From the Department of Clinical Science and Education, Södersjukhuset and Sachs´ Children´s Hospital, Södersjukhuset
Karolinska Institutet, Stockholm, Sweden

CYTOKINE PROFILES, INFECTIONS AND IGE SENSITISATION IN CHILDHOOD

Caroline Nilsson

Stockholm 2006
All previously published papers were reproduced with permission from the publisher.

Cover drawings by Stina Wirsén

© Caroline Nilsson, 2006
ISBN 91-7140-720-0
“Learning by doing”
John Dewey

To my family
1 ABSTRACT

Background: The etiologic factors behind development of IgE-mediated allergy are incompletely understood although the interaction between allergic heredity and exposure to various environmental factors seems to be most important.

Objective: The overall aim of this thesis was to study the associations between allergic heredity, environmental factors like viral infections and IgE sensitisation and the development of allergic diseases during childhood.

Methods: A cohort of 281 infants, with different patterns of family history for allergic disease (both parents, maternal or neither parent) was followed prospectively from birth to 2 years of age using questionnaires, clinical examinations, blood sampling and skin prick tests.

Results: The associations between the number of IFN-γ, IL-4 and IL-12-producing cord blood mononuclear cells (CBMC) and parental allergic history were evaluated by the ELIspot method in 57 children. Children with two allergic parents had a statistically significantly higher IL-4/IFN-γ ratio (p < 0.05) than children without allergic parents. The number of IL-12-producing CBMC was statistically significantly higher among children without allergic parents compared to the children with only maternal allergic disease (p < 0.01). These findings suggest a strong genetic influence on the cytokine pattern in CBMC, where having a father with allergic disease has at least as much influence as a mother with allergy.

The association between the number of IFN-γ, IL-4 and IL-12-producing CBMC in 82 children and allergic outcome at 2 years of age was investigated. Compared with non-sensitised children, the IgE-sensitised children had lower number of IL-12-producing CBMC after stimulation with allergens, and this was statistically significant for cat (p = 0.002). Children with eczema had statistically significantly lower numbers of IFN-γ producing CBMC after stimulation with ovalbumin (p = 0.017) and cat (p = 0.01) compared to children without eczema. These results might indicate that different cytokine profiles in cord blood are associated with different allergic phenotypes.

The association between serostatus against 13 selected viral infections and IgE sensitisation was evaluated among 246 children at 2 years of age. IgE sensitisation (24%) was statistically significantly less prevalent at 2 years of age among infants who were seropositive against Epstein-Barr virus (EBV), (ORadj = 0.34; 95% CI 0.14 - 0.86). Seropositivity against both cytomegalovirus (CMV) and EBV gave a further reduction in the risk for IgE sensitisation, indicating an interaction between the viruses. Thus, acquisition of EBV infection during the first two years of life seems to be associated with a reduced risk of IgE sensitisation and this effect is enhanced by CMV co-infection.

The association between the phytohaemagglutinin (PHA) -induced cytokine-profile in peripheral mononuclear blood cells (PBMC), serostatus against CMV and EBV and IgE sensitisation was evaluated among 75 children at 2 years of age. CMV seropositive children had higher numbers of IFN-γ-producing PBMC (ORadj 37.48 95% CI 5.71 - 246.15) and lower number of IL-4-producing PBMC (ORadj 0.05; 95% CI 0.01 - 0.46) than seronegative children. The IgE sensitised children more often had high numbers of IL-4-producing PBMC than the non-IgE sensitised (OR 13.50; 95% CI: 1.56 - 117.13). These data support the idea that persistent viral infections may affect the immune system for a considerable period of time.

Conclusion: Our findings illustrate the complex traits of allergy in infancy where the different allergic phenotypes are influenced by both genetic and environmental factors like viral infections.
LIST OF PUBLICATIONS


IV. Nilsson C, Larsson AK, Montgomery SM, Linde A, Lilja G, Troye Blomberg M. Viral infection and IgE sensitisation: CMV seropositivity in early life is associated with reduced numbers of IL-4-producing cells. Submitted
<table>
<thead>
<tr>
<th>CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Abstract .......................................................................................... 3</td>
</tr>
<tr>
<td>2  Background .......................................................................................... 1</td>
</tr>
<tr>
<td>2.1  Introduction .................................................................................... 1</td>
</tr>
<tr>
<td>2.2  Nomenclature ................................................................................... 1</td>
</tr>
<tr>
<td>2.3  IgE-mediated allergy .................................................................... 2</td>
</tr>
<tr>
<td>2.4  Influence of heredity and the environment ................................... 3</td>
</tr>
<tr>
<td>2.4.1  Allergic heredity ...................................................................... 3</td>
</tr>
<tr>
<td>2.4.2  Tobacco smoking and furred pets ............................................. 4</td>
</tr>
<tr>
<td>2.4.3  Infant feeding ........................................................................... 5</td>
</tr>
<tr>
<td>2.4.4  Microbial gut flora .................................................................... 5</td>
</tr>
<tr>
<td>2.4.5  Lifestyle factors ....................................................................... 6</td>
</tr>
<tr>
<td>2.5  Infections .......................................................................................... 6</td>
</tr>
<tr>
<td>2.6  Immunology ....................................................................................... 9</td>
</tr>
<tr>
<td>2.6.1  Innate immunity ......................................................................... 9</td>
</tr>
<tr>
<td>2.6.2  Acquired immunity .................................................................... 10</td>
</tr>
<tr>
<td>2.6.3  T-cells ......................................................................................... 10</td>
</tr>
<tr>
<td>2.6.4  Th1 and Th2 cells ...................................................................... 11</td>
</tr>
<tr>
<td>2.6.5  B cells ......................................................................................... 11</td>
</tr>
<tr>
<td>2.6.6  Cytokines ................................................................................... 12</td>
</tr>
<tr>
<td>2.6.7  Immune responses and infections ............................................ 14</td>
</tr>
<tr>
<td>2.6.8  Immune responses and allergy .................................................. 16</td>
</tr>
<tr>
<td>2.7  Immune biology of pregnancy and allergy ..................................... 16</td>
</tr>
<tr>
<td>3  Aim ....................................................................................................... 18</td>
</tr>
<tr>
<td>4  Subjects and methods ........................................................................ 19</td>
</tr>
<tr>
<td>4.1  Study population ........................................................................... 19</td>
</tr>
<tr>
<td>4.2  Study design ................................................................................... 20</td>
</tr>
<tr>
<td>4.3  Study methods ................................................................................ 21</td>
</tr>
<tr>
<td>4.3.1  Parental questionnaire .............................................................. 21</td>
</tr>
<tr>
<td>4.3.2  Child questionnaire ................................................................... 21</td>
</tr>
<tr>
<td>4.3.3  Parents’ report of infections ..................................................... 21</td>
</tr>
<tr>
<td>4.3.4  Skin prick test ............................................................................ 22</td>
</tr>
<tr>
<td>4.3.5  Blood sampling .......................................................................... 22</td>
</tr>
<tr>
<td>4.3.6  Specific IgE ................................................................................ 22</td>
</tr>
<tr>
<td>4.3.7  Viral infections ......................................................................... 22</td>
</tr>
<tr>
<td>4.3.8  Enzyme-linked immunospot (ELISpot) assay ............................... 23</td>
</tr>
<tr>
<td>4.3.9  Clinical examination ................................................................... 23</td>
</tr>
<tr>
<td>4.4  Classification of the children ......................................................... 23</td>
</tr>
<tr>
<td>4.4.1  Atopic eczema ............................................................................ 23</td>
</tr>
<tr>
<td>4.4.2  Food allergy/acute urticaria ....................................................... 24</td>
</tr>
<tr>
<td>4.4.3  Wheezing/asthma ...................................................................... 24</td>
</tr>
<tr>
<td>4.4.4  Allergic rhino-conjunctivitis ...................................................... 24</td>
</tr>
<tr>
<td>4.4.5  IgE-sensitisation ....................................................................... 24</td>
</tr>
<tr>
<td>4.5  Statistical analyses ......................................................................... 24</td>
</tr>
<tr>
<td>5  Results ................................................................................................. 26</td>
</tr>
</tbody>
</table>
5.1 Cytokine-producing cord-blood mononuclear cells and parental allergy (Paper I) ................................................................. 26
5.2 Cytokine-producing cord-blood mononuclear cells and IgE sensitisation and allergic diseases at two years of age (Paper II) .................. 28
5.3 Infections and IgE sensitisation (paper III) .......................... 30
5.4 Cytokines at 2 years of age (paper IV) ................................. 33
6 Discussion .................................................................................. 35
7 Conclusions ................................................................................. 42
8 Svensk sammanfattning ................................................................. 43
9 Acknowledgements ...................................................................... 45
10 References .................................................................................. 47
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CBMC</td>
<td>Cord blood mononuclear cells</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>FcεRI</td>
<td>High affinity receptor</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immune-globulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Th cell</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
</tbody>
</table>
2 BACKGROUND

