. 2003. Endotoxin levels in Estonian and Swedish house dust and atopy in infancy. Clin Exp Allergy 33(3): 295-300.

Campo, P., Kalra, H. K., Levin, L., Reponen, T., Olds, R., Lummus, Z. L., Cho, S. H., Khurana Hershey, G. K., Lockey, J., Villareal, M., Stanforth, S., Lemasters, G. and Bernstein, D. I. 2006. Influence of dog ownership and high endotoxin on wheezing and atopy during infancy. J Allergy Clin Immunol 118(6): 1271-8.

Canning, M. O., Grotenhuis, K., de Wit, H., Ruwhof, C. and Drexhage, H. A. 2001. 1-alpha,25-Dihydroxyvitamin D3 (1,25(OH)(2)D(3)) hampers the maturation of fully active immature dendritic cells from monocytes. Eur J Endocrinol 145(3): 351-7.

Cavaillon, J. M. 1995. The nonspecific nature of endotoxin tolerance. Trends Microbiol 3(8): 320-4.

Chaplin, D. D. 2010. Overview of the immune response. J Allergy Clin Immunol 125(2 Suppl 2): S3-23.

Chen, C. M., Morgenstern, V., Bischof, W., Herbarth, O., Borte, M., Behrendt, H., Kramer, U., von Berg, A., Berdel, D., Bauer, C. P., Koletzko, S., Wichmann, H. E. and Heinrich, J. 2008. Dog ownership and contact during childhood and later allergy development. Eur Respir J 31(5): 963-73.

Daher, S., de Arruda Geraldes Denardi, K., Blotta, M. H., Mamoni, R. L., Reck, A. P., Camano, L. and Mattar, R. 2004. Cytokines in recurrent pregnancy loss. J Reprod Immunol 62(1-2): 151-7.

Dales, R., Miller, D., Ruest, K., Guay, M. and Judek, S. 2006. Airborne endotoxin is associated with respiratory illness in the first 2 years of life. Environ Health Perspect 114(4): 610-4.

Degenkolb, T., von Dohren, H., Nielsen, K. F., Samuels, G. J. and Bruckner, H. 2008. Recent advances and future prospects in peptaibiotics, hydrophobin, and mycotoxin research, and their importance for chemotaxonomy of Trichoderma and Hypocrea. Chem Biodivers 5(5): 671-80.

Demain, A. L. 1999. Pharmaceutically active secondary metabolites of microorganisms. Appl Microbiol Biotechnol 52(4): 455-63.

Dharmage, S., Bailey, M., Raven, J., Mitakakis, T., Cheng, A., Guest, D., Rolland, J., Forbes, A., Thien, F., Abramson, M. and Walters, E. H. 2001. Current indoor allergen levels of fungi and cats, but not house dust mites, influence allergy and asthma in adults with high dust mite exposure. Am J Respir Crit Care Med 164(1): 65-71.

Douwes, J., Pearce, N. and Heederik, D. 2002. Does environmental endotoxin exposure prevent asthma? Thorax 57(1): 86-90.

Douwes, J., van Strien, R., Doekes, G., Smit, J., Kerkhof, M., Gerritsen, J., Postma, D., de Jongste, J., Travier, N. and Brunekreef, B. 2006. Does early indoor microbial exposure reduce the risk of asthma? The Prevention and Incidence of Asthma and Mite Allergy birth cohort study. J Allergy Clin Immunol 117(5): 1067-73.

Dunstan, J. A., Mori, T. A., Barden, A., Beilin, L. J., Taylor, A. L., Holt, P. G. and Prescott, S. L. 2003. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. J Allergy Clin Immunol 112(6): 1178-84.

Dziarski, R. 2003. Recognition of bacterial peptidoglycan by the innate immune system. Cell Mol Life Sci 60(9): 1793-804.

Ege, M. J., Bieli, C., Frei, R., van Strien, R. T., Riedler, J., Ublagger, E., Schram-Bijkerk, D., Brunekreef, B., van Hage, M., Scheynius, A., Pershagen, G., Benz, M. R., Lauener, R., von Mutius, E. and Braun-Fahrländer, C. 2006. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. J Allergy Clin Immunol 117(4): 817-23.

Ege, M. J., Frei, R., Bieli, C., Schram-Bijkerk, D., Waser, M., Benz, M. R., Weiss, G., Nyberg, F., van Hage, M., Pershagen, G., Brunekreef, B., Riedler, J., Lauener, R., Braun-Fahrländer, C. and von Mutius, E. 2007. Not all farming environments protect against the development of asthma and wheeze in children. J Allergy Clin Immunol 119(5): 1140-7.

Ege, M. J., Herzum, I., Büchele, G., Krauss-Etschmann, S., Lauener, R. P., Roponen, M., Hyvärinen, A., Vuitton, D. A., Riedler, J., Brunekreef, B., Dalphin, J. C., Braun-

Fahrländer, C., Pekkanen, J., Renz, H. and von Mutius, E. 2008. Prenatal exposure to a farm environment modifies atopic sensitization at birth. J Allergy Clin Immunol 122(2): 407-12, 412 e1-4.

Falkinham, J. O., 3rd 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin Microbiol Rev 9(2): 177-215.

Foley, S. C., Prefontaine, D., D'Antoni, M. and Hamid, Q. 2007. Images in allergy and immunology: regulatory T cells in allergic disease. J Allergy Clin Immunol 120(2): 482-6.

Gabrielsson, S., Söderlund, A., Nilsson, C., Lilja, G., Nordlund, M. and Troye-Blomberg, M. 2001. Influence of atopic heredity on IL-4-, IL-12- and IFN-gamma-producing cells in in vitro activated cord blood mononuclear cells. Clin Exp Immunol 126(3): 390-6.

