Early life cytokines, viral infections and IgE-mediated allergic disease

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To my small, but growing family:

“Det finns bara möjligheter”
SUMMARY

Background: The reasons why some individuals become IgE-sensitised and allergic are largely unknown, though genetic- and early life environmental factors seem to be of importance.

Objective: The overall aim of this thesis was to investigate the relationship between IgE-sensitisation and allergic disease, viral infections, genetic markers and early life cytokines.

Results: IgE-sensitised children were found to have reduced numbers of IL-12 producing cord blood mononuclear cells (CBMC), whereas children diagnosed with eczema were found to have reduced numbers of IFN-\(\gamma\) producing CBMC. When dividing the children into early onset of IgE-sensitisation and late onset of IgE-sensitisation we found that the children with an early onset had low numbers of PHA-induced IL-4, IL-12 and IFN-\(\gamma\) secreting CBMC. At the age of two there was a general exacerbation of cytokine responses in the IgE-sensitised children, and the results were similar for the children with early onset IgE-sensitisation. Children with a late onset IgE-sensitisation were more similar to the non-sensitised children, but with a specific increase in the response to cat allergen (IL-4 and IFN-\(\gamma\)). The mothers of IgE-sensitised children, were just as their children, found to have an exaggerated cytokine response as compared to mothers of non-sensitised children. Maternal responses correlated well to the responses seen in the child, though the samples were taken two years after delivery.

Cytomegalovirus (CMV) infection in early life was associated to reduced numbers of IL-4, and increased numbers of IFN-\(\gamma\) producing cells at the age of two. No association between CMV seropositivity and IgE-sensitisation was seen. Epstein-Barr virus (EBV) infection, on the other hand, was inversely correlated with IgE-sensitisation, whereas no statistically significant association to cytokine production could be seen.

We also showed that the IL12B 1188 C-allele was associated to having a positive skin prick test at the age of two. The rare alleles of the three SNPs investigated (IL12B 1188C, IL12RB1132C and IRF1 1688A) were all associated to low IL-12 production at birth.

Conclusions: Our results indicate that allergic diseases are complex traits, and that both the genetic and the cytokine background differ between the different allergic diseases. We can also conclude that the time of onset seem to play a role when investigating IgE-sensitisation, and that perhaps early and late onset IgE-sensitisation have partly different causes. CMV and EBV infection early in life are associated to a protective cytokine profile and to protection from IgE-sensitisation, respectively, again indicating the heterogeneity and the complexity of allergic diseases.
This thesis is based on the following papers, which will be referred to in the text by their roman numerals:


## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY</td>
<td>V</td>
</tr>
<tr>
<td>ORIGINAL PAPERS</td>
<td>VII</td>
</tr>
<tr>
<td>CONTENTS</td>
<td>VIII</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>XI</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>13</td>
</tr>
<tr>
<td>The immune system</td>
<td></td>
</tr>
<tr>
<td>Innate immunity</td>
<td>13</td>
</tr>
<tr>
<td>Acquired immunity</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>14</td>
</tr>
<tr>
<td>Type 1 and type 2 responses</td>
<td>15</td>
</tr>
<tr>
<td>Regulatory T cells</td>
<td>15</td>
</tr>
<tr>
<td>Dendritic cells and antigen presentation</td>
<td>17</td>
</tr>
<tr>
<td>B cells and immunoglobulin production</td>
<td>18</td>
</tr>
<tr>
<td>Immunoglobulin E</td>
<td></td>
</tr>
<tr>
<td>The high affinity IgE receptor</td>
<td>18</td>
</tr>
<tr>
<td>The low affinity IgE receptor</td>
<td>19</td>
</tr>
<tr>
<td>The cytokines</td>
<td>19</td>
</tr>
<tr>
<td>Interleukin-4</td>
<td>19</td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>20</td>
</tr>
<tr>
<td>Interleukin-12</td>
<td>20</td>
</tr>
<tr>
<td>Interleukin-13</td>
<td>21</td>
</tr>
<tr>
<td>Interleukin-23</td>
<td>21</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>22</td>
</tr>
<tr>
<td>IgE-mediated allergic diseases</td>
<td>22</td>
</tr>
<tr>
<td>Diagnosis of IgE-mediated allergy</td>
<td>23</td>
</tr>
<tr>
<td>Sensitisation and the IgE-mediated allergic reaction</td>
<td>23</td>
</tr>
<tr>
<td>Genetic influence on allergy development</td>
<td>25</td>
</tr>
<tr>
<td>Single nucleotide polymorphisms in IL-12 related genes</td>
<td>25</td>
</tr>
<tr>
<td>Environmental factors and early allergy development</td>
<td>27</td>
</tr>
<tr>
<td>Passive smoking</td>
<td>27</td>
</tr>
<tr>
<td>Breast-feeding</td>
<td>27</td>
</tr>
<tr>
<td>Furred pets and livestock</td>
<td>28</td>
</tr>
<tr>
<td>Childhood infections</td>
<td>28</td>
</tr>
<tr>
<td>The gut flora</td>
<td>30</td>
</tr>
<tr>
<td>Prenatal influence on development of early allergy</td>
<td>31</td>
</tr>
<tr>
<td>AIM OF THE STUDY</td>
<td>33</td>
</tr>
<tr>
<td>SUBJECTS</td>
<td>34</td>
</tr>
<tr>
<td>Five year follow-up</td>
<td>35</td>
</tr>
<tr>
<td>METHODS</td>
<td>36</td>
</tr>
<tr>
<td>Clinical evaluation</td>
<td>36</td>
</tr>
<tr>
<td>Plasma sampling and handling of cells</td>
<td>36</td>
</tr>
<tr>
<td>Selection of allergens</td>
<td>37</td>
</tr>
<tr>
<td>Determination of total and allergen-specific IgE</td>
<td>37</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Virological serostatus</td>
<td>37</td>
</tr>
<tr>
<td>The ELISpot assay</td>
<td>37</td>
</tr>
<tr>
<td>Quantification of IL-12p40 mRNA</td>
<td>37</td>
</tr>
<tr>
<td>Statistical analyses</td>
<td>37</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>38</td>
</tr>
<tr>
<td>Cord blood cytokines in relation to IgE-sensitisation allergic disease (Paper I)</td>
<td>38</td>
</tr>
<tr>
<td>Cytokines in two-year old children and their mothers (Paper II)</td>
<td>40</td>
</tr>
<tr>
<td>Viral infections, IgE-sensitisation and cytokines (Paper III)</td>
<td>42</td>
</tr>
<tr>
<td>IL-12 SNPs in relation to IL-12 production and allergic disease (Paper IV)</td>
<td>44</td>
</tr>
<tr>
<td>Early life cytokines and time point of onset of IgE-sensitisation (Unpublished results)</td>
<td>45</td>
</tr>
<tr>
<td>CONCLUDING REMARKS</td>
<td>49</td>
</tr>
<tr>
<td>FUTURE PERSPECTIVES</td>
<td>51</td>
</tr>
<tr>
<td>SVENSK SAMMANFATTNING</td>
<td>53</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>58</td>
</tr>
</tbody>
</table>
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEDS</td>
<td>Atopic eczema/dermatitis syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<td>BcR</td>
<td>B-cell receptor</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CBMC</td>
<td>Umbilical cord blood mononuclear cells</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ELISpot</td>
<td>Enzyme-linked immuno spot</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IM</td>
<td>Infectious mononucleosis</td>
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<td>IRF</td>
<td>Interferon regulatory factor</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>NK</td>
<td>Natural killer</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PHA</td>
<td>Phytohaemagglutinin</td>
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<td>PPD</td>
<td>Purified protein derivative</td>
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<td>PRR</td>
<td>Pattern-recognition receptor</td>
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<td>QRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>RSV</td>
<td>Respiratory syncytial virus</td>
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<td>sIgE</td>
<td>Allergen-specific IgE</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SPT</td>
<td>Skin-prick test</td>
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<td>TcR</td>
<td>T-cell receptor</td>
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<tr>
<td>Th</td>
<td>T-helper cell</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<td>Treg</td>
<td>Regulatory T cell</td>
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<td>UTR</td>
<td>Untranslated region</td>
</tr>
</tbody>
</table>
INTRODUCTION

During the last decades, there has been a dramatic increase in the prevalence of IgE-mediated allergic disorders [1-3], though some recent studies indicate that this increase has not continued during the 1990ies [4-6]. Today, about one fourth of Swedish children are affected [7-9]. Although IgE-mediated allergies certainly have a genetic component, the reason for this rapid increase in prevalence is most likely to be found in the environment. In IgE-mediated allergic diseases, the immune system reacts adversely to innocuous substances in our environment. Thus, it is important to increase our knowledge in immunology in order to find be able to explain the underlying mechanisms in allergy.

The immune system

The immune system protects the human body from intruders, such as viruses, bacteria and fungi, and it is usually divided in to two branches: the innate and the acquired immune system. These two systems are closely interrelated and dependent on each other, and dysfunction in one will almost certainly affect the function of the other.

Innate immunity

The innate immune system is the first line of defence against foreign intruders. It is made up of chemical and mechanical barriers (skin and mucosa, mucous production, antibacterial peptides, low pH etc.) that prevent the passage of potentially harmful micro-organisms into the body. The innate immune system also consists of cells, such as phagocytic cells that are able to ingest and destroy pathogens, and cells that are able to release anti-microbial substances, e.g. basophils and natural killer (NK) cells. Besides, the complement proteins, as well part of the innate defence mechanisms, interact in highly regulated enzymatic reactions. The complement cascade-reaction facilitates antigen clearance, and generation of inflammatory responses.
Pathogen-associated molecular patterns (PAMP), that are present on a variety of micro-organisms, can be recognised by so-called pattern-recognition receptors (PRR) [10]. PRR are genome-encoded receptors, and activation of these receptors leads to various immune responses, e.g. cytokine production. The most well characterised class of PRR is the Toll-like receptors (TLR), of which 13 are known today in vertebrates [11]. The ligands for the TLR range from bacterial and viral DNA sequences to bacterial cell wall components and viral RNA, but also endogenous ligands [12, 13]. TLRs can be located either extra- or intracellularly and are mainly expressed on antigen-presenting cells, but have also been shown to be expressed on e.g. T cells.