2.1 INTRODUCTION

There has been a tremendous increase in the incidence of allergic diseases during the last decades especially in the industrialised countries. The increase is most pronounced among children (1-3). Today, allergic diseases affect 1/3 of the Swedish children and have become a major health problem. Parental allergy is known to be a risk factor for allergy in offspring (4) but cannot explain this change in incidence. Altered environmental exposures and changes in lifestyle have been hypothesised to be of importance (5-9). Furthermore, the associations between changes in the intestinal microflora (10) and in the incidence of infections (“the hygiene hypothesis”) (11) in early childhood in relation to allergy receive much attention. However, this theory is not undisputable (12).

There are also data from several studies suggesting that allergic diseases might begin \textit{in utero} since mononuclear cells from cord blood can be activated \textit{in vitro} by different allergens (13-15) Thus, the “allergic march” might start \textit{in utero} and continue with eczema or food allergy during infancy, wheezing in the preschool child and rhinoconjunctivitis and asthma from pollen and furred pets in school children (16).

2.2 NOMENCLATURE

The word “allergy” (from Greek “changed reactivity”) was first used by von Pirquet in 1906 for describing hypersensitivity reactions from the immune system and in 1923 Coca and Cooke used the word “atopy” (from Greek out “of place” or “strange disease”) to describe the heredity syndrome of asthma and hay fever. In 1967 the serum factor mediating classical allergy, immune-globulin E, was discovered (17, 18).

During the last century definitions of allergic diseases have altered and have been inconsistent in epidemiological and immunological studies. However, in 2001 the European Academy of Allergology and Clinical Immunology (EAACI) defined a nomenclature for allergic and related reactions that can be used independently of target organ and age and is based on the mechanisms that initiate and mediate allergic reactions (19) (Fig. 1). This terminology has been revised and accepted by the World Allergy Organization (WAO) (20).
According to WAOs terminology **allergy** is defined as a hypersensitivity reaction initiated by immunological mechanisms. One of the immunological reactions is **IgE-mediated allergy** which is defined as the occurrence of allergen specific IgE (positive *in vitro* test for IgE or positive skin prick test) in combination with classical allergic symptoms. **Atopy** is defined as a personal or familial tendency to become sensitised and to produce IgE antibodies, while **IgE sensitisation** is the presence of specific IgE antibodies in blood or skin irrespective of allergic symptoms.

### 2.3 IgE-mediated allergy

For reasons still unknown some people start to produce specific IgE antibodies when they encounter common antigens, the sensitisation phase. Antigens causing this reaction are called allergens. T helper (Th) cells are activated by antigen-presenting cells (APC) and start to produce Th2 type cytokines, such as interleukin-4 (IL-4) in predisposed humans. The allergens are usually common proteins in our environment. This allergen activation results in IgE production and growth and differentiation of eosinophils and mast cells as well as other cells. IgE antibodies can circulate in the blood unbound, but is mainly bound by the high affinity receptor (FcεRI) expressed on the cell-membrane of mast cells and basophils.
In an IgE-sensitised individual with specific IgE antibodies bound to mast cells or basophils, allergen re-exposure can lead to an allergic reaction. The release of histamine, leukotriens and prostaglandins following cross linking of allergen-specific IgE antibodies on the surface of these cells induces the development of the allergic symptoms (Fig. 2). Depending on where in the human body there is a high frequency of the mast cells and basophils, different symptoms (i.e. bronchial asthma, eczema, rhinoconjunctivitis etc.) occur. This is called the early response.

The late phase response in an allergic reaction is caused mainly by eosinophils and neutrophils activated by chemotactic factors leading to epithelial disruption and continued swelling and erythema.

![Fig. 2. The allergic reaction. Antigen presenting cell (APC), T-helper cell 2 (Th2), B-cell (B), mast cell (M).]

### 2.4 INFLUENCE OF HEREDITY AND THE ENVIRONMENT

#### 2.4.1 Allergic heredity

Several studies have shown that allergy in parents is one of the most important factors influencing the development of allergic diseases among children (21, 22). In children without an allergic family history around 10% will develop allergic disease. The corresponding figures among children with single (one parent) and double parental (both parents) allergic history have been shown to be 20-30 % and 50 %, respectively.
A stronger association has been suggested for the presence of the same allergic symptom in both parents (24). During the 1990s several studies have claimed that there is a greater risk of a child developing allergy if the mother rather than if the father is allergic (25, 26).

Genetic research to find candidate genes for allergic diseases is ongoing. Many loci on 15 different chromosomes have been reported from candidate gene studies. However, the strongest evidence points towards four regions on: 5q, 6p, 11q and 12q (27). A recently published study has showed that polymorphisms in G-protein-coupled receptor (GPR154) on chromosome 7 are associated with allergic diseases in European children (28). Another region for which there is strong evidence for linkage with asthma and allergy (especially to serum-IgE levels) is chromosome 5q31-q33. This region contains multiple candidate genes for allergy and asthma (29). However, the striking increase in asthma cannot be explained by genetic factors since these most likely have not changed dramatically during the past half decade. Environmental and other factors must be in focus when trying to understand why certain children develop early allergy and others do not.

### 2.4.2 Tobacco smoking and furred pets

Exposure to different environmental factors such as tobacco smoke and animal dander and their influence on the development of allergic diseases among children is discussed. Maternal smoking during pregnancy is associated with reduced respiratory function in early infancy and an increased risk of recurrent wheezing during infancy and early childhood (30). In addition, parental smoking (particularly maternal) during infancy has been shown to be associated with an increased prevalence of wheezing and asthma among children up to six years of age. It has also been suggested that exposure to passive smoking increase the risk of sensitisation to allergens in children (reviewed in (31)).