Garantziotis, S., Hollingsworth, J. W., Zaas, A. K. and Schwartz, D. A. 2008. The effect of toll-like receptors and toll-like receptor genetics in human disease. Annu Rev Med 59: 343-59.

Garrett, M. H., Rayment, P. R., Hooper, M. A., Abramson, M. J. and Hooper, B. M. 1998. Indoor airborne fungal spores, house dampness and associations with environmental factors and respiratory health in children. Clin Exp Allergy 28(4): 459-67.

Gehring, U., Bischof, W., Fahlbusch, B., Wichmann, H. E. and Heinrich, J. 2002. House dust endotoxin and allergic sensitization in children. Am J Respir Crit Care Med 166(7): 939-44.

Gehring, U., Bolte, G., Borte, M., Bischof, W., Fahlbusch, B., Wichmann, H. E. and Heinrich, J. 2001. Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. J Allergy Clin Immunol 108(5): 847-54.

Gent, J. F., Ren, P., Belanger, K., Triche, E., Bracken, M. B., Holford, T. R. and Leaderer, B. P. 2002. Levels of household mold associated with respiratory symptoms in the first year of life in a cohort at risk for asthma. Environ Health Perspect 110(12): A781-6.

Gereda, J. E., Klinnert, M. D., Price, M. R., Leung, D. Y. and Liu, A. H. 2001. Metropolitan home living conditions associated with indoor endotoxin levels. J Allergy Clin Immunol 107(5): 790-6.

Gereda, J. E., Leung, D. Y., Thatayatikom, A., Streib, J. E., Price, M. R., Klinnert, M. D. and Liu, A. H. 2000. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. Lancet 355(9216): 1680-3.

Gern, J. E., Reardon, C. L., Hoffjan, S., Nicolae, D., Li, Z., Roberg, K. A., Neaville, W. A., Carlson-Dakes, K., Adler, K., Hamilton, R., Anderson, E., Gilbertson-White, S., Tisler, C., Dasilva, D., Anklam, K., Mikus, L. D., Rosenthal, L. A., Ober, C., Gangnon, R. and Lemanske, R. F., Jr. 2004. Effects of dog ownership and genotype on immune development and atopy in infancy. J Allergy Clin Immunol 113(2): 307-14.

Gold, D. R., Bloomberg, G. R., Cruikshank, W. W., Visness, C. M., Schwarz, J., Kattan, M., O'Connor, G. T., Wood, R. A., Burger, M. S., Wright, R. J., Witter, F., Lee-Parritz, A., Sperling, R., Sadovsky, Y., Togias, A. and Gern, J. E. 2009. Parental characteristics, somatic fetal growth, and season of birth influence innate and adaptive cord blood cytokine responses. J Allergy Clin Immunol 124(5): 1078-87.

Goodridge, H. S., Wolf, A. J. and Underhill, D. M. 2009. Beta-glucan recognition by the innate immune system. Immunol Rev 230(1): 38-50.

Guan, R. and Mariuzza, R. A. 2007. Peptidoglycan recognition proteins of the innate immune system. Trends Microbiol 15(3): 127-34.

Halonen, M., Lohman, I. C., Stern, D. A., Spangenberg, A., Anderson, D., Mobley, S., Ciano, K., Peck, M. and Wright, A. L. 2009. Th1/Th2 patterns and balance in cytokine production in the parents and infants of a large birth cohort. J Immunol 182(5): 3285-93.

Haugland, R. A., Varma, M., Wymer, L. J. and Vesper, S. J. 2004. Quantitative PCR analysis of selected Aspergillus, Penicillium and Paecilomyces species. Syst Appl Microbiol 27(2): 198-210.

Heaton, T., Rowe, J., Turner, S., Aalberse, R. C., de Klerk, N., Suriyaarachchi, D., Serralha, M., Holt, B. J., Hollams, E., Yerkovich, S., Holt, K., Sly, P. D., Goldblatt, J., Le Souef, P. and Holt, P. G. 2005. An immunoepidemiological approach to asthma: identification of in-vitro T-cell response patterns associated with different wheezing phenotypes in children. Lancet 365(9454): 142-9.

Heinrich, J., Gehring, U., Douwes, J., Koch, A., Fahlbusch, B., Bischof, W. and Wichmann, H. E. 2001. Pets and vermin are associated with high endotoxin levels in house dust. Clin Exp Allergy 31(12): 1839-45.

Herzum, I., Blümer, N., Kersten, W. and Renz, H. 2005. Diagnostic and analytical performance of a screening panel for allergy. Clin Chem Lab Med 43(9): 963-6.

Hesselmar, B., Aberg, B., Eriksson, B., Björkstén, B. and Aberg, N. 2003. High-dose exposure to cat is associated with clinical

tolerance--a modified Th2 immune response? Clin Exp Allergy 33(12): 1681-5.

Heumann, D. and Roger, T. 2002. Initial responses to endotoxins and Gram-negative bacteria. Clin Chim Acta 323(1-2): 59-72.

Holt, P. G. 2005. Developmental factors as determinants of risk for infections and atopy in childhood. Eur Respir Rev 14(95): 69-73.

Holt, P. G., Clough, J. B., Holt, B. J., Baron-Hay, M. J., Rose, A. H., Robinson, B. W. and Thomas, W. R. 1992. Genetic 'risk' for atopy is associated with delayed postnatal maturation of T-cell competence. Clin Exp Allergy 22(12): 1093-9.

Holt, P. G. and Jones, C. A. 2000. The development of the immune system during pregnancy and early life. Allergy 55(8): 688-97.