**Acquired immunity**

The second branch of the immune system is antigen-specific and possesses both high specificity and memory, and is comprised of cells bearing receptors with affinity for specific antigens. B and T cells bear B-cell receptors (BcR, also called antibodies or immunoglobulins) and T-cell receptors (TcR), respectively. The BcR recognises specific epitopes on whole antigen molecules, whereas the TcR only recognise small peptide fragments (approximately 8-25 amino acids long) of the antigen, when displayed in the context of so-called major histocompatibility complex (MHC) molecules. The MHC molecules can be found on a broad spectrum of cells, called antigen-presenting cells (APCs). BcRs and TcRs are unique in that they can obtain a tremendous variety of specificities. This specificity is achieved mainly through genomic recombination of gene segments encoding distinct parts of the receptors. There are, as an example, more than $10^8$ possible specificities for a BcR.

The second feature of the acquired immune system is that it possesses memory. Immunological memory means that the cells of the acquired immune system can mount a quicker, and more long-lasting immune response of higher affinity, upon a second encounter with antigen. Memory is achieved through special memory cell formation, and the higher affinity in a memory response is achieved through a process called affinity maturation.
T cells

T cells can be sub-divided into cytotoxic T-lymphocytes (CTL) and helper T (Th) cells, which bear the CD8 and the CD4 receptors, respectively. In simple terms, the CTL-cells recognise and destroy cells that are infected with intracellular pathogens, when fragments of these are displayed on the surface of the infected cell, in the context of MHC class I molecules. Th cells, on the other hand, recognise extracellular antigens that are taken up by so called professional APCs, degraded and displayed on their surface in association with MHC class II molecules. When the Th cell recognises the antigen, the Th cell is activated, and among many other things, starts to produce cytokines. These cytokines will help the CTL-cells to kill their targets, and will also help B cells to produce antibodies.

Type 1 and type 2 responses

Depending on the nature of the APC that presents antigen to the T cells, the T cells will polarise in different directions. The factors determining the polarisation of the T cell include, besides genetics, e.g. the presence or absence of specific co-stimulatory molecules [14], the antigen dose, and other environmental factors, such as cytokines.

It has long been believed that Th cells can be polarised into either of two directions called Th1 and Th2 [15]. Th1 development is antigen dependent and induced by IFN-γ and by the activation of the transcription factor T-bet, and is maintained by IL-12 signalling and activation of the transcription factor STAT4 [16]. Th1 cells are characterised by the production of Th1-type of cytokines for example IFN-γ and IL-2 and promote mainly cellular immunity [17].

The Th2 lineage is also antigen-dependent and develops in the presence of IL-4 and the activation of STAT6, and it is thought that the Th2 lineage is maintained through the transcription factor GATA-3, whose gene is a direct target of its own product [16]. Th2 cells promote foremost humoral immune responses, with IgE and IgG4 production, through the production of Th2-type of cytokines. The Th2-type of cytokines include e.g. IL-4, -5, -6 and –13. Th1-type of cytokines suppresses the production of Th2-type of cytokines and vice versa [17]. IgE-mediated allergic
diseases are claimed to result from an imbalance between Th1 and Th2-type of immune responses, with a preferential skewing towards a Th2-response.

Since the initial discovery of the two Th cell subsets, it has become clear that the polarisation of Th cells is more complex [18], and that Th1- and Th2-type of cytokines can even be co-expressed in the same cell [19]. It is becoming clear that Th1 and Th2 cells are not exclusive in their production of Th1- and Th2-type of cytokines, which has led to the conclusion that not only Th cells, but also for example CTL cells, dendritic cells (DC) and natural killer (NK) cells, can be divided into type 1 and type 2, depending on their cytokine production [20-22]. It is also clear that there are definitely other Th-cell lineages than Th1 and Th2. For example regulatory Th cells have been extensively studied during the last few years, and recently, the existence of an IL-17 producing Th cell lineage, called Th-17, has also been proposed [23] (see Figure 1).

**Figure 1**
Precursor T helper cells (Th0) can develop in either of four directions, Th1, Th2, Treg or Th17, depending on e.g. the surrounding cytokine milieu. Tregs are a heterogeneous group of cells that develop after e.g. IL-4/TGF-β (Th3) or IL-10 (Tr1) stimulation.
**Regulatory T cells**

Recently, much focus has been on a subset of T cells called regulatory or suppressor T cells (Treg). These cells can be either CD4+ or CD8+, and work as suppressors of immune responses by various mechanisms, including both paracrine mechanisms and cell-cell contact. The Tregs are a largely heterogeneous group, and consists of many subpopulations, including e.g. Th3, Tr1, CD4\(^+\)CD25\(^{\text{high}}\) T cells, \(\gamma\delta\) T cells and aged CD4+CD25- cells [24]. However, expression of the Foxp3 transcription factor seems to be a common trait of these cells.

The Th3 cells are characterised by a preferential secretion of TGF-\(\beta\), and target mainly APCs with the effect of Th1 and Th2 down-regulation. Th3 cell differentiation is mainly dependent on IL-4 and TGF-\(\beta\). The Tr1 population is induced after IL-10 stimulation and these cells rarely divide because of their own high production of IL-10. They suppress immune responses via cell-cell contact and/or via secretion of IL-10 and TGF-\(\beta\). The CD4\(^+\)CD25\(^{\text{high}}\) suppressors mainly respond to self-antigens, and their elimination is sufficient to induce autoimmune disease in animal models [24].

**Dendritic cells and antigen presentation**

When an extracellular antigen enters the body it will be presented to Th cells in the context of MHC class II molecules. MHC class II molecules are present on the surface of professional APCs such as DCs. Immature DCs are densely scattered in skin and mucosa, areas that all antigens have to pass in order to gain entry to their host.

Immature DCs are very efficient in capturing and endocytosing antigens. Upon capturing the antigen, DCs migrate into regional lymph nodes where they present the antigen to Th cells. During migration, the DCs start a maturation process where they loose the ability to capture antigen through the down-regulation of endocytic receptors. Instead they become efficient in presenting the antigen through the up-regulation of MHC- and co-stimulatory molecules (e.g. CD 80 and CD86), which are needed for efficient presentation of the antigen to the Th cells in the regional lymph nodes [14, 25].
DCs are thought to be the only APCs that are able to present antigen to and activate naïve T cells, and thereby enable these cells to become effector- or memory cells [25].

**B cells and immunoglobulin production**

B cells first express IgM antibodies. However, upon the encounter with antigen and activation by cytokines in the surroundings, the B cell can switch from IgM to the production of other Ig isotypes. Different isotypes perform distinct effector functions, e.g. neutralisation of antigen, mediating phagocytosis and antibody-mediated cytotoxicity or activating the complement cascade.

*Immunoglobulin E*

The Th2-type of cytokines IL-4 and IL-13 (described below) are able to make B cells switch to IgE production [26, 27]. The switch to IgE requires also a second signal, namely the ligation of the CD40 molecule on the surface of the B cell with its ligand (CD154), expressed on activated T cells [28, 29]. The IgE molecule is a monomeric antibody, composed of two heavy and two light chains. It has a molecular weight of 190 kDa, which is slightly higher than IgG, because of the addition of a fourth constant region. The half-life of serum IgE is only 2-3 days, but its half-life is greatly prolonged when the antibody binds to Fc receptors on the surface of mast cells and basophils. There are two Fcε receptors: the high affinity FcεRI [30, 31] and the low affinity FcεRII.

*The high affinity IgE receptor*

FcεRI is the high affinity IgE receptor that is present primarily on mast cells and basophils, but it is also expressed in an alternative form on monocytes (trimeric as compared to the tetrameric that is expressed by mast cells and basophils) [32, 33]. Cross-linking of bound IgE up-regulates the expression of the receptor, indicating a mechanism for augmenting the biological effects of IgE when antigen is present [34]. The high affinity IgE receptor is greatly involved in atopic disease because the cross-linking of two adjacent receptors leads to mast cell degranulation and release of inflammatory mediators responsible for the IgE-mediated allergic reaction. The high affinity IgE receptors present on APCs, deliver antigen into MHC class II antigen presentation pathways [33].
Early life cytokines, viral infections and IgE-mediated allergic disease

The low affinity IgE receptor
FcεRII, also called CD23, is present on B cells, monocytes, DCs, eosinophils and many other cell types [35, 36]. The low affinity receptor has a 100-1000 fold lower affinity for IgE, than the high affinity receptor FcεRI. FcεRII is not directly involved in IgE-mediated allergy reactions, but serves as a regulator of IgE synthesis [37]. Blocking of the receptor diminishes IgE synthesis by B cells [35], and studies show a correlation between serum IgE levels and the number of CD23 positive cells [38]. CD23 exists both in a membrane-bound form and as a soluble receptor that is generated through auto-proteolysis of the membrane-bound receptor [36].

The cytokines
Cytokines are a heterogeneous group of molecules, produced by most cells and used in the communication between cells. Here, only a few of them are mentioned, i.e. those of particular interest for this study.

Interleukin-4
IL-4, the signature Th2-type cytokine, is responsible for the isotype switching of B cells from IgM/D to production of IgE and IgG4, and it also augments IgE production [27]. IL-4 is mainly produced by Th2 lymphocytes, but can be produced by other cells, such as mast cells, basophils, macrophages and B cells [39]. The induction of isotype switching in B cells is not the only functional property of IL-4. IL-4 can as well promote the differentiation of Th cells into the Th2 lineage, and inhibit the differentiation of Th1-type cells [18].