The most common indoor allergens in Sweden are those from cats and dogs, in contrast with many other parts of the world, where allergens from house dust mites and cockroaches are widespread. The association between having furred pets at home and allergic disease, especially asthma, has been intensively discussed and studied, but the results are not consistent. Some studies indicate a protective effect from keeping furred pets with decreased risk of having wheeze/asthma or eczema (reviewed in (32)). Other
studies claim there is an increased risk for allergic diseases with exposure to furred pets at home (33-35).

2.4.3 Infant feeding

The association between breastfeeding and allergic disease has been studied intensively, but the results are inconsistent. Some recently published data, from a prospectively followed birth cohort in Stockholm (the BAMSE cohort), shows that exclusive breastfeeding for at least four months seems to reduce the risk for both asthma and eczema during infancy (36, 37). These observations are in agreement with previous studies (38-40). However, other studies have failed to confirm a protective effect of breastfeeding against allergy or even suggest an increased risk of asthma and eczema associated with breastfeeding, in particular if the mother has asthma or eczema herself (41, 42).

In addition to breastfeeding, in recent years there has been a focus on vitamins, antioxidants and fatty acids as well as consumption of fruits and vegetables in the context of reducing the risk for development of allergic disease (43-47).

2.4.4 Microbial gut flora

The establishment of the intestinal microflora is proposed as a major factor driving maturation of the immune system in newborns. It is now generally accepted that the bacterial microflora of the human gut is an integral component of the immune defence. An association between the microbial gut flora and the development of allergy has been addressed in several studies (reviewed in (48). *Bifidobacteria* has been reported to be less prevalent while *Clostridia* comprised a higher proportion of the intestinal microflora among children with allergic diseases (10). In addition, prospective studies of probiotics containing lactobacilli show promising results in the prevention of eczema although no effect on IgE sensitisation was observed (49). One theory explaining why the composition of our gut flora matters in Th1/Th2 diseases is the “old friends” hypothesis, where contact with “old friends” (harmless microorganisms such as lactobacilli, saprophytic mycobacteria and helminths for example) is diminished in our modern society (50).
2.4.5 Lifestyle factors

To be born or to live on a livestock farm during infancy has been shown to protect against the development of allergic disease (51, 52). It is suggested that the protective effect is induced after early and high exposure to microbial lipopolysaccharide (LPS) which seems to stimulate Th1 activity (51, 53).

Extensive use of antibiotics during the first year of life, especially macrolides, has been proposed as a risk for allergy, possibly through effects on the gut flora (54, 55). However, other studies have not confirmed an association between antibiotic use and allergy (56).

Children of anthrophosophic families that frequently consume fermented food containing lactobacilli and avoid vaccinations and antibiotics are less prone to develop allergy (57, 58). In addition, their gut flora has a different bacterial composition (59). However, it is still partly unknown how these factors influence the immune system, and further studies are needed.

2.5 INFECTIONS

Viral infections are the most common infections in childhood and often precede bacterial infections of the respiratory tract. Symptoms of viral respiratory tract infections are mainly nasal catarrh and cough. The herpes viruses and the vaccine preventable diseases, measles, mumps and rubella often cause systemic infections with symptoms from various organs. For more information about viruses, see Box 1.

The concept of a protective effect of infectious diseases on the development of allergy, the “hygiene hypothesis”, was raised in the late 1980s by Strachan who reported an inverse association between family size and hay fever (11). Such an association had already been observed in 1976 among white families and Native Americans in Northern Canada. Serum-IgE levels and the prevalence of asthma and eczema were higher in the white population whereas helminthic, viral and bacterial infections were more common among Native Americans (60).

Other studies, using various markers as an indication of increased burden of infections, have also demonstrated protection against allergy but these studies have often had a retrospective design.
Box 1. Common viruses in childhood

**Rhinovirus:** Rhinoviruses are the most frequent cause of the “common cold” and > 100 subtypes of rhinovirus have been described. In children, 75% of rhinovirus infections are linked to symptoms like rhinitis and pharyngitis. Rhinoviruses have been associated with severe lower respiratory symptoms in infants and are also strongly associated with exacerbations of asthma.

**Coronavirus:** Coronaviruses cause 5-15% of “common colds”.

**Influenzavirus:** Three types A, B and C have been described. Influenza types A and B are the main influenza pathogens. Influenza is a severe, febrile respiratory illness with acute onset. Among children around 25% may have otitis media as a bacterial complication.

**Parainfluenzavirus:** Parainfluenzaviruses are particularly associated with laryngotracheitis, bronchitis and croup. There are three main types; 1, 2 and 3. By age 3 years most children have been exposed to all of the three parainfluenza subtypes.

**Adenovirus:** Adenoviruses cause a wide array of clinical syndromes including acute respiratory disease and gastroenteritis. There are 49 different serotypes but only 1/3 of these have been associated with clinical disease. The first adenovirus infections usually occur early in childhood.

**Respiratory syncytial virus (RSV):** RSV is the major cause for bronchiolitis and pneumonia in children below 1 year of age. In older children and adults it causes common cold. A severe RSV-infection in childhood is often followed by a propensity for wheezing during childhood. Most children have been infected with RSV at 2 years of age.

**Metapneumovirus:** The four subtypes of metapneumovirus discovered during the past five years are a cause of severe respiratory tract infections in childhood, though more infrequently than RSV. Asthma is a frequent complication of metapneumovirus-infections.

**Rotavirus:** Rotavirus is the most common worldwide cause of gastroenteritis in children. This virus can cause disease in all mammals and birds and is classified in five subgroups.

**Calicivirus:** Caliciviruses are the most common cause of gastroenteritis in older children and adults.

**Herpes simplex virus (HSV):** HSV consists of two types. Primary infection can be asymptomatic or with symptoms such as fever and stomatitis, probably depending on the host’s immune response. Reactivation of the dormant virus can occur at anytime. Around 70% of adults have antibodies against HSV, but the infection has become less frequent among children in the western world during the past century.

**Cytomegalovirus (CMV):** CMV is the most common cause of congenital infections. The infection is almost always subclinical in small children. In older children CMV may cause mononucleosis-like syndrome. CMV exposure produces a lifelong latent infection. Around 70% of Swedish adults carry CMV.

**Varicella Zoster virus (VZV):** The primary infection chickenpox, results in a lifelong latent infection. Reactivation of VZV causes herpes zoster (shingles). In temperate countries 90-95% of the population acquire VZV in childhood but infection is less common in a tropical climate.

**Epstein-Barr virus (EBV):** EBV infects > 95% of the world’s population. Small children usually get no symptoms from the primary infection but 50% of older children and teenagers may develop mononucleosis. The latent lifelong EBV-infection was the first virus infection to be associated with malignancies.

**Human herpes virus 6 (HHV6):** HHV6 causes exanthema subitum, fever for three days followed by a rash in among 30% of infected children. Among children 3-5 years of age 80-100% have antibodies against HHV6. (61, 62)
Thus, starting day care at an early age (63, 64) or having older siblings (11, 65) have been shown to protect against allergy and asthma. The proposed mechanism underlying these associations is more frequent exposure to childhood infections. Compared with children from the former West Germany, those living in the former East Germany had more respiratory tract-infections, probably due to childcare at an early age, large families and overcrowded housing conditions. When measuring the prevalence of asthma in these populations, asthma was more prevalent in children living in the former West Germany (66). In the former East Germany, changes towards a more western lifestyle have occurred since unification. A significant increase in IgE sensitisation among children in this part of Germany has been shown some years after unification (67).