Holt, P. G., Rudin, A., Macaubas, C., Holt, B. J., Rowe, J., Loh, R. and Sly, P. D. 2000. Development of immunologic memory against tetanus toxoid and pertactin antigens from the diphtheria-tetanus-pertussis vaccine in atopic versus nonatopic children. J Allergy Clin Immunol 105(6 Pt 1): 1117-22.

Horner, A. A. 2006. Toll-like receptor ligands and atopy: a coin with at least two sides. J Allergy Clin Immunol 117(5): 1133-40.

Hugg, T. T., Jaakkola, M. S., Ruotsalainen, R., Pushkarev, V. and Jaakkola, J. J. 2008. Exposure to animals and the risk of allergic asthma: a population-based cross-sectional study in Finnish and Russian children. Environ Health 7: 28.

Huttunen, K., Hyvärinen, A., Nevalainen, A., Komulainen, H. and Hirvonen, M. R. 2003. Production of proinflammatory mediators by indoor air bacteria and fungal spores in mouse and human cell lines. Environ Health Perspect 111(1): 85-92.

Huttunen, K., Jussila, J., Hirvonen, M. R., Iivanainen, E. and Katila, M. L. 2001. Comparison of mycobacteria-induced cytotoxicity and inflammatory responses in human and mouse cell lines. Inhal Toxicol 13(11): 977-91.

Hyvärinen, A., Sebastian, A., Pekkanen, J., Larsson, L., Korppi, M., Putus, T. and Nevalainen, A. 2006a. Characterizing microbial exposure with ergosterol, 3-hydroxy fatty acids, and viable microbes in house dust: determinants and association with childhood asthma. Arch Environ Occup Health 61(4): 149-57.

Hyvärinen, A., Roponen, M., Tiittanen, P., Laitinen, S., Nevalainen, A. and Pekkanen, J. 2006b. Dust sampling methods for endotoxin - an essential, but underestimated issue. Indoor Air 16(1): 20-7.

Härtel, C., Adam, N., Strunk, T., Temming, P., Müller-Steinhardt, M. and Schultz, C. 2005. Cytokine responses correlate differentially with age in infancy and early childhood. Clin Exp Immunol 142(3): 446-53.

Iossifova, Y. Y., Reponen, T., Bernstein, D. I., Levin, L., Kalra, H., Campo, P., Villareal, M., Lockey, J., Hershey, G. K. and LeMasters, G. 2007. House dust (1-3)-beta-D-glucan and wheezing in infants. Allergy 62(5): 504-13.

Jacob, B., Ritz, B., Gehring, U., Koch, A., Bischof, W., Wichmann, H. E. and Heinrich, J. 2002. Indoor exposure to molds and allergic sensitization. Environ Health Perspect 110(7): 647-53.

Jirapongsananuruk, O., Melamed, I. and Leung, D. Y. 2000. Additive immunosuppressive effects of 1,25-dihydroxyvitamin D3 and corticosteroids on TH1, but not TH2, responses. J Allergy Clin Immunol 106(5): 981-5.

Johansson, S. G., Bieber, T., Dahl, R., Friedmann, P. S., Lanier, B. Q., Lockey, R. F., Motala, C., Ortega Martell, J. A., Platts-Mills, T. A., Ring, J., Thien, F., Van Cauwenberge, P. and Williams, H. C. 2004. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. J Allergy Clin Immunol 113(5): 832-6.

Jones, C. A., Holloway, J. A. and Warner, J. O. 2000. Does atopic disease start in foetal life? Allergy 55(1): 2-10.

Jussila, J., Komulainen, H., Huttunen, K., Roponen, M., Hälinen, A., Hyvärinen, A., Kosma, V. M., Pelkonen, J. and Hirvonen, M. R. 2001. Inflammatory responses in mice after intratracheal instillation of spores of *Streptomyces californicus* isolated from indoor air of a moldy building. Toxicol Appl Pharmacol 171(1): 61-9.

Jussila, J., Komulainen, H., Huttunen, K., Roponen, M., Iivanainen, E., Torkko, P., Kosma, V. M., Pelkonen, J. and Hirvonen, M. R. 2002a. *Mycobacterium terrae* isolated from indoor air of a moisture-damaged building induces sustained biphasic inflammatory response in mouse lungs. Environ Health Perspect 110(11): 1119-25.

Jussila, J., Komulainen, H., Kosma, V. M., Nevalainen, A., Pelkonen, J. and Hirvonen, M. R. 2002b. Spores of *Aspergillus versicolor* isolated from indoor air of a moisture-damaged building provoke acute inflammation in mouse lungs. Inhal Toxicol 14(12): 1261-77.

Jussila, J., Pelkonen, J., Kosma, V. M., Mäki-Paakkanen, J., Komulainen, H. and Hirvonen, M. R. 2003. Systemic immunoresponses in mice after repeated exposure of lungs to spores of *Streptomyces*

californicus. Clin Diagn Lab Immunol 10(1): 30-7.

Karvonen, A. M., Hyvärinen, A., Roponen, M., Hoffmann, M., Korppi, M., Remes, S., von Mutius, E., Nevalainen, A. and Pekkanen, J. 2009. Confirmed moisture damage at home, respiratory symptoms and atopy in early life: a birth-cohort study. Pediatrics 124(2): e329-38.

Keski-Nisula, L., Hirvonen, M. R., Roponen, M., Heinonen, S. and Pekkanen, J. 2003. Maternal and neonatal IL-4 and IFN-gamma production at delivery and 3 months after birth. J Reprod Immunol 60(1): 25-33.

Keski-Nisula, L., Hirvonen, M. R., Roponen, M., Heinonen, S. and Pekkanen, J. 2004. Spontaneous and stimulated interleukin-6 and tumor necrosis factor-alpha production at delivery and three months after birth. Eur Cytokine Netw 15(1): 67-72.