It is of importance to remember that although IL-4 and IL-13 are responsible for the switch to IgE, and therefore can be classified as “the bad guys” in atopic diseases, they are extremely important for other immunological processes. Thus, mastocytosis, eosinophilia, IgE synthesis and mucous production that are induced by the Th2-type of cytokines are of importance for elimination of infesting worms [40].
Interleukin-10

IL-10 is a cytokine that contributes to the down-regulation and termination of immune responses through its ability to down-regulate both Th1- and Th2-type of responses [41]. IL-10 was first recognised as CSIF, or cytokine synthesis inhibitory factor. In mice, IL-10 is classified as a Th2-type of cytokine since it has more powerful down-regulating activities on Th1- than on Th2-type of responses. However, in humans IL-10 is usually not classified as either Th1- or Th2-type since its effects are similar on both sides [42]. IL-10 is produced in large quantities by certain regulatory T cell subsets, but can also be produced by many other cell types. In humans, monocytes and B-cells are the major producers of IL-10 [43].

Interleukin-12

IL-12 is a heterodimer, consisting of two disulfide-linked subunits. The p35 subunit is constitutively expressed but not secreted, while the p40 subunit is inducible and is the rate-limiting step in IL-12p70 production. The subunits, p35 and p40, are encoded by genes on different chromosomes, 3p12-3q13.2 and 5q31-33 [44], respectively. IL-12 is produced primarily by macrophages and DCs is involved in the regulation of both innate and adaptive immune responses [45].

IL-12 is mainly produced after microbial stimulation [45], but also after engagement of the CD40 molecule with its ligand (CD154), that is present on activated Th cells, and can also be induced by IFN-γ [46]. IL-12 exerts its biological effect through binding to specific IL-12 receptors (IL-12R). The functional high-affinity IL-12R is composed of two subunits (IL-12Rβ1 and IL-12Rβ2), each independently exhibiting low affinity for IL-12. The presence of the two subunits of the IL-12R is necessary for the IL-12/IL-12R system to be functional [47].

IL-12 was originally called natural killer cell stimulatory factor (NKSF) because of its ability to stimulate NK cells [48]. A molecule with the ability to influence CTL cells, termed cytotoxic lymphocyte maturation factor, was found to be identical with NKSF, and both are now called IL-12 [49]. IL-12 is a key factor in the induction of macrophage and NK cell activation, generation of Th1 cells and CTLs, generation of opsonising as well as complement fixing antibodies, and resistance to intracellular
Early life cytokines, viral infections and IgE-mediated allergic disease

Infections [45]. IL-12 favours Th1 differentiation through stimulation of IFN-γ production by T and NK cells [45]. IL-12 is an important bridge between the innate and the adaptive immune system, since it is produced upon microbial stimuli and pattern recognition, but exerts its effects on the adaptive immune response.

Tumours and infectious agents often evade immune responses by producing IL-12 inhibiting agents, such as IL-10 and TGF-β [50].

Interleukin-13

IL-13 is important in IgE-mediated allergies as one of its receptor subunits (the α subunit) is shared with the IL-4 receptor [51]. IL-13 is, just as IL-4, able to induce IgE class switch in B cells [26]. Functional IL-13 receptors are not expressed by T cells, and therefore IL-13 can not, in contrast to IL-4, induce Th2 differentiation [52]. IL-13 is produced by T cells and DCs [52].

Interleukin-23

IL-23 is another member of the IL-12 family of heterodimeric cytokines. The IL-23 heterodimer is composed of the IL-12p40 subunit and a novel subunit, structurally very similar to the IL-12p35 subunit, called p19 [53]. Just as for IL-12, the synthesis of both subunits within the same cell is required for the production of the bioactive cytokine. IL-23 is mainly expressed by activated DCs and phagocytic cells [53]. IL-23 seems to be more readily induced by TLR2 agonists, than by TLR4 agonists such as LPS [54].

IL-23 exerts its effects through binding to its dimeric receptor, composed of the IL-12Rβ1 subunit and a novel receptor called the IL-23R [55]. IL-23R shares many features with the IL-12Rβ2 subunit, and they mainly signal through the same signalling molecules, though some distinctions exist. The IL-23R is highly expressed on activated/memory T cells and NK cells, whereas monocytes, macrophages and DCs express low levels [55].

IL-12 and IL-23 seem to have both overlapping, but also distinct, effects despite their many structural and signalling similarities. One of the main effects of IL-23 is that it
appears to be crucial for the development of a new Th-cell subset. This new Th-cell subset is characterised by the production of the pro-inflammatory cytokine IL-17, and has been designated the name Th17 [23] (see Figure 1). IL-12 on the contrary, appears to inhibit IL-23 induced IL-17 production [56, 57]. Th17 cells trigger potent pro-inflammatory responses by up-regulating chemokine production in cells such as fibroblasts. The effects of IL-23 on B cells, are largely unknown [58].

**Interferon-γ**

The most typical Th1-type of cytokine is IFN-γ, a heavily glycosylated 34 kD homodimer. IFN-γ augments cytotoxic responses to intracellular organisms and tumours and is produced by T lymphocytes and NK cells after antigenic stimuli. IFN-γ can also be induced after IL-12 stimulation [46]. IFN-γ is a potent activator of macrophages and neutrophils, and it has opposing effects to IL-4 in that it promotes differentiation of Th0 cells into the Th1 lineage, and inhibits the differentiation of Th2-type of cells [17]. IFN-γ has been shown to inhibit the production of IgE [27].

**IgE-mediated allergic diseases**

The word allergy is derived from Greek and means altered reaction (allos = altered or changed, ergon = reaction). The term was originally intended to mean “deviation from the original state” after antigenic stimulation, irrespectively of whether the response was beneficial or harmful to the host. According to the nomenclature task force of the European Academy of Asthma and Clinical Immunology (EAACI), the term allergy should be used to describe a hypersensitivity reaction, initiated by immunological mechanisms [59]. In most allergic diseases, the reaction is antibody mediated, and the antibody typically responsible is of the IgE isotype, but allergic disease can also be cell mediated.

The word allergy is generally used interchangeably with the term atopy, a word that, according to the EAACI nomenclature task force, should be used to describe a personal or familial predisposition to produce IgE antibodies to low doses of otherwise harmless antigens, called allergens. Thus, the term atopy should only be used when IgE sensitisation has been documented [59].
Early life cytokines, viral infections and IgE-mediated allergic disease

**Diagnosis of IgE-mediated allergy**

There are several methods for diagnosis of IgE-mediated allergic diseases. The most common, and simple way of diagnosis in the clinic is the skin prick test (SPT). In the SPT, allergens are placed on the skin of the patient, usually on the forearm, and a lancet is used to prick the surface of the skin in order to make the allergen penetrate. If IgE is present, mast cells in the skin will degranulate and cause a weal and flare reaction, of which the weal size can be estimated. There are also *in vitro* methods available to establish the involvement of IgE, where the amount of allergen-specific IgE (sIgE) in serum is quantified with the help of radioactively labelled anti-IgE antibodies.

**Sensitisation and the IgE-mediated allergic reaction**

During the first encounter with an allergen, in the atopic individual, IgE antibodies are produced that bind to high affinity Fcε receptors on mast cells present in the mucosal membranes. This is called the sensitisation phase. Upon the second encounter with the allergen, the specific IgE molecules will recognise and bind the allergen (see Figure 2). Cross-linking of two nearby IgE antibodies on the cell surface of the mast cell leads to its degranulation. The mast cell granules contain preformed mediators, such as histamine, that cause contraction of smooth muscle, increased vascular permeability and increased mucous production. In addition to these effects, histamine has profound effects on immune regulation [60]. The cross-linking of the surface IgE will also start the synthesis of other mediators such as prostaglandins and leukotrienes that further increase bronchoconstriction, mucous production and vascular permeability.

The effects of mast cell degranulation are usually divided into an early or acute and a late phase. The acute phase is the direct effect of the release of the pre-formed mediators from the mast cell granules, and the late phase is the consequence of the cytokines that are also synthesised and released upon IgE cross-linking. The cytokines mediate the recruitment of inflammatory cells to the affected area (i.e. the airways in allergic asthma or the conjunctiva in allergic conjunctivitis), which in turn leads to a delayed long-lasting inflammation. The IgE-mediated allergic reactions cause
symptoms of varying complexity, ranging from hay fever and eczema, to life-threatening conditions such as systemic anaphylaxis.

**Figure 2**
When an allergen enters the body for the first time, it is captured by dendritic cells (DC). The DC processes and presents peptide fragments of the allergen through MHC class II molecules on their surface. The fragment is recognised by T helper (Th) cells, which for unknown reasons, in certain individuals induces the production of interleukin-4 (IL-4). IL-4 induces class switch to IgE and IgG4 in B cells. The IgE attaches to the high affinity IgE-receptors on the surface of e.g. mast cells. This cascade of events is called the sensitisation phase. Upon a second encounter with the allergen, the allergen binds to and cross-links the IgE molecules on the mast cell surface, which leads to the degranulation of the mast cell and the release of both preformed and newly synthesised mediators causing symptoms of allergy.

The reason why some people start to produce IgE antibodies upon encounter with allergens, while others do not, is not known. Allergens are seemingly common antigens, that are in the normal case non-hazardous, but which for some reason induce Th2-type of responses in certain individuals, that can lead to detrimental effects. As to yet there are no common traits revealed for allergen structure or function. What is known is that the genetic constitution of the individual, and the amount of antigen during sensitisation is critical. High antigen exposure upon the first encounter is said
Early life cytokines, viral infections and IgE-mediated allergic disease

to result in immunological unresponsiveness, while low allergen doses result in sensitisation [61]. It has also been proposed that it is not the balance between Th1- and Th2-like immune responses, but the balance between Th2- and regulatory T cells that may be decisive in the development of IgE-mediated allergy [62].