Interest has recently been focused on viral infections since they have been implicated in influencing IgE-mediated sensitisation (68, 69) through causing an imbalance between Th1- and Th2-immune responses (11, 65, 70). Matricardi et al. showed that students undergoing military training who were seropositive against hepatitis A had a low prevalence of allergic diseases (71). Others have observed that children with an increased number of respiratory tract infections in early life reported by parents have a higher risk of developing asthma (72). In a prospective study, among children from Norway, using questionnaires and diaries to report episodes of upper respiratory tract infections, similar findings were reported (73). Hospital admission due to respiratory syncytial virus (RSV) infection is linked to the development of bronchial asthma, allergy and induction of IgE synthesis (74, 75). Rhinovirus and parainfluenza are other respiratory viral infections particularly associated with asthma exacerbations in children (76-78). Rhinovirus alone is detected in approximately 50 % of virus induced acute attacks of asthma (79). Inoculation with rhinovirus in experimental studies shows a reduction in peak flow and FEV\textsubscript{1} in asthmatics compared with non-asthmatic subjects (80, 81).

Other viruses of interest are cytomegalovirus (CMV) and Epstein-Barr virus (EBV) which are persistent viruses, shed for a long time after primary infections (82). The chronic nature of both viruses has been shown to exert an effect on the immune system (83). In developing countries the majority of young children usually have asymptomatic CMV and EBV infections early in life (84, 85). This is in contrast to what is seen in industrialized countries where primary EBV infection is often delayed.
until puberty (86, 87), resulting in infectious mononucleosis (IM) in about half of those infected at this age. Interestingly, the prevalence of allergy is considerably lower in developing countries (88). 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 serostatus to the viruses and clinical allergic symptoms. However, IgE sensitisation against air-born and food allergen was positively associated with CMV seropositivity among children who were seronegative against EBV (89).

It has been proposed that vaccinations against some of the viral and bacterial infections influence the outcome of allergic disease. Research in this field does not show any evidence that vaccines such as Bacille Calmette-Guerin, pertussis, influenza, measles, mumps, rubella or smallpox have an effect on the risk of developing allergy later in life (reviewed in (90)).

### 2.6 IMMUNOLOGY

To understand the mechanism behind allergic reactions and to be able to study the impact of environmental factors, immunology is indispensable. The immune system protects us from foreign invaders and without it we cannot survive. There are two branches of the immune system, the innate (natural) and the acquired (adaptive) which are dependent of each other. The acquired immune defence cannot function without the innate immune defence and the latter system is more effective operating in conjunction with the acquired immune system.

#### 2.6.1 Innate immunity

The innate part of the immune system serves as a rapid first-line host defence. It contains evolutionary conserved receptors and molecules (91) as well as chemical and mechanical barriers (skin and mucosa, mucous production, antibacterial peptides, low pH etc.) that prevent the passage of micro-organisms into the body (92). The innate immune defence also consists of phagocytic cells that are able to destroy micro-organisms and has cells that are able to release anti-microbial substances (93). Examples of such cells are basophils and natural killer (NK) cells (94, 95). NK cells have the ability to both lyse target cells and provide early source of immunoregulatory cytokines. NK cells express the surface molecule CD 56 and are negative for the conventional T cell marker. A subdivision of NK cells is described and CD56 bright NK
cells produce a high amount of IFN-γ during the innate immune response while the CD57dim cells exert cytolytic functions (96).

One of the mechanisms by which the innate immune system senses the invasion of pathogenic micro-organisms is through the Toll-like receptors (TLRs) which recognize pathogens-associated molecular patterns (PAMPs). PAMPs are microbial components present in all microbial organisms, not only the pathogenic ones. One PAMP that has gained a lot of attention in research is lipopolysaccharide (LPS) present on gram-negative bacteria. Stimulation of different TLRs induces distinct patterns of gene expressions, which not only lead to the activation of innate immunity but are also instrumental in the activation of antigen-specific acquired immunity. The first discovered TLR in humans was TLR4. Today, 12 members of the TLR family have been identified in mammals. TLRs are expressed on various cells, including macrophages, dendritic cells, B-cells and specific types of T-cells. TLRs can be divided into two classes, the TLRs that recognise bacteria and are mainly located on the surface of the cell, and those that recognise viruses and are intracellularly localised (reviewed in (97)). The TLRs suggested as being of importance for allergic diseases are TLR2 and TLR4 (98).

The complement system with its intrinsic cascade reaction is also a component of the innate immune system.

### 2.6.2 Acquired immunity

The acquired part of the immune system involves antigen recognition by specific receptors present on two cell types. T- and B-lymphocytes are unique cells in that they possess memory, diversity and have the capacity to distinguish between self and non-self. Both T-lymphocytes and B-lymphocytes originate from the bone marrow but T-lymphocytes develop in the thymus where they learn to discriminate between self and non-self peptides. The main task for B-lymphocytes is to produce antibodies against antigens (*humoral immunity*) (99) while T-cells are the main effectors of the cellular adaptive immune response (100).

### 2.6.3 T-cells

CD3+ T-cells can be divided into cytotoxic T-cells (CD8+) and T-helper (Th) cells (CD4+). The cytotoxic T-cells destroy cells infected, with intracellular pathogens,
through recognition of fragments of the pathogen presented via class I major histocompatibility complex (MHC) present on the infected cells. The CD4+ T-helper cells recognise fragments of exogenous antigens in association with MHC class II molecules. MHC molecules class I are highly polymorphic molecules present on the surface of all nucleated cells while MHC II are present on professional antigen presenting cells (APC) i.e. dendritic cells, monocytes/macrophages and B cells. To activate the T cells, at least two signals are required one through the interaction between the MHC and T-cell receptor and the other through co-stimulatory molecules of which CD28 (present on T cells) and the CD80 or CD86 (present on APC) are considered the most important. The activation events also lead to cytokine production, both in T cells and APCs. The cytokines influence both the T-cell and the B-cell components of the immune system.

2.6.4 Th1 and Th2 cells

In 1986 a subdivision of Th cells was based on their cytokine production (101). Th2 cells mainly produce Th2-type cytokines: interleukin (IL) -4, IL-5 and IL-13 and these regulate the humoral arm of the immune system. Th1 cells produce Th1-type cytokines, i.e. interferon-gamma (IFN-γ) and IL-2 (102) and these regulate the cell-mediated immune system. There is interplay between Th1- and Th2-type cytokines by which they suppress and activate each other; IL-4 inhibits the production of IFN-γ and IFN-γ inhibits the production of Th2 cytokines. This can lead to a polarized immune response, since once the development along one of the pathways has started, it tends to progress in that direction. However, in contrast to previous knowledge, new data shows that the polarisation of Th-cells is more complex than what was first believed (103). Th1- and Th2-types of cytokine can even be co-expressed in the same cell (104).

2.6.5 B cells

B-cells are the producers of the many different antibodies involved in antigen recognition. An enormous number of new B cells are released in the body every day that can recognise different antigens. Before leaving the bone-marrow the B cells are controlled so that they do not recognise self-antigen; cells that do die. The B cells mature in the spleen and start thereafter to circulate in the body. The mature B cells present their specific antibody on their cell surface. The contact between B-cells and pathogenic antigens initiates the clonal selection whereby the B cells divide and start to
produce a high quantity of the antibody against the intruder. The antibodies can facilitate phagocytosis and activate the complement system (reviewed in (105)).