Keski-Nisula, L., Katila, M. L., Remes, S., Heinonen, S. and Pekkanen, J. 2009. Intrauterine bacterial growth at birth and risk of asthma and allergic sensitization among offspring at the age of 15 to 17 years. J Allergy Clin Immunol 123(6): 1305-11.

Kihlstrom, A., Lilja, G., Pershagen, G. and Hedlin, G. 2003. Exposure to high doses of birch pollen during pregnancy, and risk of sensitization and atopic disease in the child. Allergy 58(9): 871-7.

Kimura, M., Yamaide, A., Tsuruta, S., Okafuji, I. and Yoshida, T. 2002. Development of the capacity of peripheral blood mononuclear cells to produce IL-4, IL-5 and IFN-gamma upon stimulation with house dust mite in children with atopic dermatitis. Int Arch Allergy Immunol 127(3): 191-7.

Kjaer, H. F., Eller, E., Andersen, K. E., Host, A. and Bindslev-Jensen, C. 2009. The association between early sensitization patterns and subsequent allergic disease. The DARC birth cohort study. Pediatr Allergy Immunol 20(8): 726-34.

Knudsen, T. B., Thomsen, S. F., Ulrik, C. S., Fenger, M., Nepper-Christensen, S. and Backer, V. 2007. Season of birth and risk of atopic disease among children and adolescents. J Asthma 44(4): 257-60.

Kopp, M. V., Zehle, C., Pichler, J., Szepfalusi, Z., Moseler, M., Deichmann, K., Forster, J. and Kuehr, J. 2001. Allergen-specific T cell reactivity in cord blood: the influence of maternal cytokine production. Clin Exp Allergy 31(10): 1536-43.

Kuoppala, T., Tuimala, R., Parviainen, M., Koskinen, T. and Ala-Houhala, M. 1986. Serum levels of vitamin D metabolites, calcium, phosphorus, magnesium and alkaline phosphatase in Finnish women throughout pregnancy and in cord serum at delivery. Hum Nutr Clin Nutr 40(4): 287-93.

Kutzner, K.J., 1986. The Family Streptomycetaceae. In: Starr M.P., Stolp H, Truper H.G., Balows A. and Schlegel H.G., (eds.). The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria, vol 2. Springer-Verlag, New York, 2028-2090.

Larsson, A. K., Nilsson, C., Höglind, A., Sverremark-Ekstrom, E., Lilja, G. and Troye-Blomberg, M. 2006. Relationship between maternal and child cytokine responses to allergen and phytohaemagglutinin 2 years after delivery. Clin Exp Immunol 144(3): 401-8.

Lauener, R. P., Birchler, T., Adamski, J., Braun-Fahrländer, C., Bufe, A., Herz, U., von Mutius, E., Nowak, D., Riedler, J., Waser, M. and Sennhauser, F. H. 2002. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. Lancet 360(9331): 465-6.

Lendor, C., Johnson, A., Perzanowski, M., Chew, G. L., Goldstein, I. F., Kelvin, E., Perera, F. and Miller, R. L. 2008. Effects of winter birth season and prenatal cockroach and mouse allergen exposure on indoor allergen-specific cord blood mononuclear cell proliferation and cytokine production. Ann Allergy Asthma Immunol 101(2): 193-9.

Levy, O. 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol 7(5): 379-90.

Levy, O., Coughlin, M., Cronstein, B. N., Roy, R. M., Desai, A. and Wessels, M. R. 2006. The adenosine system selectively inhibits TLR-mediated TNF-alpha production in the human newborn. J Immunol 177(3): 1956-66.

Levy, O., Zarember, K. A., Roy, R. M., Cywes, C., Godowski, P. J. and Wessels, M. R. 2004. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. J Immunol 173(7): 4627-34.

Lignell, U., Meklin, T., Rintala, H., Hyvärinen, A., Vepsäläinen, A., Pekkanen, J. and Nevalainen, A. 2008. Evaluation of quantitative PCR and culture methods for detection of house dust fungi and streptomycetes in relation to moisture damage of the house. Lett Appl Microbiol.47(4): 303-8.

Lindfors, A., van Hage-Hamsten, M., Rietz, H., Wickman, M. and Nordvall, S. L. 1999. Influence of interaction of environmental risk factors and sensitization in young asthmatic

children. J Allergy Clin Immunol 104(4 Pt 1): 755-62.

Ling, E. M., Smith, T., Nguyen, X. D., Pridgeon, C., Dallman, M., Arbery, J., Carr, V. A. and Robinson, D. S. 2004. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 363(9409): 608-15.

Litonjua, A. A., Milton, D. K., Celedon, J. C., Ryan, L., Weiss, S. T. and Gold, D. R. 2002. A longitudinal analysis of wheezing in young children: the independent effects of early life exposure to house dust endotoxin, allergens, and pets. J Allergy Clin Immunol 110(5): 736-42.

Loser, K. and Beissert, S. 2009. Regulation of cutaneous immunity by the environment: an important role for UV irradiation and vitamin D. Int Immunopharmacol 9(5): 587-9.

Ly, N. P., Ruiz-Perez, B., Onderdonk, A. B., Tzianabos, A. O., Litonjua, A. A., Liang, C., Laskey, D., Delaney, M. L., DuBois, A. M., Levy, H., Gold, D. R., Ryan, L. M., Weiss, S. T. and Celedon, J. C. 2006. Mode of delivery and cord blood cytokines: a birth cohort study. Clin Mol Allergy 4: 13.

Macaubas, C., de Klerk, N. H., Holt, B. J., Wee, C., Kendall, G., Firth, M., Sly, P. D. and Holt, P. G. 2003. Association between antenatal cytokine production and the development of atopy and asthma at age 6 years. Lancet 362(9391): 1192-7.