**Genetic influence on allergy development**

It is well known that the risk for a child to develop allergy is increased if one of the parents is allergic, and that the risk increases even more if both parents are allergic [63, 64]. The risk of developing allergy for a child with no family history is up to 20%, whereas if both parents are allergic the risk is 50-75% [65], so there is no doubt that the genetic heritage is of major importance for the development of allergic diseases. The genetic influence is multifactorial, i.e. no single atopy gene has been identified. In the year 2000, over 22 loci on 15 chromosomes had been reported from candidate gene studies. However, the strongest evidence points towards four main regions: 5q, 6p, 11q and 12q [66]. The 5q31-33 region encodes a cytokine cluster, including the genes for IL-4 and IL-13, and the 11q13 region includes e.g. the gene for the β-chain of the high affinity IgE receptor. Within the 12q15-24.1 region, the genes for IFN-γ and STAT-6 are found, and the 6p21 and HLA-D region contains genes that are involved in antigen recognition and immune responsiveness [66].

**Single nucleotide polymorphisms in IL-12 related genes**

Genes encoding cytokines are prime candidates for the genetic analysis in diseases resulting from helper T cell imbalances. Atopic diseases are, as mentioned earlier, claimed to result from an imbalance between Th1 and Th2 type of cytokines, with a skewing towards a preferential Th2-response. Many single nucleotide polymorphisms (SNPs), in many different genes, have been described that are associated with IgE-sensitisation and allergic diseases. IL-12 it is the major Th1 promoting cytokine, and has been implicated in the pathogenesis of atopic disease [67-69]. A number of SNPs have been described in the IL-12p40 gene and IL-12 related genes, that might affect the production of IL-12, and there by also skew the immune system towards or away from atopic disease.
One of the described SNPs is the IL12B (A1188C) in the 3’ untranslated region (UTR) of the IL-12p40 gene. This SNP has been associated with susceptibility to both Th2 and Th1 driven diseases, such as atopic dermatitis and psoriasis vulgaris [70], inability to resolve herpes virus C infection [71] and to multiple sclerosis [72]. However, others have reported on a lack of association between IL12B 1188 genotype and immunological disease [72, 73]. Thus, the 3’UTR polymorphism has been reported to correlate to both increased IL-12p70 secretion [74] and decreased IL-12p40 secretion [75] in vitro.

Several SNPs are found in the IL-12Rβ1 and IL-12Rβ2 genes [76, 77]. In the IL-12Rβ1 gene a SNP exists at position 1132 in exon 10, causing an amino acid substitution (G→R), which has been found to be in complete linkage disequilibria with three other SNPs in this gene at positions 641, 684 and 1094 [76]. In the same study, homozygotes for the CC genotype of the IL-12Rβ1 (G1132C) SNP were shown to have lower levels of IL-12 induced signalling [76]. The IL-12Rβ1 SNP has been found to be linked to tuberculosis [76], but not to endometriosis [78].

Interferon regulatory factor (IRF)-1 is a member of the IRF family of transcription factors, that are involved in the regulation of genes that control cell growth, differentiation and death. IRF-1 is involved in regulation and expression of IFN-α, β and γ inducible genes such as MHC class II. In addition, IRF-1 is involved in several steps of the immune response, including the polarization of the cytokine response in CD4+ T cells. The gene for IRF-1 is located on chromosome 5q31 [79], which is linked to atopy and asthma in genome wide researches [80]. IRF-1 deficiency results in an elevation of Th2 cytokines and is related to a failure to mount a Th1 response, which can be attributed to impaired IL-12 production [81, 82]. IRF-1 binding sites are present in the IL12B promoter region (Ma et al., 1996), and IRF-1 serves as a critical transcription factor for high levels of IL-12 production in macrophages [83]. A G- to A polymorphism at position 1688 in the 3’UTR of IRF1, linked to Juvenile Idiopathic Arthritis, was discovered by Donn et al. [84].
Environmental factors and early allergy development

There are differences in the prevalence of atopic diseases in different parts of the world, and the sharp increase in prevalence came earlier in the “westernised” world. A unique opportunity was offered to researchers during the reunification of East and West Germany. Before the unification, the allergy prevalence was higher in West Germany [85, 86]. As the standard of living has increased in the former East, the prevalence of allergy is raising, and approaching the same levels as in former West Germany [87-89]. Considering that the two populations have a similar genetic background, this indicates that the way of living certainly affects the probability of allergy development, independently of genetic predisposition. Environmental stimuli might affect gene expression and thereby also the phenotypic outcome. It has been shown that environmental factors might be able to elicit different and even opposite phenotypes on the same genetic background [90].

Further evidence for the hypothesis that life style factors affect the prevalence of allergy came from studies showing that children brought up in an anthroposophic environment have lower prevalence of allergic diseases [91, 92]. The Th1/Th2 balance has also been shown to be affected by psychological stress [93, 94].

Passive smoking

One environmental factor well known to enhance the risk for wheezing and asthma, is postnatal maternal smoking [95] (Raherison C et al. article in press). Also maternal smoking during pregnancy is associated with increased allergy morbidity and decreased lung function in the child [96, 97]. One study has shown that cord blood from children born to smoking mothers have increased levels of IL-13 [98], whereas another study showed lower levels of both IL-4 and IFN-\(\gamma\) and an increased risk of wheezing in children at the age of 6 [99].

Breast-feeding

It is not controversial that breast-feeding is the preferred method of infant nutrition, because of its nutritional, immunological and psychological effects. The effects of breast-feeding on the development of allergic disease are more disputed [100]. A bulk of evidence suggests, that exclusive breast-feeding for at least four months seem to
protect against eczema and wheezing in childhood [100-102]. However, there are studies showing the opposite [103], and one study shows that mothers with eczema and a long duration of breast-feeding increase the risk of allergy in the child [104]. In line with these results, milk of atopic mothers has been shown to have a different composition from that of non-sensitised mothers e.g. concentration of IL-4 was higher in breast milk of atopic mothers [105].

**Furred pets and livestock**

Most of the data available on the effects of early exposure to pets on allergies are conflicting. However, for dogs the data seem to point towards a protective effect of early exposure, but for cats the data are more inconsistent [106]. Several publications show that children growing up on farms that keep livestock are less prone to develop allergic diseases [92, 107-111] and there is evidence that these children loose their sensitisation more frequently than other children [112]. However, these data are not undisputed [106]. Important to remember is that how much of this effect that can be attributed to animal allergens *per se*, and not to e.g. endotoxin and other microbial products, is still unclear. A delay in the Th1 shift during early life has been observed in atopic children and a recent Finnish study showed that the change in IFN-γ responses during the first months of life was associated with farming, endotoxin in house dust and cat and dog exposure [113], indicating that early contact with animals may influence allergy development.

**Childhood infections**

There has been much focus on patterns of early childhood infections, and available data suggest that the decreased load of infections in the modern society prevents the development of a strong Th1-type immunity. The resulting “under-developed” Th1 immunity leads to a skewing towards a Th2-type of protection, which in turn would increase the risk for developing allergic diseases later in life. There is lots of epidemiological evidence for this theory, generally called “the hygiene hypothesis”.

The first evidence was published in 1989, where it was shown that a large family size was inversely related to the risk of developing hay fever [114]. Later studies show that children with many elderly siblings [115] and children attending early day-care [116]
have a lower prevalence of allergic diseases, probably because of the greater risk of infection. One Italian study has shown the inverse relationship between orofecal and food-borne infections to asthma and total IgE levels [117].

Today, early childhood infections are often viral, and not bacterial. Viral infections trigger an NK- and later a T cell response with high production of IFN-γ and accordingly, repetitive viral infections are claimed to protect from childhood allergic disease. Matricardi et al. showed that students undergoing military training who were seropositive against hepatitis A had a low prevalence of allergic diseases [117]. Despite this, certain individual viruses, mainly respiratory, have been reported to increase the risk for allergic disease. Children with an increased number of parentally reported respiratory tract infections in early life, were reported to have a higher risk of developing asthma [118]. A severe respiratory syncytial virus (RSV) infection in the first year of life has been linked to an increased risk for asthma, high serum allergen-specific IgE and a positive SPT at the age of 13 [119]. Also rhinovirus and metapneumovirus have been associated to childhood wheezing. An explanation for this effect could be that respiratory viruses damage the barrier function of the airway epithelium, leading to enhanced absorption of aeroallergens across the airways, thus increasing the risk for sensitisation.

Other viruses, such as herpes viruses, might also have an effect on allergic diseases. Both cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are DNA-viruses of the herpes family. They infect via the respiratory tract, and whereas CMV mainly infects monocytes, the primary target of EBV is B-cells. CMV is often transmitted to the child during delivery or later through the breast milk [120]. In developing countries, EBV and CMV primary infections are often asymptomatic. In developed countries, the primary infection is often delayed into puberty, resulting in infectious mononucleosis (EBV).

CMV and EBV are persistent viruses. They evade the immune system through e.g. keeping a restricted gene expression and interfering with the host antigen processing and presentation. Through their chronic nature it would be possible that both viruses exerts effects on the immune system.
In a recent study, the serostatus for CMV and EBV in four-year old children in relation to IgE sensitisation was studied in a cohort in Sweden with prospective data collection (The BAMSE-study). There were no associations between the viral serostatus and clinical allergic symptoms. However, IgE-sensitisation against air-born and food allergens was positively associated with CMV seropositivity among children who were seronegative against EBV [121]. A study from our group, showed that EBV infection was negatively associated with IgE-sensitisation, and that this association was further enhanced by CMV co-infection [122]. A few other studies have also been performed where the relationship between allergies and CMV/EBV infection have been studied [123-125]. Unfortunately, the results have been inconclusive.