2.6.5.1 Immunoglobulins

Immunoglobulins (Ig), or antibodies as they are called, exist as secreted or membrane bound forms. The Ig molecule consists of a tetramer of two heavy and two light chains which are held together by disulphide bonds. A highly variable amino acid part of the Ig heavy and light chains determine which antigenic fragment that is recognised. The carboxy part of the Ig molecule determines the antibody subclass. By certain processes referred to as class switching, a B cell is capable of changing the Ig subclass without changing the specificity of the antibody. The Ig subclass involved in allergic reactions is IgE (reviewed in (105)).

2.6.6 Cytokines

Cytokines are potent hormone-like effector molecules produced by many different cell-types, whereby cells communicate with each other within and between the innate and the acquired immune systems. In this presentation only cytokines relevant to our study are discussed.

2.6.6.1 Th1-type cytokines

IL-12 has strong Th1 inducing activities (106) through stimulation of IFN-\(\gamma\)-production by NK or T-cells (107) and is a key factor in regulating cell-mediated immunity. IL-12 is produced mainly by phagocytic cells, macrophages and dendritic cells (106). Th1-type cytokines promote mainly cellular immunity (108). IL-12 is induced after stimulation with microbial products probably via TLR dependent mechanisms or after CD40 CD40L ligation (i.e. contact between T-cells and APC). IL-12 is composed of the p35 and p40 subunits and co-expressed in the same cell in order to make a functional IL-12 p70 (109). The IL-12 receptor (IL-12R) is expressed mainly on NK-cells and T-cells (110). However, the receptor is not exclusively for or specific to IL-12. A new report suggests that IL-12p40 in cord blood could be the same as IL-23 and have a role in the immune system of newborns (111).

IFN-\(\gamma\) is the most important Th1-type cytokine. It is produced by lymphocytes and NK-cells following antigenic stimulation. IFN-\(\gamma\) can also be induced by IL-12 stimulation
IFN-γ is a potent activator of macrophages and neutrophils and because of its down-regulating effect on Th2-cells it can inhibit the production of IgE (113).

2.6.6.2 Th2-type cytokines

Th2-type cytokines are involved in the regulation of B-cell maturation. The Th2 cytokine IL-4 is involved in humoral immune responses and regulates the switching from IgM/IgG to IgE in B-cells (113). Thus, IL-4 mediates important functions in allergy, including the promotion of Th2 lymphocyte differentiation, induction of IgE production and up-regulation of IgE receptors (Fig.3). IL-4 is mainly produced by T lymphocytes, but can also be produced by other cells such as mast cells, basophils, macrophages and B cells (114, 115). The effect of Th2-type cytokines is not only harmful. Thus, eosinophilia, mucous production and IgE synthesis induced by the Th2-type cytokines are important in protection from helmintic parasites (116).

IL-13 is important in IgE-mediated allergies as one of its receptor sub-units is shared with the IL-4 receptor (117). IL-13 is, as IL-4, able to induce IgE class switching in B cells (118). Functional IL-13 receptors are not expressed by T cells, and therefore IL-13 cannot, in contrast to IL-4, induce Th2 differentiation (119). IL-13 is produced by T-cells and dendritic cells (119).

2.6.6.3 T regulatory cytokines

IL-10 is an important anti-inflammatory cytokine produced by both Th1 and Th2 cells as well as by macrophages and dendritic cells (120). IL-10 inhibits Th1-cytokine production via inhibition of IL-12 (121), restrains the activation of mast cells (122) and cytokine production by eosinophils (123). It also obstructs APC function (124). IL-10 has an important role in the regulation of Th2-type cytokine responses by blocking the activation of Th2 cells (125-127). An interesting finding in an in vitro experiment is that corticosteriods can interfere with the immune system and seems to increase the IL-10 production by T-cells and macrophages (128).

2.6.6.4 Regulatory T-cells

The regulatory T-cells (Tregs) were previously called suppressor T-cells. Several subsets of Tregs have been described. Naturally occurring CD4⁺CD25⁺ Tregs and type 1 Tregs are the most investigated Tregs (129). CD4⁺CD25⁺ Tregs and type 1 Tregs that release IL-10 are characterized by abolished allergen-induced specific T-cell
proliferation and suppressed Th1- and Th2-type cytokine secretion. The increased levels of IL-10 and transforming growth factor-Beta (TGF-Beta), produced by the type Th3 Treg potently suppress IgE production, while simultaneously increasing production of IgG4 and IgA. In addition, Treg cells directly or indirectly suppress effector cells of allergic inflammation such as mast cells, basophils, and eosinophils (reviewed in (128, 130)). It has been hypothesised that harmless micro-organisms drive Treg cells, creating a balance between Th1- and Th2- type cytokines (50).

IL-10 producing Tregs has been implicated in the prevention of IgE sensitisation. It has been shown that non-allergic individuals have an increased number of IL-10 producing Treg-cells compared to allergic individuals and that these IL-10 producing Treg-cells could specifically inhibit allergen-activated IL-4 producing cells (reviewed in (128)).

Fig. 3. Different stimuli and genetic predispositions lead to production of various cytokines.

2.6.7 Immune responses and infections

Different pathogens are handled in different ways by the immune system.
2.6.7.1 *Intracellular bacteria*

After presentation of intracellular bacteria via class II MHC, Th1 cell activation starts to produce Th1-type cytokines including IFN-γ, and in turn, IFN-γ recruits and activates macrophages. The activated macrophages can phagocytose the free bacteria. Alternatively the activated macrophages can destroy intracellular pathogens through the production of H₂O₂ or NO derivatives (reviewed in (105)).

2.6.7.2 *Viruses*

The defence against viruses starts with the innate immune branch, such as NK-cells and complement. Thereafter the acquired immunity is triggered, and antibodies in combination with phagocytic cells can kill the virus-infected cells. Antibodies alone can prevent a virus to gain access to the body while cytotoxic T-cells are necessary to limit an established virus infection by destruction of virus-infected cells.

Associations between cytokine profiles, and the number of respiratory tract infections have been seen both in cord blood and in one-year olds (76). Friedlander *et al.* have shown that low levels of PHA-induced IFN-γ in cord blood were associated with a high number of respiratory tract infections during the first year of life. However, they also noted that a high number of respiratory tract infections during the first year were associated with high levels of IFN-γ at age one year (76). RSV, a virus connected with asthma and allergy, is associated with many different dominating immune responses. Both Th1 and Th2-type immune responses have been observed during infection. It has been shown that almost all children who get infected with RSV develop virus-specific IgE. The amount of RSV-specific IgE, and the persistence and the duration of this response are critical in determining which patients are going to develop bronchiolitis and wheezing (reviewed in (131)).

The successful coexistence of herpes viruses with their host requires a variety of mechanisms for anti-viral immune evasion. Symptomatic primary infections with CMV and EBV among adults seem to induce Th1-type cytokines (132, 133). However, *in vitro* stimulation of EBV-infected B-cells from allergic patients has been shown to generate IL-4 production (134). NK cells seem to be of importance in protection against chronic active EBV-infection (135).
2.6.8 Immune responses and allergy

Increased Th1 activity with synthesis of IFN-\(\gamma\) has long been believed to dominate among non-sensitised individuals while an increase in allergen-specific Th2 activities with synthesis of IL-4 dominate among IgE sensitised individuals (136). Even as early in life as during the perinatal period, there are immunological differences in neonates at high risk of developing allergy compared to low-risk neonates with no family history of allergy (137). Previous studies have shown that the production of IFN-\(\gamma\) is lower in cord blood than in adult blood (138) and that the secreted levels of IFN-\(\gamma\) are decreased in cord blood among children who will later develop allergy (137, 139). In support of these findings, other researchers have shown that children developing allergic disease at one year of age had a higher amount of IL-13 in their cord blood (140). Interestingly, the majority of children in these studies developed atopic eczema as the dominating clinical manifestation of allergic disease. Another publication has showed an association between IgE sensitisation in children and high production of IL-4 in peripheral blood mononuclear cells (141).