Malamitsi-Puchner, A., Protonotariou, E., Boutsikou, T., Makrakis, E., Sarandakou, A. and Creatsas, G. 2005. The influence of the mode of delivery on circulating cytokine concentrations in the perinatal period. Early Hum Dev 81(4): 387-92.

Manicassamy, S. and Pulendran, B. 2009. Modulation of adaptive immunity with Toll-like receptors. Semin Immunol 21(4): 185-93.

Marchini, G., Berggren, V., Djilali-Merzoug, R. and Hansson, L. O. 2000. The birth process initiates an acute phase reaction in the fetus-newborn infant. Acta Paediatr 89(9): 1082-6.

Marodi, L. 2006. Innate cellular immune responses in newborns. Clin Immunol 118(2-3): 137-44.

Martinez, F. D. 2001. The coming-of-age of the hygiene hypothesis. Respiratory Research 2(3): 129-132.

Martinez, F. D. and Holt, P. G. 1999. Role of microbial burden in aetiology of allergy and asthma. Lancet 354 Suppl 2: SII12-5.

Matsuoka, T., Matsubara, T., Katayama, K., Takeda, K., Koga, M. and Furukawa, S. 2001. Increase of cord blood cytokine-producing T cells in intrauterine infection. Pediatr Int 43(5): 453-7.

Medzhitov, R. 2001. Toll-like receptors and innate immunity. Nat Rev Immunol 1(2): 135-45.

Melen, E., Wickman, M., Nordvall, S. L., van Hage-Hamsten, M. and Lindfors, A. 2001. Influence of early and current environmental exposure factors on sensitization and outcome of asthma in pre-school children. Allergy 56(7): 646-52.

Michel, G., Gailis, A., Jarzebska-Deussen, B., Muschen, A., Mirmohammadsadegh, A. and Ruzicka, T. 1997. 1,25-(OH)2-vitamin D3 and calcipotriol induce IL-10 receptor gene expression in human epidermal cells. Inflamm Res 46(1): 32-4.

Moore, M. M., Rifas-Shiman, S. L., Rich-Edwards, J. W., Kleinman, K. P., Camargo, C. A., Jr., Gold, D. R., Weiss, S. T. and Gillman, M. W. 2004. Perinatal predictors of atopic dermatitis occurring in the first six months of life. Pediatrics 113(3 Pt 1): 468-74.

Müller-Alouf, H., Carnoy, C., Simonet, M. and Alouf, J. E. 2001. Superantigen bacterial toxins: state of the art. Toxicon 39(11): 1691-701.

Nehama, H., Wientroub, S., Eisenberg, Z., Birger, A., Milbauer, B. and Weisman, Y. 1987. Seasonal variation in paired maternal-newborn serum 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D concentrations in Israel. Isr J Med Sci 23(4): 274-7.

Ng, T. W., Holt, P. G. and Prescott, S. L. 2002. Cellular immune responses to ovalbumin and house dust mite in egg-allergic children. Allergy 57(3): 207-14.

Noakes, P. S., Hale, J., Thomas, R., Lane, C., Devadason, S. G. and Prescott, S. L. 2006. Maternal smoking is associated with impaired neonatal toll-like-receptor-mediated immune responses. Eur Respir J 28(4): 721-9.

Noakes, P. S., Holt, P. G. and Prescott, S. L. 2003. Maternal smoking in pregnancy alters neonatal cytokine responses. Allergy 58(10): 1053-8.

Obihara, C. C., Bollen, C. W., Beyers, N. and Kimpen, J. L. 2007. Mycobacterial infection and atopy in childhood: a systematic review. Pediatr Allergy Immunol 18(7): 551-9.

Omori, A., Takahashi, K., Hazawa, M., Misaki, N., Ohba, H., Manabe, M., Sato, H., Kudo, K., Takahashi, T. A. and Kashiwakura, I. 2008. Maternal and neonatal factors associated with the high yield of mononuclear low-density/CD34+ cells from placental/umbilical cord blood. Tohoku J Exp Med 215(1): 23-32.

Osborne, M., Reponen, T., Adhikari, A., Cho, S. H., Grinshpun, S. A., Levin, L., Bernstein, D. I. and LeMasters, G. 2006. Specific fungal

exposures, allergic sensitization, and rhinitis in infants. Pediatr Allergy Immunol 17(6): 450-7.

Ownby, D. R., Johnson, C. C. and Peterson, E. L. 2002. Exposure to dogs and cats in the first year of life and risk of allergic sensitization at 6 to 7 years of age. Jama 288(8): 963-72.

Paramasivam, K., Michie, C., Opara, E. and Jewell, A. P. 2006. Human breast milk immunology: a review. Int J Fertil Womens Med 51(5): 208-17.

Park, J. H., Spiegelman, D. L., Gold, D. R., Burge, H. A. and Milton, D. K. 2001a. Predictors of airborne endotoxin in the home. Environ Health Perspect 109(8): 859-64.

Park, J. H., Gold, D. R., Spiegelman, D. L., Burge, H. A. and Milton, D. K. 2001b. House dust endotoxin and wheeze in the first year of life. Am J Respir Crit Care Med 163(2): 322-8.

Pasare, C. and Medzhitov, R. 2004. Toll-like receptors: linking innate and adaptive immunity. Microbes Infect 6(15): 1382-7.

Pfefferle, P. I., Büchele, G., Blümer, N., Roponen, M., Ege, M. J., Krauss-Etschmann, S., Genuneit, J., Hyvärinen, A., Hirvonen, M. R., Lauener, R., Pekkanen, J., Riedler, J., Dalphin, J. C., Brunekeef, B., Braun-Fahrländer, C., von Mutius, E. and Renz, H. 2010. Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy: the PASTURE Study. J Allergy Clin Immunol 125(1): 108-15 e1-3.