Although there are a lot of epidemiological data supporting the hygiene hypothesis, the immunological basis for this hypothesis remains controversial, and more recent studies have e.g. shown increased risk for eczema in children attending day-care [126]. If an impaired microbial stimulation would result in a general shift of the immune response towards a Th2-type of response, with increased prevalence of IgE-mediated allergic diseases as a result, we would also expect Th1-like diseases to decrease in prevalence. The truth is that Th1-associated diseases, such as autoimmunity (e.g. multiple sclerosis and inflammatory bowel disease), are actually also on the rise.

The gut flora
The effector Th1 and Th2 type of cells are controlled by specialised subsets of regulatory T cells. These regulatory cells are mainly triggered by usually harmless micro-organisms such as helminths, bifidobacteria and lactobacilli, which are nowadays virtually absent in the westernised society [127]. In contrast to the hygiene hypothesis, deficient exposure to these “old friends” might explain the increase in general, in not only Th2 associated disease such as IgE-mediated allergy, but also Th1 associated disease.

The establishment of the intestinal micro-flora is proposed to be a major factor driving the maturation of the immune system in newborns. It is now generally accepted that the bacterial micro-flora of the human gut is an integral component of
Early life cytokines, viral infections and IgE-mediated allergic disease

the immune defence, where it serves as a constant stimuli to keep the immune system alert [128]. An association between the microbial gut flora and the development of allergy has been found in several studies [128]. Bifidobacteria have been reported to be less prevalent, while Clostridia comprised a higher proportion of the intestinal micro-flora in children with allergic diseases [129]. In addition, prospective studies of probiotics containing lactobacilli, show promising results in the prevention of eczema, although no effect on IgE sensitisation was observed [130]. Altogether the recent results on composition and colonisation of the intestinal micro-flora indicate that these factors might affect early allergy development.

Prenatal influence on development of early allergy

It has until recently been regarded as a fact that the intra-uterine environment is strongly skewed towards a Th2-type of immunity, and that Th1-type immunity is not compatible with a successful pregnancy [131]. During the last years it has become clear that a balanced Th1/Th2 immunity is necessary for pregnancy to succeed [132, 133], though the foetus is still considered to be Th2-skewed [134]. Pregnancy is by no means an immune-suppressed state, as was previously believed, though potentially dangerous T cell mediated responses are down-regulated, and components of the innate immune system are more active [133].

It has been shown that allergen sensitisation occurs already in utero, the evidence being that the umbilical cord blood mononuclear cells (CBMC) of newborn children proliferate in response to allergen challenge in vitro [135-138]. The mechanism for the prenatal priming is unknown, but there are speculations suggesting the transfer of soluble factors from the mother to the child. Such factors could be allergen, allergen in complex with antibody or anti-idiotypic antibodies. There are several studies showing that both free allergen and allergen in complex with IgG antibodies can pass from the maternal to the foetal side of the placenta [139-141], and that cells and co-stimulatory molecules needed for priming of the immune system, are present in the foetus already before birth [142]. Another study suggests that maternal IgE can pass the foetal membranes and sensitise the child [143].
Many studies have been performed to find out the effects of prenatal exposure on the development of early atopy. One recent Dutch study shows that there is at least a short-lasting protective effect of prenatal exposure to pets [144]. Another study on the effects of high and low-dose exposure to birch pollen during pregnancy concludes no major differences on the sensitisation rates in the children, rather they conclude that the atopic state of the mother is of greater importance for development of sensitisation in the child than is the in utero exposure level [145]. In another study the same authors also show that early postnatal birch pollen exposure seem to be of more importance than the prenatal in utero exposure [146].

During the 1990:s several reports claimed that there was a greater risk for a child to develop atopic disease, if the mother was atopic than if the father was atopic [147-149]. The excessive maternal influence on the child is further supported by the fact that infant total IgE-levels correlate with maternal, but not paternal total IgE-levels [150]. One may speculate that atopic mothers, that are systemically more Th2 skewed than non-atopic mothers, therefore would provide a more Th2 skewed in utero milieu, and hence predispose the immune system of their fetuses towards an atopic cytokine profile. Our group, and others, have shown that the cord blood of children with an atopic mother is more polarised towards a Th2-type of cytokine profile, as reflected by elevated IL-4 levels [151], elevated IL-4/IFN-γ ratios and fewer IL-12 producing cells [69], or by lower levels of IFN-γ [64, 152-154]. Many groups have also shown a depression of both Th1 and Th2 responses in cord blood of atopic children, but the time for diagnosing allergic outcome time has often been during the first years of life. In a study following children up to the age of six, Macaubas et al. showed that sensitisation to inhalant allergens and asthma at six years of age was associated with low cord blood IL-4 and IFN-γ [99].

Another more speculative explanation for the preferential maternal inheritance could be genetic factors passed on from mother to child through the mitochondrial DNA. Mitochondrial DNA is only inherited from the mother.
AIM OF THE STUDY

The over-all aim of this study was to evaluate the association between IgE-sensitisation, allergic disease and viral infections in young children and early life cytokine secretion.

More specifically we wanted to evaluate:

- the association between cord blood cytokine profiles and IgE-sensitisation/allergic disease among children at two years of age (Paper I)
- the cytokine profiles in mothers of IgE-sensitised and mothers of non-sensitised children, when the child was two years of age (Paper II)
- cytokine profiles among IgE-sensitised and non-sensitised children at two years of age (Paper II)
- the association between cytokine profiles and CMV and EBV serostatus to IgE-sensitisation among children at two years of age (Paper III)
- the association between three IL-12 related SNPs and IL-12 production, IgE-sensitisation and allergic disease in early life (paper IV)
- the association between early life cytokine profiles and different time points of onset of IgE-sensitisation
SUBJECTS

All papers in this thesis utilise material from the same, larger longitudinal and prospective study cohort.

Families living in the south of Stockholm who where expecting a child, were asked by midwives at the maternity wards if they were interested in participating in the study. The invitation was addressed to families where both or none of the parents were allergic. Besides, families where only the mother was allergic were also invited to participate. The last group of women were invited in order to be able to study the impact of only the mother’s allergy on the immunology of the foetus/newborn infant. A total of 717 families showed interest in the study and received further information and were interviewed by telephone. Some 330 parental couples fulfilled the selection criteria for participating in the study and were invited to the out patient ward at Sachs’ Children’s Hospital for a further interview and skin prick testing (SPT).

Only those parents whose SPT results confirmed a positive or a negative history of respiratory allergy (bronchial asthma and/or allergic rhino-conjunctivitis) to pollen and/or furred pets were invited to continue the study (n=281). The children were born from September 1997 until August 2000. One hundred and twenty children had two allergic parents (group dh = double heredity), 84 children had an allergic mother but not an allergic father (group mh = maternal heredity) and 77 children had no allergic parents (group nh = no parental heredity).

- In paper I, the association between cytokine profiles in cord blood and IgE-sensitisation and presence of allergic diseases at 2 years of age was evaluated in 82 of the 281 children. The children participating in paper I were originally recruited for a study investigating the impact of family history of allergy on cord blood cytokines [69], and were consecutively selected. The selection was based on whether the amount of CBMC obtained was sufficient for the
ELISpot assay and if an even distribution in relation to allergic heredity was achieved.

- **In paper II**, cytokine profiles in IgE-sensitised and non-sensitised children, when the child was two years of age, were evaluated by the ELISpot assay. Mononuclear cells in peripheral blood from 77 (94%) of the 82 children in paper I were available for analysis. Besides, mononuclear cells from peripheral blood from the mothers of the 77 children were also included for analysis.

- **In paper III**, the association between cytokine profile in PBMC and serostatus against EBV and CMV at two years of age was evaluated. Seventy-five (97%) of the children in paper I were evaluated.

- **In paper IV**, the association between three IL-12 related SNPs, IL-12 production and allergic disease was evaluated. One hundred and eighty-four (65%) of the 281 children were included for genotyping. The children were selected on basis of the availability of pelleted cells from the serum samplings at 12 month of age.

**Five year follow-up**

Two hundred and forty (86%) children attended the five-year surveillance visit to the clinic. Clinical data as well as results on IgE sensitisation (SPT and blood sampling) at 2 and 5 years of age are available for 226 (80%) of the children.

The five years follow-up allowed us to evaluate the association between early life cytokines and IgE sensitisation over time among 70 (85%) of the children participating in paper I. The children were divided in three categories; those with early (persistent) IgE-sensitisation, i.e. sensitisation both at 2 and 5 years of age; late IgE sensitisation, i.e. sensitisation only at 5 years of age; and children that had never been sensitised.
METHODS

Clinical evaluation

The children were examined at Sachs’ Children’s Hospital at 6, 12, 18, 24 and 60 months of age by the same paediatrician.

- **Eczema**, previously called atopic eczema/dermatitis syndrome (AEDS) was defined according to Hanifin and Rajka [155].

- **Asthma** during the first two years was defined as at least 3 episodes of wheezing or signs of hyper-reactivity (wheezing or severe coughing at exaltation, infections, exercise and exposure to cold weather or disturbing coughing at night) and in addition respiratory symptoms treated with inhaled glucocorticoids. The child was also classified as having wheeze/asthma if having any episode of wheezing or hyper-reactivity in combination with a family history of allergic disease or allergic symptoms in the child.

- **Allergic rhino-conjunctivitis** was diagnosed if rhinitis and/or conjunctivitis appeared at least twice after exposure to a particular allergen and was unrelated to infection.

- **IgE-sensitisation** was defined as a positive SPT (≥3mm) and/or the presence of allergen-specific IgE antibodies (≥0.35 kU/l) in plasma, in accordance with Johansson et al. [59].