Synthesis of IL-12 p70 is markedly reduced in the neonatal period and is still lower than in adults at 12 years of age (142). Theoretically, lower IL-12 production would lead to skewing against Th2-type of cytokines. Prescott et al. showed that reduced IL-12 production in APC during the perinatal period was associated with reduced T-cell activation, a stronger neonatal Th2 response and a weaker Th1 in response to allergen (143).

The innate branch of the immune system also seems to be important when discussing allergy development. Thus, low microbial stimulation leads to reduced stimulation of TLRs. In a recent study, in vivo administration of a TLR4 or TLR2 agonist, to mice that were sensitised against ovalbumin, modulated allergic immune responses. Inflammatory parameters like pulmonary eosinophilia, IL-13 in bronchoalveolar lavage, total serum IgE and airway hyper-responsiveness were decreased (98). This strongly indicates that TLRs are involved in the allergic immune response.

2.7 IMMUNE BIOLOGY OF PREGNANCY AND ALLERGY

Pregnancy is an immunological interesting condition where the mother carries non-self biological material, the foetus. The barrier between the mother and the foetus is the
placenta which consists of a foetal part; the membranes amnion and chorion, the umbilical cord and the chorionic villi. The maternal part is composed of the decidua and the intervillous space.

The Th1/Th2 balance during pregnancy is intricate and has been debated over the last decade. Until recently, a Th2-type of immunity was thought to be required for a successful pregnancy (144, 145). However, a combination of old and newer knowledge indicate that both Th1- and Th2-type cytokines, in a complex interplay, are important for pregnancy (146, 147). During pregnancy, immune responses belonging to the adaptive part of immune function are suppressed while the innate branch of the maternal immune system is activated (148). Granulocytes increase in numbers (149) and both granulocytes and monocytes intensify their phagocytic capacity (150).

It has been demonstrated that the foetus is able to produce IgE antibodies as early as week 22 of gestation (151). In a recent study, Sverremark-Ekström et al. have shown that IgE positive cells were more frequently located in the foetal part than in the maternal part of the placenta (152). Interestingly, placental IgE was expressed on macrophages and independent of whether the mother had an allergic disease.

Maternal IgG can be transported across the placenta; it begins slowly and accelerates during the pregnancy (153). Several publications have reported that both respiratory and food-allergens can be transported through the placenta from the mother to the foetus (14, 154) perhaps facilitated by IgG. It is thought that the allergens may be able to prime foetal T-cells and as a consequence cytokine responses can be detected in mononuclear cells from cord blood after stimulation with allergens/mitogens (155). Hypothetically these observations indicate that IgE sensitisation may occur as early as in utero.
3 AIM

The overall objective of this thesis was to study the associations between cytokine profiles, viral infections and IgE sensitisation among children during infancy. The specific aims were to evaluate the associations:

- of the maternal history of allergic disease with the cytokine profiles for IL-4, IL-12 and IFN-γ in mitogen and allergen stimulated cord blood mononuclear cells (CBMC).

- of the cytokine profiles for IL-4, IL-12 and IFN-γ in mitogen and allergen stimulated CBMC with IgE sensitisation/ various allergic phenotypes among infants at 2 years of age.

- between viral infections during early infancy and IgE sensitisation among infants at 2 years of age.

- of viral infections during infancy with the cytokine profiles for IL-4, IL-12, IL-10 and IFN-γ in mitogen stimulated peripheral blood cells (PBMC) at 2 years of age.
4 SUBJECTS AND METHODS

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

4.1 STUDY POPULATION

Families expecting a child and who were living in the southern part of Stockholm (in the city and the suburbs) 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 neither of the parents had symptoms of allergy or an allergic disease. Families where only the mother was allergic were also invited to participate in the study. 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 negative or a positive history of respiratory allergy (bronchial asthma and/or allergic rhino-conjunctivitis) to pollen and/or furred pets were invited to continue (n=281) the study. The children were born between September 1997 and August 2000. One hundred and twenty children had two allergic parents (group dh = double heredity), 84 children had an allergic mother but no allergic father (group mh = maternal heredity) and 77 children had no allergic parents (group nh = no parental heredity).

In Paper I the association of the mother’s allergy with the cytokine profile in CBMC was evaluated among 57 of the 281 children. The children were selected consecutively and selection was based on whether the amount of CBMC in cord-blood was sufficient for the ELIspot method. Besides, selection was designed to produce an even distribution in relation to allergic heredity (20 newborns with two allergic parents, 18 with an allergic mother but no allergic father and 19 newborns without allergic parents).

The same children (including the subset involved in Paper I) provided the material used for Paper II (the association between cytokine profile in CBMC with IgE sensitisation at two years of age) and Paper IV (the association between cytokine profile in PBMC with serostatus against EBV and CMV at two years of age). There were 82 children...
available for analysis in Paper II and 75 children available for analysis in Paper IV. The selection of children was identical to the selection procedure used for Paper I, e.g. that the amount of CBMC (cord blood) and PBMC (at 2 years of age) was sufficient for the ELIspot method.

A total of 264 (94 %) of the children were evaluated at 24 months of age and among these 246 (88 %) provided a blood sample and SPT. Thus, 246 children were included in the analysis for Paper III (the association of serostatus against 13 different viruses with IgE sensitisation at 2 years of age).

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.

Table I. The selected study population.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telephone interview</td>
<td>717</td>
<td></td>
</tr>
<tr>
<td>Primary evaluation</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>All study participants</td>
<td>281</td>
<td>(100)</td>
</tr>
<tr>
<td>2-year visit</td>
<td>264</td>
<td>(94.0)</td>
</tr>
<tr>
<td>Complete data (clinical evaluation + SPT + blood analysis) at 2 years of age</td>
<td>246</td>
<td>(87.5)</td>
</tr>
<tr>
<td>5-year visit</td>
<td>240</td>
<td>(85.5)</td>
</tr>
<tr>
<td>Complete data (clinical evaluation + SPT + blood analysis) at 5 years of age</td>
<td>226</td>
<td>(80.5)</td>
</tr>
</tbody>
</table>

4.2 STUDY DESIGN

Study design is presented in Table II and included repeated measurements using: questionnaires (parents and child), skin prick testing and blood sampling (child). At the first visit the parents were provided with instructions and material for cord blood sampling at the delivery department. The cord blood samples were obtained by midwives.
### 4.3 STUDY METHODS

#### 4.3.1 Parental questionnaire

Parental symptoms related to allergic diseases were collected prenatally (usually in the third trimester of pregnancy). Information about environmental factors such as living conditions, smoking in the household, number of siblings, exposure to furred pets, socioeconomic status (estimated using the father’s occupation) was collected when the child was 6 months of age.

#### 4.3.2 Child questionnaire

The parents answered standardized questions at every visit (6, 12, 18 and 24 months) about symptoms related to allergic diseases, vaccination status and duration of exclusive and partial breast feeding in the child.