Phipatanakul, W., Celedon, J. C., Raby, B. A., Litonjua, A. A., Milton, D. K., Sredl, D., Weiss, S. T. and Gold, D. R. 2004. Endotoxin exposure and eczema in the first year of life. Pediatrics 114(1): 13-8.

Piirainen, L., Pesola, J., Pesola, I., Komulainen, J. and Vaarala, O. 2009. Breastfeeding stimulates total and cow's milk-specific salivary IgA in infants. Pediatric Allergy and Immunology 20(3): 295-8.

Platts-Mills, T., Vaughan, J., Squillace, S., Woodfolk, J. and Sporik, R. 2001. Sensitisation, asthma, and a modified Th2 response in children exposed to cat allergen: a population-based cross-sectional study. Lancet 357(9258): 752-6.

Platts-Mills, T. A., Erwin, E., Heymann, P. and Woodfolk, J. 2005. Is the hygiene hypothesis still a viable explanation for the increased prevalence of asthma? Allergy 60 Suppl 79: 25-31.

Power, L. L., Popplewell, E. J., Holloway, J. A., Diaper, N. D., Warner, J. O. and Jones, C. A. 2002. Immunoregulatory molecules during pregnancy and at birth. J Reprod Immunol 56(1-2): 19-28.

Prescott, S. L. 2003. Early origins of allergic disease: a review of processes and influences during early immune development. Curr Opin Allergy Clin Immunol 3(2): 125-32.

Prescott, S. L. 2008. Effects of early cigarette smoke exposure on early immune development and respiratory disease. Paediatr Respir Rev 9(1): 3-9; quiz 10.

Prescott, S. L., Barden, A. E., Mori, T. A. and Dunstan, J. A. 2007. Maternal fish oil supplementation in pregnancy modifies neonatal leukotriene production by cord-blood-derived neutrophils. Clin Sci (Lond) 113(10): 409-16.

Prescott, S. L., King, B., Strong, T. L. and Holt, P. G. 2003. The value of perinatal immune responses in predicting allergic disease at 6 years of age. Allergy 58(11): 1187-94.

Prescott, S. L., Macaubas, C., Holt, B. J., Smallacombe, T. B., Loh, R., Sly, P. D. and Holt, P. G. 1998. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. J Immunol 160(10): 4730-7.

Prescott, S. L., Macaubas, C., Smallacombe, T., Holt, B. J., Sly, P. D. and Holt, P. G. 1999. Development of allergen-specific T-cell memory in atopic and normal children. Lancet 353(9148): 196-200.

Prescott, S. L., Wickens, K., Westcott, L., Jung, W., Currie, H., Black, P. N., Stanley, T. V., Mitchell, E. A., Fitzharris, P., Siebers, R., Wu, L. and Crane, J. 2008. Supplementation with *Lactobacillus rhamnosus* or *Bifidobacterium lactis* probiotics in pregnancy increases cord blood interferon-gamma and breast milk transforming growth factor-beta and immunoglobin A detection. Clin Exp Allergy 38(10): 1606-14.

Pålsson-McDermott E.M. and O'Neill, L.A. 2007. Building an immune system from nine domains, Biochem Soc Trans 35(pt 6): 1437–44.

Raghupathy, R. 1997. Th1-type immunity is incompatible with successful pregnancy. Immunol Today 18(10): 478-82.

Raunio, P., Pasanen, A. L., Reiman, M. and Virtanen, T. 1998. Cat, dog, and house-dust-mite allergen levels of house dust in Finnish apartments. Allergy 53(2): 195-9.

Rausch-Fan, X., Leutmezer, F., Willheim, M., Spittler, A., Bohle, B., Ebner, C., Jensen-Jarolim, E. and Boltz-Nitulescu, G. 2002. Regulation of cytokine production in human peripheral blood mononuclear cells and allergen-specific th cell clones by 1alpha,25-dihydroxyvitamin D3. Int Arch Allergy Immunol 128(1): 33-41.

Remes, S. T., Castro-Rodriguez, J. A., Holberg, C. J., Martinez, F. D. and Wright, A. L. 2001.

Dog exposure in infancy decreases the subsequent risk of frequent wheeze but not of atopy. J Allergy Clin Immunol 108(4): 509-15.

Remes, S. T., Iivanainen, K., Koskela, H. and Pekkanen, J. 2003. Which factors explain the lower prevalence of atopy amongst farmers' children? Clin Exp Allergy 33(4): 427-34.

Renz, H. and Herz, U. 2002. The bidirectional capacity of bacterial antigens to modulate allergy and asthma. Eur Respir J 19(1): 158-71.

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A. A. and Fanelli, C. 2010. Natural functions of mycotoxins and control of their biosynthesis in fungi. Appl Microbiol Biotechnol 87(3): 899-911.

Riedler, J., Braun-Fahrländer, C., Eder, W., Schreuer, M., Waser, M., Maisch, S., Carr, D., Schierl, R., Nowak, D. and von Mutius, E. 2001. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 358(9288): 1129-33.

Rintala, H., Hyvärinen, A., Paulin, L. and Nevalainen, A. 2004. Detection of streptomycetes in house dust--comparison of culture and PCR methods. Indoor Air 14(2): 112-9.

Rintala, H. and Nevalainen, A. 2006. Quantitative measurement of streptomycetes using real-time PCR. J Environ Monit 8(7): 745-9.

Rintala, H., Pitkäranta, M., Toivola, M., Paulin, L. and Nevalainen, A. 2008. Diversity and seasonal dynamics of bacterial community in indoor environment. BMC Microbiol 8: 56.