Plasma sampling and handling of cells

Plasma was separated from whole blood samples by centrifugation at 6, 12, 18, 24 and 60 months. Thereafter mononuclear cells from cord blood, 24 and 60 months samples were separated through gradient centrifugation. The blood samples were obtained from the umbilical cord vein after delivery and from the child and the mother two years later. Further detailed descriptions on the handling and preparation of cells are provided in papers I-IV.
Early life cytokines, viral infections and IgE-mediated allergic disease

Selection of allergens

For allergen stimulation three allergens were used; birch pollen, cat dander and hens egg albumin (ovalbumin). The allergens were chosen already during the cord blood study (paper I), when the allergic status of the children was unknown. They were selected in order to detect a general predisposition to develop IgE-sensitisation, and not to detect individual antigen-specific responses. We chose to include two inhaled allergens, one perennial (cat) and one seasonal (birch), and one food (ovalbumin) allergen. We decided to continue with the same allergens also for the stimulation of PBMC in the two-year olds, in order to be able to compare the results. PHA, a polyclonal T cell stimulator was used as the positive control, and in paper IV LPS was included in order to reach a maximum release of IL-12.

Determination of total and allergen-specific IgE

Descriptions of IgE quantification methodology can be found in the methods section of papers I and II.

Virological serostatus

Methodology for IgG serostatus against CMV and EBV is described in paper III.

The ELISpot assay

The ELISpot assay is described in full in the methods section of paper I and paper II.

Quantification of IL-12p40 mRNA

The methods for RNA preparation and IL-12p40 mRNA quantification can be found in the methods section of paper IV.

Statistical analyses

The statistical methodology is described in each material and methods section.
RESULTS AND DISCUSSION

Cord blood cytokines in relation to IgE-sensitisation allergic disease (Paper I)

In the first paper, we aimed at investigating if the cytokine profile in cord blood was associated with the development of IgE sensitisation, or atopic disease at the age of two years. The ELISpot technique was used for the enumeration of IFN-γ, IL-4 and IL-12 producing CBMC, after allergen and PHA stimulation. We found that the IgE-sensitised children had fewer allergen-induced IL-12-producing CBMC at birth, than their non-sensitised counterparts. The low numbers of IL-12 producing cells seen in the cord blood of the IgE-sensitised children are in line with an earlier study by our group, showing lower numbers of IL-12 producing cells in the cord blood of children at risk of developing atopic disease [69], and is also in line with what has been observed by others [68]. A Spanish study reported that infants that developed severe bronchiolitis after RSV infection had lower levels of IL-12 in cord blood [156]. This is of interest since RSV is known to induce synthesis of IgE and have been putatively linked to allergy [157, 158].

Upon allergen stimulation, IL-12 is produced by APCs during presentation to T cells, mainly due to the ligation of CD40 with its ligand CD154. IL-12 is also produced after TLR-stimulation, with bacterial derivatives such as LPS, which are probably also present, both during natural in vivo exposure and in our in vitro stimulation. The numbers of IL-12 producing cells in our study were lower after PHA stimulation, than after allergen stimulation. This is probably due to that PHA is not an optimal inducer of IL-12 since it stimulates mainly T cells, and IL-12 is preferentially produced by monocytes and DCs [45]. The IL-12 production seen after PHA stimulation is probably a secondary effect, induced by the IFN-γ produced by T cells. One possible explanation for the low numbers of IL-12 producing cells seen in the cord blood of IgE-sensitised children in this study, might be explained by a lack of or defect APCs. A low number or defect APCs would decrease the allergen presentation to T-cells,
and low allergen exposure have previously been linked to increased sensitisation [61]. A second possible explanation is that differences in genetic markers in the IL-12 genes or in genes governing the expression of IL-12, might be responsible for the differences seen. This possibility has been further investigated in paper IV.

The involvement of IFN-\(\gamma\) in atopic diseases has been extensively discussed, mainly in the context of the so called “hygiene hypothesis”. Several studies support the involvement of IFN-\(\gamma\) in atopy. Reduced levels of cord blood IFN-\(\gamma\), in response to both allergens and mitogens, in children developing, or at risk of developing atopic allergy, have been described [64, 152-154]. Though, in our study, there was a statistically significant correlation between IL-12 and IFN-\(\gamma\) indicating a close co-regulation between the two cytokines, the numbers of IFN-\(\gamma\) producing cells did not differ between the sensitised and the non-sensitised children. However, the children with a clinical diagnosis of eczema had lower numbers of IFN-\(\gamma\)-producing cells after ovalbumin, birch and cat stimulation than children that did not develop eczema. Eczema is usually the first manifestation of an atopic disease.

Our observations that the numbers of IL-12 producing cells are reduced in IgE-sensitised children, where as children with eczema had low numbers of IFN-\(\gamma\) producing cells, indicate that different allergic phenotypes are associated to different cytokine profiles in cord blood.

There was also a tendency for lower numbers of cord blood IL-4-producing cells in the IgE sensitised children. These results are in line with the generally suppressed cytokine responses in cord blood of atopic children described by others [159].

The low levels of IL-12 and IFN-\(\gamma\) seen in atopic children, and in children at risk of developing atopic disease, have been suggested to lead to a dysregulation in the cytokine balance, with elevated Th2-type of cytokines, and is considered to be a prediction for future development of atopic diseases [64, 152-154].
Cytokines in two-year old children and their mothers (Paper II)

In the second paper, we examined the numbers of IFN-γ, IL-4, IL-10 and IL-12-producing PBMC from two-year old children and their mothers in response to allergens and PHA, using the ELISpot method. Given the fact that not only children with allergic parents, but also children with no family history of allergic disease, become IgE-sensitised, we decided to divide the mothers in this study on the basis of the IgE-sensitisation status of their children. The hypothesis behind this being that differences in the cytokine profile of the mothers would indicate differences in the possibility to deviate the cytokine responses of their children towards or away from atopic diseases, through genetics or through in utero effects.

We found a general increase in cytokine responses, of both Th1 (IFN-γ and IL-12) and Th2 (IL-4 and IL-10) type, in mothers of IgE-sensitised children as compared to mothers of non-sensitised children. In the IgE-sensitised children the same increase could be seen as in their mothers, though not as pronounced as in the case of the mothers. The maternal and the child cytokine responses were statistically significantly correlated, though the samples were taken two years after delivery.

Most studies on allergic individuals have shown Th2-skewed immune responses to allergens and mitogens. However, there are several studies showing mixed Th1/Th2 responses with elevated levels of IFN-γ in both adult atopic asthmatics [160, 161] and in atopic children [162-167]. In a recent study, following children from birth until one year of age, an increase in the IFN-γ levels in children of allergic mothers during the first year of life was noted [168]. This phenomenon was also seen in our study, where the numbers of PHA-induced IFN-γ producing cells in the IgE-sensitised children were 2.4‰ (238/100 000 CBMC, paper I) in the cord blood and 6.4‰ (128/20 000 PBMC) at two years of age, whereas in the non-sensitised children the numbers were more similar (2.6‰ in the cord blood and 3.6‰ in the two-year olds). The IFN-γ responses seen in the children were still low as compared to the adults (7.4‰).
The numbers of IL-12 producing cells were similar in the adults as compared to the two-year olds, whereas in the cord blood the numbers were lower. The numbers of IL-12 producing cells were higher in the IgE-sensitised children and their mothers, than in the non-sensitised children and their mothers. In the children the difference was more pronounced in response to ovalbumin, than to cat allergen, which could be explained by the fact that at two years of age children are more commonly sensitised to food- than to inhalant allergens [169]. In the present study 13 children were sensitised to food allergens (four against hens egg, and the others to milk, peanut, soya and wheat) and four to animal allergens (three against cat and two against dog).

In response to allergen the numbers of IL-4 producing cells were very low, which resulted in difficulties to compare the two groups. The numbers of IL-4-producing cells were found to be higher after PHA stimulation in the IgE sensitised children and their mothers, than in the non-sensitised children and their mothers, though the numbers in the children were still lower than the maternal. IL-4 is one of the cytokines responsible for IgE switching [27], and for polarisation of Th cells into a Th2-type of cytokine production [18]. It was therefore not surprising to see elevated numbers of IL-4 producing cells in the sensitised children and their mothers, as compared to the non-sensitised children and their mothers.

IL-10 production from PBMC at 12 months of age, has been found to be selectively induced after allergen challenge in non-atopic children, where as in the non-atopic controls no IL-10 was induced upon allergen stimulation [64]. In contrast to these results, another group found that 6 year-old atopic children had higher levels of IL-10 than their non-sensitised counterparts [164]. In our study, IL-10 production was induced in both IgE sensitised and non-sensitised children at the age of two and the numbers of IL-10-producing cells were higher in sensitised children and their mothers than in their non-sensitised counterparts and their mothers. IL-10 is produced in large amounts from Tregs [24]. The increased numbers of IL-10 producing cells seen in the IgE-sensitised children and their mothers might reflect “over-active” Tregs, trying to dampen an exaggerated immune response. The fact that IgE-sensitised children seem to have a dampened immune response at birth, as evidenced by the low numbers of IFN-γ and IL-4 producing cells (paper I), where as the response seems to be
exaggerated at the age of two, also supports the idea of a dysregulated immune system.

Correlated cytokine responses between mother and child were seen in this study. We believe that these correlations are likely to result from a genetic inheritance, since the correlations persist two years after delivery. In this study, we have not investigated the cytokine profiles of the fathers, which would of course have been interesting in this context.

We provide evidence for a dysregulated immune system in IgE-sensitised children and their mothers. Taken together, we believe that genetic inheritance and possibly environmental factors are important contributors to the development of IgE-sensitisation, and that the cytokine response pattern of the mother, rather than the allergy status might be the link between maternal and child atopy. This might explain why also children with no atopic parents become IgE-sensitised.