#### 4.3.3 Parents' report of infections

The parents were asked to record every infection that their child had in a structured diary from birth up to and including 24 months of age. This included symptoms: runny nose, cough, vomiting, diarrhoea, fever and a doctor’s diagnosis where relevant. In an attempt to identify illnesses not included in the diary, the parents were asked at each visit: “Has your child had any illness since your last visit?” If additional illnesses were mentioned, they were added to the diary.
4.3.4 Skin prick test
SPT against food allergens (ALK, Copenhagen, Denmark) were performed when the children were 6, 12, 18, 24 and 60 months of age: egg white (Soluprick weight to volume ratio 1/100); cod (Soluprick 1/20); peanut (Soluprick 1/20); cow’s milk (3 % fat, standard milk); and soy bean protein (Soja Semp® Semper AB, Stockholm, Sweden). SPT was also performed for inhalant allergens: cat; dog; Dermatophagoides farinae; birch; and timothy (Soluprick 10 HEP). The parents were skin prick tested against the same inhalant allergens as the children but also against horse, rabbit and mugwort. Histamine chloride (10 mg/ml) was the positive control and the allergen diluent was the negative control. The SPT was considered positive if the wheal diameter was > 3 mm after 15 minutes.

4.3.5 Blood sampling
Cord blood from the newborns and peripheral venous blood from the children at ages 2 and 5 years were collected by the aspiration technique. For blood sampling at 2 and 5 years of age the skin was pre-treated with local anaesthesia cream (EMLA®). Plasma was collected by centrifugation and thereafter blood mononuclear cells were obtained by Ficoll-Paque (Pharmacia-Upjohn Upssala, Sweden) gradient centrifugation, performed twice. Ten million cells/ml were frozen in tissue-culture media (TCM). Cells were stored in liquid nitrogen until thawed. After thawing, cells were tested for viability with trypan blue exclusion. The viability was greater than 90% for all samples assayed.

Further detailed descriptions of the preparation of cells and the subsequent allergen stimulation are provided in Papers I, II and IV.

4.3.6 Specific IgE
Circulating IgE antibodies against cow’s milk, egg white, peanut, cod fish, soy bean, cat dander, dog dander, birch pollen, timothy pollen and *Dermatophagoides farinae* were determined in plasma (CAP-FEIA™ Phadia, Uppsala, Sweden) at two and five years of age. A positive test was defined as an IgE antibody level > 0.35 kU/l.

4.3.7 Viral infections
The serostatus against 13 viruses was investigated, including respiratory tract infections - adenovirus, influenza (A/H1, A/H3 and influenza B), parainfluenza (type 1, 2, 3) and respiratory syncytial virus (RSV)) - and herpes viruses: cytomegalovirus (CMV),
Epstein-Barr virus (EBV), herpes simplex virus (HSV), human herpesvirus 6 (HHV6) and varicella-zoster virus (VZV).

The presence of IgG against the EBV capsid antigen and human herpesvirus 6 was determined according to previously published immunofluorescence assays (156, 157). For HSV, CMV and VZV IgG enzyme-linked immunosorbent assays (ELISA) were used (158, 159).

The presence of IgG-antibodies against influenza A H1, H3 and influenza B were determined with ELISAs, using recombinant influenza antigens (160). IgG antibodies against parainfluenza (serotype 1, 2, 3), RSV and adenoviruses were measured by ELISA designed to be for diagnostic purposes.

The serological methods are described in detail in Paper III.

4.3.8 Enzyme-linked immunospot (ELISpot) assay
The principle of the ELISpot assay is the detection of cells that produce a product rather than levels of the secreted product itself. Briefly, the cells were cultured in an antibody-coated 96-well membrane plate for 40 hours. When the cytokine of interest is released from the cell, it is bound to the antibody in the bottom of the well. Cells were washed away, and the detection performed by a secondary antibody. Blue spots were formed at the site of cytokine-secreting cells. The spots were then counted using a computerized ELISpot counter. For more detailed information, see Papers I, II and IV.

4.3.9 Clinical examination
The children were examined clinically in the allergy department at Sachs’ Children’s Hospital at, 6, 12, 18, 24 and 60 months of age by the same paediatrician (CN). Each child was clinically classified with respect to wheezing/asthma, allergic rhinoconjunctivitis, food allergy/acute urticaria and atopic eczema.

4.4 CLASSIFICATION OF THE CHILDREN
4.4.1 Atopic eczema
Eczema, previously called atopic eczema / dermatitis syndrome (AEDS), was defined according to Hanifin and Rajka (161).
4.4.2 Food allergy/acute urticaria
Food allergy/acute urticaria was diagnosed as acute onset of symptoms such as skin reactions, wheezing, vomiting, or diarrhoea on more than one occasion after ingestion of, or contact with, a particular food or allergen.

4.4.3 Wheezing/asthma
Wheezing/asthma during the first 2 years of life was defined as three or more episodes of wheezing or signs of hyper-reactivity (wheezing or severe coughing at infections, exercise and exposure to cold weather or disturbed sleep because of coughing at night) and also respiratory symptoms treated with inhaled glucocorticoids. The child was also classified as having wheeze/asthma if any episode of wheezing or hyper-reactivity was combined with a family history of allergic disease or allergic symptoms in the child.

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

4.4.5 IgE-sensitisation
The child was classified as IgE sensitised (19) if at least one or more SPT was positive ($\geq 3$ mm) and/or if specific IgE against at least one or more of the selected allergens was $\geq 0.35$ kUA/l. In order to optimise the classification in IgE sensitised and non IgE-sensitised children the results were combined.

4.5 STATISTICAL ANALYSES
Descriptive statistics were used to characterize the data. The Chi-squared test and the Student’s T-test (two tailed) were used for comparison of IgE sensitised and non IgE-sensitised children where appropriate.

Cells spontaneously producing cytokines were subtracted from all values shown in paper I, II and IV. As the distribution of the number of cytokine producing cells was not norm, the Mann-Whitney U-test was used to compare the ELISpot results in different study groups (Paper I and II). Correlations between cytokines were tested with Spearman’s rank test. P-values $< 0.05$ were considered as statistically significant.
For the analysis used in paper IV the distribution of the cytokine producing cells were divided into thirds (low, medium and high numbers of cytokine producing cells). Three sets of logistic regression models used either seropositivity against CMV or EBV, or IgE sensitisation as the dependent variable. The independent variables in these analyses were IFN-\(\gamma\), IL-4, IL-10 and IL-12. Odds ratios and 95 % confidence intervals were calculated. Where the association between a cytokine and the dependent variable was altered notably by adjustment for the other cytokines, these were included in the model one by one to identify potential confounding or modifying factors. Additional adjustment for the other dependent measures, CMV seropositivity, EBV seropositivity, and IgE sensitisation, was performed as appropriate. Additionally, the cytokine distributions were transformed using natural logarithms to remove skewness, thus allowing us to model these distributions as continuous variables.

In the analysis for paper III the number of serologically verified infections was normally distributed and this distribution was divided into quarters defined by quartiles using the statistical program. There was a variation in the size of the groups due to characteristics of the distribution. Odds ratios and 95 % confidence intervals were calculated for the development of IgE sensitisation. Adjustments were made for sex, parental allergy (none, single or 2 allergic parents), maternal age, parental smoking, furred pets at home, month of birth, older siblings, duration of breast feeding, socioeconomic status, parentally reported infections and seropositivity against viruses. The interaction of seropositivity for CMV with seropositivity for EBV was investigated using logistic regression, with adjustment for the main effects. Ninety-five percent confidence intervals that do not cross 1.00 were considered as statistically significant.