Rogers, B. B., Alexander, J. M., Head, J., McIntire, D. and Leveno, K. J. 2002. Umbilical vein interleukin-6 levels correlate with the severity of placental inflammation and gestational age. Human Pathology 33(3): 335-340.

Romagnani, S. 2000. The role of lymphocytes in allergic disease. J Allergy Clin Immunol 105(3): 399-408.

Romagnani, S. 2004a. Immunologic influences on allergy and the TH1/TH2 balance. J Allergy Clin Immunol 113(3): 395-400.

Romagnani, S. 2004b. The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? Immunology 112(3): 352-63.

Romagnani, S. 2007. Coming back to a missing immune deviation as the main explanatory mechanism for the hygiene hypothesis. J Allergy Clin Immunol 119(6): 1511-3.

Roponen, M., Hyvärinen, A., Hirvonen, M. R., Keski-Nisula, L. and Pekkanen, J. 2005. Change in IFN-gamma-producing capacity in early life and exposure to environmental microbes. J Allergy Clin Immunol 116(5): 1048-52.

Rowe, J., Macaubas, C., Monger, T., Holt, B. J., Harvey, J., Poolman, J. T., Loh, R., Sly, P. D. and Holt, P. G. 2001. Heterogeneity in diphtheria-tetanus-acellular pertussis vaccine-specific cellular immunity during infancy: relationship to variations in the kinetics of postnatal maturation of systemic th1 function. J Infect Dis 184(1): 80-8.

Saitoh, Y., Dake, Y., Shimazu, S., Sakoda, T., Sogo, H., Fujiki, Y., Shirakawa, T. and Enomoto, T. 2001. Month of birth, atopic disease, and atopic sensitization. J Investig Allergol Clin Immunol 11(3): 183-7.

Salyers, A. A. and Dixie, D. W. 2000. Microbiology: Diversity, Disease, and the Environment. Bethesda, Maryland, USA, Fitzgerald Science Press, Inc.

Saraf, A., Larsson, L., Burge, H. and Milton, D. 1997. Quantification of ergosterol and 3-hydroxy fatty acids in settled house dust by gas chromatography-mass spectrometry: comparison with fungal culture and determination of endotoxin by a *Limulus* amebocyte lysate assay. Appl Environ Microbiol 63(7): 2554-9.

Schaub, B., Lauener, R. and von Mutius, E. 2006. The many faces of the hygiene hypothesis. J Allergy Clin Immunol 117(5): 969-77; quiz 978.

Schaub, B., Liu, J., Hoppler, S., Schleich, I., Huehn, J., Olek, S., Wieczorek, G., Illi, S. and von Mutius, E. 2009. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. J Allergy Clin Immunol 123(4): 774-82 e5.

Schram-Bijkerk, D., Doekes, G., Douwes, J., Boeve, M., Riedler, J., Ublagger, E., von Mutius, E., Benz, M. R., Pershagen, G., van Hage, M., Scheynius, A., Braun-Fahrländer, C., Waser, M. and Brunekreef, B. 2005. Bacterial and fungal agents in house dust and wheeze in children: the PARSIFAL study. Clin Exp Allergy 35(10): 1272-8.

Schultz, C., Strunk, T., Temming, P., Matzke, N. and Härtel, C. 2007. Reduced IL-10 production and -receptor expression in neonatal T lymphocytes. Acta Paediatr 96(8): 1122-5.

Seroogy, C. M. and Gern, J. E. 2005. The role of T regulatory cells in asthma. J Allergy Clin Immunol 116(5): 996-9.

Simpson, A., John, S. L., Jury, F., Niven, R., Woodcock, A., Ollier, W. E. and Custovic, A. 2006. Endotoxin exposure, CD14, and allergic disease: an interaction between genes and the environment. Am J Respir Crit Care Med 174(4): 386-92.

Smart, J. M. and Kemp, A. S. 2001. Ontogeny of T-helper 1 and T-helper 2 cytokine

production in childhood. Pediatr Allergy Immunol 12(4): 181-7.

Smart, J. M. and Kemp, A. S. 2002. Increased Th1 and Th2 allergen-induced cytokine responses in children with atopic disease. Clin Exp Allergy 32(5): 796-802.

Starr, M. P., Stolp, H., Trüper, H. G., Balows, A. and Schlegel, H. G. (eds) 1981. The Prokaryotes, A Handbook on Habitats, Isolation and Identification of Bacteria. Berlin, Heidelberg, New York, USA, Springer-Verlag.

Stencel-Gabriel, K., Gabriel, I., Wiczkowski, A., Paul, M. and Olejek, A. 2009. Prenatal priming of cord blood T lymphocytes by microbiota in the maternal vagina. Am J Reprod Immunol 61(3): 246-52.

Strachan, D. P. 1989. Hay fever, hygiene, and household size. Bmj 299(6710): 1259-60.

Strachan, D. P. 2000. Family size, infection and atopy: the first decade of the "hygiene hypothesis". Thorax 55 Suppl 1: S2-10.

Sullivan Dillie, K. T., Tisler, C. J., Dasilva, D. F., Pappas, T. E., Roberg, K. A., Carlson-Dakes, K. T., Evans, M. D., Rosenthal, L. A., Gangnon, R. E., Gern, J. E. and Lemanske, R. F., Jr. 2008. The influence of processing factors and non-atopy-related maternal and neonate characteristics on yield and cytokine responses of cord blood mononuclear cells. Clin Exp Allergy 38(2): 298-304.

Tamay, Z., Akcay, A., Ones, U., Guler, N., Kilic, G. and Zencir, M. 2007. Prevalence and risk factors for allergic rhinitis in primary school children. Int J Pediatr Otorhinolaryngol 71(3): 463-71.