**Viral infections, IgE-sensitisation and cytokines (Paper III)**

It has been suggested that viruses may influence the differentiation of T cells, thereby causing inappropriate expression of Th1- and Th2-immune responses and influencing the expression of an allergic phenotype. Our group has previously shown that Epstein-Barr virus (EBV) and combined EBV and cytomegalovirus (CMV) seropositivity at two years of age is negatively related to IgE sensitisation [122].

In paper III we investigated the relationship between IgE-sensitisation, CMV/EBV serostatus and PBMC cytokines in children at two years of age. We found CMV to be associated with high numbers of IFN-γ producing cells and with low numbers of IL-4 producing cells. This is compatible with previous reports, showing that CMV induces Th1-type of cytokine responses [170]. In theory, high IFN-γ and low IL-4 could be protective against IgE-sensitisation, but in our study no association between CMV and IgE-sensitisation was observed, in line with what was reported by an Italian group [117]. Since CMV is a persistent virus, the explanation for the high IFN-γ could be an
Early life cytokines, viral infections and IgE-mediated allergic disease

elevated T- and NK cell count in response to the infection, though we do not know when the primary infection took place. T- and NK cells are known to produce IFN-γ and to be involved in the defence against CMV [171]. In this study we did not phenotype the cytokine producing cells, and therefore we do not know the source of the IFN-γ.

For EBV, we could confirm a negative association to IgE-sensitisation, though we could not detect any statistically significant differences in cytokine patterns from their non-infected counterparts. However, there was a non-statistically significant indication of higher numbers of IFN-γ, IL-4, IL-10 and IL-12 producing cells in the seropositive children. The findings are consistent with findings in young adults with infectious mononucleosis (IM), where both type I and type II interferons and IL-12 were found to be high in serum [172], and with another report showing IL-4 production from EBV transformed B cell lines [173]. Our finding of a cytokine profile similar to that of adults with IM is intriguing. These results indicate that persistent viral infections might affect the immune system for a substantial period of time.

The finding that EBV seropositivity is negatively associated to IgE sensitisation, while the cytokine profile in those seropositive against EBV were more similar to that in the IgE-sensitised than in the non-sensitised children, is paradoxical. This is in contrast to what was seen in the CMV seropositive children, where the low IL-4 and high IFN-γ theoretically could prevent IgE sensitisation. Despite this, CMV was not negatively associated with IgE sensitisation. However, in a previous study from our group, it was noted that the reduced risk of being IgE sensitised after acquisition of EBV was enhanced by CMV co-infection [122]. We therefore speculate that EBV acts by some other mechanisms than through the cytokines tested in this study. One possible mechanism is that EBV could influence B cells to a rapid maturation, which could induce a preferential IgG4 production in the B cells. IgG4 antibodies has been shown to act as allergen neutralising antibodies.
IL-12 SNPs in relation to IL-12 production and allergic disease (Paper IV)

In paper I, we found differences in the cord blood IL-12 production between IgE-sensitised and non-sensitised children. We therefore became interested in this cytokine, and wanted to investigate if genetic differences could explain the observations in paper I. In paper IV, we investigated the relationship between atopic diseases and IL-12/IFN-γ production to three different IL-12-related SNPs. The SNPs investigated were the IL12B 1188, IL12RB1 1132 and the IRF1 1688 SNPs, that have all previously been implicated in immunological diseases [70-72, 76, 84] or to alter Th1-type cytokine production [74, 75, 81-83]. Innovative for this study is that we have measured IL-12 production in three different ways: as IL-12p40 mRNA, as IL-12p70 secretion and as numbers of IL-12 secreting cells.

We found that children homozygous for the IL12B 1188 C-allele had a complete lack of IFN-γ and IL-12 secreting cells in response to birch stimulation at birth, a difference that was statistically significant from the carriers of the A-allele. In addition, we found a stepwise increased risk to develop a positive SPT if the child carried the IL12B 1188 C-allele. It is also worth noting that all of the five children homozygous for the C-allele had eczema. Since the IL12B 1188 SNP is situated in the 3’ UTR it is, at least in theory, likely to affect IL-12p40 gene expression, since the 3’ UTR is involved in regulating gene expression and accordingly several studies have shown effects of the IL12B 1188 SNP on IL-12 production [74, 75, 174]. Looking at our results it is of course tempting to speculate that SNPs in the IL-12p40 gene might be at least one of the explanations for the low numbers of IL-12 producing cells seen in the cord blood of IgE-sensitised children in paper I. In theory a low IL-12 production would of course favour a Th2 response and allergic disease.

The IL12RB1 1132 C-allele was, just as the IL12B 1188 C-allele, associated to lower numbers of IFN-γ producing cells at birth. This was observed both after birch and cat stimulation of the cord blood. There were no differences in the numbers of IL-12 producing CBMC. In theory, alterations in the IL-12 receptor function would first alter IFN-γ production, which possibly in turn would result in changes in the IL-12
production as a secondary event. A previous study showed decreased IL-12 induced signalling in IL12RB1 1132 C-carriers [76], which fits well with our results of low numbers of IFN-γ producing cells. In contrast to what was seen in the cord blood, the IL12RB1 1132 C-allele was at two years of age associated to elevated IL-12p70 secretion and increased numbers of IL-12 producing cells in response to PHA. This indicates that there might be environmental, developmental, or epigenetic factors involved in the expression of this gene, or other genes governing the expression of the IL12RB1 gene. In this study there was no association to clinical parameters and upon looking in the literature we could only find one study that found associations of this IL12RB1 SNP to immunological disease [76].

IRF-1 has been shown to be essential for the ability to mount Th1-type of immune responses [81-83]. In our study children carrying the IRF1 1688 A-allele had lower numbers of ovalbumin induced IL-12 producing cells at birth and at two years of age IL-12p40 mRNA levels and IL-12p70 secretion was lower than in the carriers of the G-allele, i.e. there was a depression of the IL-12 response which was constant over time. Despite the evidence for a depressed IL-12 production we could not find any association to signs of allergy, which could have been expected.

Taken together our data suggest that all of the investigated mutations are likely to affect IL-12 production. We also show that children carrying the IL12B mutations are more prone to develop a positive SPT. Therefore the IL12B mutation appears to be a likely candidate to explain the low numbers of cord blood IL-12 producing cells seen in paper I. We speculate that the impaired IL-12 production, possibly caused partly by the investigated mutations, may be one of the causes for the delayed Th2-switch seen in allergic children [175], and may cause allergic disease.

Early life cytokines and time point of onset of IgE-sensitisation (Unpublished results)

Since not everyone who become sensitised do become so at the same time point in life, we decided to investigate whether differences in early life cytokine patterns could explain the different time points of onset. We have hence investigated cytokine profiles in non-sensitised children and children with either an early onset and
persistent IgE-sensitisation or a late onset IgE-sensitisation. Early onset was defined as displaying IgE-sensitisation at two years and five years of age \((n=14)\) and late onset was defined as displaying IgE-sensitisation at five years of age, but not previous to five years of age \((n=13)\). We also wished to include a group of transiently sensitised children (only at 2 years) but the group became to small. The IgE sensitised children were compared to children that did not display IgE-sensitisation during the first five years of life \((n=43)\).

We found that children with an early onset of IgE-sensitisation had lower numbers of PHA-induced IL-4, IL-12 and IFN-\(\gamma\) producing cells in their cord blood, than the non-sensitised and the children with a late onset of IgE-sensitisation (see Figure 3). The children with an early onset of IgE-sensitisation also had statistically significantly lower numbers of cat induced IL-12 producing CBMC, and a similar tendency was observed after ovalbumin stimulation, which fits with our findings of low IL-12 in cord blood of IgE-sensitised children seen in paper I. This difference could be caused either by a maternal \textit{in utero} influence or a genetic difference and could be a potential cause, or at least a sign for a predisposition of an early onset of IgE-sensitisation. The differences between early and late onset of IgE-sensitisation may potentially explain controversies in the literature regarding cytokine profiles in cord blood and IgE-sensitisation and also emphasise that time is a crucial factor. We speculate that children with an early sensitisation might be induced already \textit{in utero} to develop IgE-sensitisation through effects of the mother on the foetus, in this study evidenced by the low numbers of cytokine-producing cells in response to PHA in the children with early onset of IgE-sensitisation. The children with a late onset IgE sensitisation showed no differences from the non-sensitised children in the cord blood, indicating that a predisposition can not be detected as early as at birth for those children that become IgE-sensitised “later” in life.
At birth, children with an early onset IgE-sensitisation show lower numbers of phytohaemagglutinin (PHA)-induced interferon-γ, interleukin-4 and interleukin-12 producing cells than both non-sensitised children and children with a late onset IgE-sensitisation.

Interestingly, when investigating the responses to cat and ovalbumin at the age of two, we found that the children with a late onset displayed significantly higher numbers of IFN-γ producing cells in response to cat, but not to ovalbumin and they also showed a tendency for higher numbers of IL-4 after cat stimulation. The children with an early onset had statistically significantly higher ovalbumin induced IFN. At two years of age the most common allergens that IgE-sensitised children react adversely to is food derived allergens, whereas at the age of five it is more common to be sensitised against inhaled allergens, such as cat [169]. It is worth noting that the increase in the numbers of IFN-γ producing cells in response to cat observed in this study proceeded the IgE-sensitisation that did not display until after the age of two in these children.
The children displaying early onset of IgE-sensitisation showed increased numbers of IL-12 and IL-10 producing cells after most stimuli, compared to the non-sensitised children and the children with a late onset of IgE-sensitisation (see Figure 4). In addition, children with an early onset had statistically significantly higher numbers of PHA-induced IL-4, where as the children with a late onset had numbers similar to the non-sensitised children.

**Figure 4**

Children with an early onset IgE-sensitisation have higher numbers of interleukin-10 and interleukin-12 producing peripheral blood mononuclear cells in response to ovalbumin (ova), cat and phytohaemagglutinin (PHA) stimulation at the age of two.
CONCLUDING REMARKS

IgE-sensitisation and allergic disease often develop and manifest in early life. A key to understanding the underlying mechanisms must be sought for in early immunological divergence. We have focused on investigating early life cytokines in relation to allergic disease and IgE-sensitisation. We have also investigated the relationship between early viral infections, cytokines and IgE-sensitisation.

According to the results of our study IL-12 seems to be a crucial cytokine in the development of early allergic disease and IgE-sensitisation. We have provided evidence that cord blood IL-12 appears to be low in children displaying IgE-sensitisation at the age of two. At two years of age IgE-sensitised children had an exacerbation of not only IL-12, but also IL-4, IL-10 and IFN-γ producing cells. We have also shown the importance of three IL-12 related SNPs on IL-12 production and to some extent also on development of allergic disease.

We have also shown that SNPs in the IL12B, the IL12RB1 and the IRF-1 genes seem to be associated with low numbers of IL-12 producing cells at birth, and we could also conclude that at least the IL12B mutation was associated to an increased prevalence of positive SPT. The association between the IL12B mutation, cord blood IL-12 production and a positive SPT might at least partly explain the low numbers of cord blood IL-12 producing cells seen in paper I. We speculate that the impaired IL-12 production at birth, possibly caused by the investigated mutations, may lead to allergic disease.

We have in this study quantified IL-12 in several ways. It is important to keep in mind that when measuring the IL-12p40 subunit, it is not necessarily IL-12 itself that we are measuring, though it might seem appropriate to assume so. The IL-12p40 subunit is also part of IL-23 together with the IL-23p19 subunit [53], and in cord blood the IL-12p40 subunit has been shown to preferentially dimerise with the p19 subunit of IL-23 [176].
Looking at our results on cord blood and two-year old children, it seems likely that the IgE-sensitised children might have some kind of a regulatory defect. In the cord blood of the children with early and persistent IgE-sensitisation PHA-induced cytokine-producing cells were low, and looking at children sensitised at the age of two there was a depressed CB IL-12 response. In the two-year old children the numbers of cytokine producing cells were high. It is hence reasonable to suspect a Treg deficiency, resulting in a dysregulated cytokine response. However, this was not evaluated further in our study.

We could also conclude that there are differences in the cytokine profiles of children that become IgE-sensitised at an early age and children that become sensitised later in life. These differences could be seen both at birth and at the age of two, indicating that there might be differences in the mechanisms of onset depending on when in life you become sensitised.

In addition, we have shown that early EBV infection is negatively associated to IgE-sensitisation at the age of two, not knowing what is the hen and what is the egg. The presumed protective effect of EBV is probably mediated through other mechanisms than a Th1 skewing of the immune response. Our speculation is that EBV causes a rapid B-cell maturation, which in turn results in the production of IgG4 antibodies, as opposed to IgE antibodies. CMV infection was not associated to IgE-sensitisation, though a Th1 skewed immune response after CMV infection could be proved.
FUTURE PERSPECTIVES

In our study we have found evidence of a dysregulation of cytokine responses in IgE-sensitised children. Since Tregs are involved in dampening and terminating immune responses it seems reasonable to suspect a dysfunction in this cell population, and it would hence be interesting to investigate numbers and function of Tregs in IgE-sensitised children. Previous studies on the subject have indicated differences in the amount and function of Tregs in allergic individuals [62, 177].

The genotyping studies made, indicate that the IL12B, IL12RB1 and IRF-1 SNPs investigated are related to IL-12 production and that the IL12 1188 SNP is also associated to the development of allergic disease. We were able to show this in a rather limited group of children, and hence the results can, although convincing, be questioned from a statistical point of view. It would hence be interesting to firmly establish these associations in either a second, or a larger cohort of children.

We found that there were differences in the cytokine patterns of children with early and persistent IgE-sensitisation, as compared to those with a later onset of their IgE-sensitisation. Also in this material, the number of children was rather limited, and it would be interesting to do these experiments in a larger cohort, since there are few other studies investigating these differences. We aimed at including a group of children with a transient sensitisation, which was unfortunately not possible, but would certainly have been interesting.

We used the ELISpot technique to enumerate cytokine-producing cells, mainly because of its superiority in investigating IL-4. It would be interesting to also quantify the actual amount of cytokines by ELISA or by cytometric bead assay, since numbers of cytokine-producing cells and actual cytokine amount do not necessarily correlate.

In the virus study (paper III) we found an association between seropositivity for EBV and IgE-sensitisation, whereas CMV infection was associated to an “anti-allergic
cytokine profile”. In this study we can only speculate that EBV seems to protect against IgE-sensitisation, not knowing what is the hen and the egg. Since primary infections with both CMV and EBV are often asymptomatic, it will be difficult to find out if there is a protective effect, or if the IgE-sensitisation with its cytokine milieu makes the child more susceptible to infection with EBV. It would of course also be of great interest to try to find out what mediates the protective effect of EBV. Our only speculation on what mediates this assumed protective effect is an EBV-mediated rapid maturation of B cells. It would certainly be interesting to see if EBV could cause such a maturation, and if this would increase a restricted profile of IgG sub-classes (i.e. a preferential increase in blocking IgG4 antibodies).
SVENSK SAMMANFATTNING

Under de senaste årtiondena har de allergiska sjukdomarna ökat lavinartat, framför allt i den industrialiserade delen av världen. I Sverige är minst en fjärdedel av alla barn drabbade. IgE-medierade allergier, som vi till största delen fokuserat på i denna avhandling, orsakas av att immunförvaret reagerar på till synes ofarliga proteiner i vår omgivning, så kallade allergener. Det är ännu okänt varför detta händer.

Genom att en antikropp, IgE, känner igen allergenet och binder till det, aktiveras mastceller i hud och slemhinnor. Aktiverade mastceller släpper ifrån sig inflammatoriska substanser, så som histamin. Detta leder till t ex hösnuva och magtarmbesvär, men också till allvarligare symtom som astma och anafylaktisk chock.

Eftersom den allergiska debuten ofta sker tidigt i livet, har vi valt att fokusa på att karaktärisera särdrag i immunförsvaret hos små barn som blivit allergiska. Vi har undersökt hur cytokiner (substanser som t ex används för kommunication mellan cellerna i immunförsvaret) redan vid födseln, samt vid två års ålder, är relaterade till utvecklandet av IgE-medierad allergi under de första levnadsåren.

Vi fann att IgE-sensibiliserade barn (barn där IgE-antikroppar mot vanliga allergen kan påvisas i blodet) redan vid födseln har ett lägre antal interleukin (IL)-12-producerande celler än icket-sensibiliserade barn. Vi kunde också visa att barn med eksem hade ett lägre antal interferon (IFN)-γ-producerande celler. Både IL-12 och IFN-γ är cytokiner som motverkar produktionen av IgE. När vi undersökte cytokinproduktionen vid två års ålder, fann vi att de IgE-sensibiliserade barnen hade högre antal av alla de cytokinproducerande celler som undersöktes, dvs både av cytokiner som gynnar IgE-produktionen och de som hämmer den. Detta indikerar att IgE-sensibiliserade barn har ett hyperreaktivt immunförsvår. Vi kunde också visa att detta framför allt gäller barn som sensibiliserats tidigt, och att barn som uppvisar sin sensibilisering senare inte alls har samma cytokinprofil.
Vi undersökte även mammorna till barnen och fann att mammor till IgE-
sensibiliserade barn också verkade ha ett hyperreaktivt svar på allergenen. Att vi
kunde se dessa skillnader visar att mammans förmåga att svara på allergenstimulering
kanske är en viktigare komponent för att förutsäga barnets allergi, jämfört med
mammans faktiska allergi.

Vi har i denna studie också undersökt hur tidiga Epstein-Barrvirus (EBV) och
cytomegalovirus (CMV) infektioner är relaterade till IgE-sensibilisering och tidiga
cytokinprofiler. Det visade sig att CMV inte är associerat med IgE-sensibilisering,
men med en cytokinprofil som teoretiskt kan verka skyddande mot allergi. EBV
visade sig vara negativt associerat till IgE-sensibilisering. En förmodat skyddande
effekt av EBV-infektion tycks inte verka genom en förändrad cytokinmiljö.

I många gener finns ett antal punktmutationer som kan påverka uttrycket från genen.
Vi har också studerat punktmutationer i tre IL-12-relaterade gener. Vi kunde
konstatera att alla dessa hade mer eller mindre förmåga att påverka produktionen av
IL-12 och att en av dem även var associerade till utveckandet av IgE-sensibilisering.
På grund av dessa fynd tror vi att IL-12 kan vara viktigt för utvecklandet av allergi
och IgE-sensibilisering.

Våra resultat visar på ett komplext samspel mellan arv och miljö i den allergiska
sjukdomsprocessen. Vi har visat på genetikens och IL-12:s betydelse för IgE-
sensibilisering, samt att vissa virusinfektioner verkar kunna påverka cytokinprofilen
eller ha en skyddande effekt på IgE-sensibilisering. Resultaten av undersökningarna i
navelsträngsblood och i perifert blod vid två års ålder indikerar en defekt i regleringen
av cytokinsvaret hos IgE-sensibiliserade barn.
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Early life cytokines, viral infections and IgE-mediated allergic disease


Early life cytokines, viral infections and IgE-mediated allergic disease


Early life cytokines, viral infections and IgE-mediated allergic disease


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Early life cytokines, viral infections and IgE-mediated allergic disease


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