Tariq, S. M., Matthews, S. M., Hakim, E. A., Stevens, M., Arshad, S. H. and Hide, D. W. 1998. The prevalence of and risk factors for atopy in early childhood: a whole population birth cohort study. J Allergy Clin Immunol 101(5): 587-93.

Torvinen, E., Torkko, P. and Rintala, A. N. 2010. Real-time PCR detection of environmental mycobacteria in house dust. J Microbiol Methods 82(1): 78-84.

Triantafilou, M. and Triantafilou, K. 2005. The dynamics of LPS recognition: complex orchestration of multiple receptors. J Endotoxin Res 11(1): 5-11.

Täubel, M., Rintala, H., Pitkäranta, M., Paulin, L., Laitinen, S., Pekkanen, J., Hyvärinen, A. and Nevalainen, A. 2009. The occupant as a source of house dust bacteria. J Allergy Clin Immunol 124(4): 834-40 e47.

Uekert, S. J., Akan, G., Evans, M. D., Li, Z., Roberg, K., Tisler, C., Dasilva, D., Anderson, E., Gangnon, R., Allen, D. B., Gern, J. E. and Lemanske, R. F., Jr. 2006. Sex-related differences in immune development and the expression of atopy in early childhood. J Allergy Clin Immunol 118(6): 1375-81.

Upham, J. W. and Holt, P. G. 2005. Environment and development of atopy. Curr Opin Allergy Clin Immunol 5(2): 167-72.

Upham, J. W. and Stumbles, P. A. 2003. Why are dendritic cells important in allergic diseases of the respiratory tract? Pharmacol Ther 100(1): 75-87.

van der Velden, V. H., Laan, M. P., Baert, M. R., de Waal Malefyt, R., Neijens, H. J. and Savelkoul, H. F. 2001. Selective development of a strong Th2 cytokine profile in high-risk children who develop atopy: risk factors and regulatory role of IFN-gamma, IL-4 and IL-10. Clin Exp Allergy 31(7): 997-1006.

van Strien, R. T., Engel, R., Holst, O., Bufe, A., Eder, W., Waser, M., Braun-Fahrländer, C., Riedler, J., Nowak, D. and von Mutius, E. 2004. Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health. J Allergy Clin Immunol 113(5): 860-7.

Vercelli, D. 2006. Mechanisms of the hygiene hypothesis--molecular and otherwise. Curr Opin Immunol 18(6): 733-7.

Vitoratos, N., Papadias, C., Economou, E., Makrakis, E., Panoulis, C. and Creatsas, G. 2006. Elevated circulating IL-1beta and TNF-alpha, and unaltered IL-6 in first-trimester pregnancies complicated by threatened abortion with an adverse outcome. Mediators Inflamm 2006(4): 30485.

von Mutius, E. 2007. Asthma and allergies in rural areas of Europe. Proc Am Thorac Soc 4(3): 212-6.

von Mutius, E., Braun-Fahrländer, C., Schierl, R., Riedler, J., Ehlermann, S., Maisch, S., Waser, M. and Nowak, D. 2000a. Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin Exp Allergy 30(9): 1230-4.

von Mutius, E., Pearce, N., Beasley, R., Cheng, S., von Ehrenstein, O., Björkstén, B. and Weiland, S. 2000b. International patterns of tuberculosis and the prevalence of symptoms of asthma, rhinitis, and eczema. Thorax 55(6): 449-53.

von Mutius, E. and Schmid, S. 2006. The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe. Allergy 61(4): 407-13.

Warner, J. A., Jones, C. A., Jones, A. C. and Warner, J. O. 2000. Prenatal origins of allergic disease. Journal of Allergy and Clinical Immunology 105(2): S493-S496.

Warner, J. O. 2004. The early life origins of asthma and related allergic disorders. Arch Dis Child 89(2): 97-102.

Waser, M., von Mutius, E., Riedler, J., Nowak, D., Maisch, S., Carr, D., Eder, W., Tebow, G., Schierl, R., Schreuer, M. and Braun-Fahrländer, C. 2005. Exposure to pets, and the association with hay fever, asthma, and atopic sensitization in rural children. Allergy 60(2): 177-84.

West, M. A. and Heagy, W. 2002. Endotoxin tolerance: A review. Crit Care Med 30(1 Supp): S64-S73.

World Health Organization. 2009. WHO guidelines for indoor air quality: dampness and mould, Copenhagen, Denmark: WHO Regional Office for Europe.

Wright, R. J., Visness, C. M., Calatroni, A., Grayson, M. H., Gold, D. R., Sandel, M. T., Lee-Parritz, A., Wood, R. A., Kattan, M., Bloomberg, G. R., Burger, M., Togias, A., Witter, F. R., Sperling, R. S., Sadovsky, Y. and Gern, J. E. 2010. Prenatal maternal stress and cord blood innate and adaptive cytokine responses in an inner-city cohort. Am J Respir Crit Care Med 182(1): 25-33.

Yerkovich, S. T., Wikstrom, M. E., Suriyaarachchi, D., Prescott, S. L., Upham, J. W. and Holt, P. G. 2007. Postnatal development of monocyte cytokine responses to bacterial lipopolysaccharide. Pediatr Res 62(5): 547-52.

Yoo, Y., Yu, J., Kang, H., Kim, D. K., Koh, Y. Y. and Kim, C. K. 2005. Birth month and sensitization to house dust mites in asthmatic children. Allergy 60(10): 1327-30.

Zittermann, A., Dembinski, J. and Stehle, P. 2004. Low vitamin D status is associated with low cord blood levels of the immunosuppressive cytokine interleukin-10. Pediatr Allergy Immunol 15(3): 242-6.