The data were analyzed using Stata (7.0 Stata Corporation, TX, USA), SPSS 11.0 for Windows and the SAS System for Windows release 8.02.
5 RESULTS

5.1 CYTOKINE-PRODUCING CORD-BLOOD MONONUCLEAR CELLS AND PARENTAL ALLERGY (PAPER I)

The associations of maternal history of allergic disease with the number of IFN-γ, IL-4 and IL-12-producing cord blood mononuclear cells (CBMC) were evaluated by the ELISpot method in 57 children after in vitro stimulation with birch, ovalbumin, cat and phytohaemagglutinin (PHA). The children were divided into three groups, double allergic heredity (dh; n = 20), maternal allergic heredity (mh; n = 18) or no allergic heredity (nh; n = 19).

After allergen stimulation the number of IL-4 producing CBMC was very low and independent of family history of allergy. In response to PHA, there was an induction of IL-4-producing cells and the numbers of these cells were statistically significantly higher in the dh group compared to the mh group (p < 0.05). The number of IFN-γ-producing CBMC in response to allergens tended to be highest in the nh group and lowest in the dh group however these differences are not statistically significant. There were large inter-individual variations in the numbers of IFN-γ- and IL-4-producing cells after PHA stimulation. However, there was a statistically significant correlation between the number of IL-4 and IFN-γ-producing cells (r_s = 0.72, p< 0.0001). The inter-individual variations lead us to calculate the ratio between the numbers of IL-4 and IFN-γ producing CBMC (Th2/Th1). In response to PHA, a significantly higher ratio was observed in the dh group than in the nh group (p<0.05) (Fig 4).

High numbers of IL-12-producing cells in response to allergens were induced in all groups. The group without allergic heredity exhibited significantly higher numbers of IL-12-producing CBMC compared to the group with only maternal heredity, which displayed the lowest number of IL-12-producing CBMC. There was a statistically significantly difference between the groups for birch and for cat (p < 0.01) (Fig. 5).
Figure 4. Ratios between IL-4 and IFN-γ-producing cells /100 000 CBMC in response to PHA from children with different allergic heredity. □ dh = double heredity, ■ = maternal heredity,  □ = no heredity. Spontaneous production is subtracted and mean ratio +/- s.e.m. is shown. * = p < 0.05

Figure 5. Frequencies of IL-12-producing cells /100 000 CBMC in response to birch, ovalbumin and cat allergens. Spontaneous production is subtracted and mean ratio +/- s.e.m. is shown. ** = p < 0.01
5.2 CYTOKINE-PRODUCING CORD-BLOOD MONONUCLEAR CELLS AND IGE SENSITISATION AND ALLERGIC DISEASES AT TWO YEARS OF AGE (PAPER II)

The associations of the number of IFN-γ, IL-4 and IL-12-producing CBMC and IgE sensitisation with various allergic phenotypes (wheezing/asthma, allergic rhinoconjunctivitis and atopic eczema) were evaluated at 2 years of age in 82 children. Sixteen (19.5%) children were IgE sensitised, 17 (21%) had wheezing/asthma, 30 (37%) had atopic eczema (AEDS) and none of the children had allergic rhinoconjunctivitis at 24 months of age. The cumulative incidence of atopic eczema was 65% (n=53) during the 24-month follow-up. There were no statistically significant differences in sex, family history of allergy, parental smoking habits, delivery mode, presence of furred pets at home and number of siblings between IgE sensitised and non-sensitised children. However, non-sensitised children were more often attending day care than sensitised children (p=0.002).

Compared with the non-sensitised children, IgE sensitised children had a lower number of IL-12-producing CBMC after stimulation with birch, ovalbumin and cat and this difference is statistically significant for cat stimulation (p = 0.002) (Fig.6).

Fig. 6. Frequency of IL-12-producing cells/100 000 CBMC after stimulation with birch pollen □, ovalbumin ■ and cat □ among IgE sensitised (n=16) and non-sensitised (n=66) infants at two years of age. Results are illustrated using the box plot model. Boxes cover the middle 50% of the data values, between the 25th (q1) and 75th (q3) percentiles, the central dot being the median. Lines extend out to non-outlier maximum and non-outlier minimum values. ** = p < 0.01.
There were no associations between the number of IFN-γ-producing cells and IgE sensitisation. The number of IL-4-producing CBMC was very low except after stimulation with PHA. The non-sensitised group of children showed an increased number of IL-4-producing CBMC after stimulation with PHA (p=0.038) compared to the children in the sensitised group. Thus, it was not children with a low IFN-γ/IL-4 ratio that developed sensitisation but those with a low number of IL-12-producing CBMC.

Children with eczema, during their first two years of life, had statistically significantly lower numbers of IFN-γ producing CBMC after stimulation with ovalbumin (p = 0.017) and cat (p = 0.010) compared to children without eczema (Fig. 7). No statistically significantly associations were seen with wheezing/asthma for the studied cytokine profiles in cord blood.

Since IL-12 has been shown to induce IFN-γ-production and IFN-γ, on the other hand, promotes IL-12 it was of interest to evaluate the correlation between the two cytokines. There was a statistically significant positive correlation between the number of IFN-γ and IL-12-producing CBMC after stimulation with PHA among all children (r_s = 0.68, p<0.001).
5.3 INFECTIONS AND IGE SENSITISATION (PAPER III)

The associations with IgE sensitisation for serostatus against 13 selected viral infections and parentally reported infections were evaluated among 246 children at 2 years of age. Fifty-nine (24%) children were classified as being IgE sensitised. The majority (n=49; 83 %) were sensitised against food allergens. Demographic data are presented in Paper III. There were no statistically significant differences in sex, having a furred pet at home or having smoking parents between IgE sensitised/non-sensitised children. However, the non-sensitised children were statistically significantly more likely to have been born during the summer than sensitised children and more frequently had more than one sibling. The sensitised children were also statistically significantly more likely to have two parents with allergic disease.

The median number of parentally-reported infections was 13 (range 4 - 24) during the first 2 years of life. There was no association between the number of parentally reported infections and IgE sensitisation at 2 years of age (Fig. 8).

![Parentally reported infections and IgE sensitisation](image_url)

Fig. 8. Parentally reported infections among sensitised and non-sensitised children at 2 years of age. The X-axis shows the number of parentally-reported infections during the first two years of life for each child. The Y-axis shows percentage of children having different numbers of parentally-reported infections.

The number of children who were seropositive against the investigated viruses is presented in Table III. The median number of viruses the children showed...
seropositivity against was 5 (range 0 - 13) during the first 2 years of life. There was no statistically significant correlation between the number of parentally reported infections and the number of viruses identified through serology.

Table III. Seropositivity against the investigated viruses in IgE-sensitised and non sensitised children.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Whole cohort</th>
<th>IgE sensitised</th>
<th>Non sensitised</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>246</td>
<td>59</td>
<td>187</td>
</tr>
<tr>
<td>n (%)</td>
<td></td>
<td>n (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td>Parainfluenza 1</td>
<td>70 (28.5)</td>
<td>16 (27.1)</td>
<td>54 (28.9)</td>
</tr>
<tr>
<td>Parainfluenza 2</td>
<td>30 (12.2)</td>
<td>7 (11.9)</td>
<td>23 (12.3)</td>
</tr>
<tr>
<td>Parainfluenza 3</td>
<td>166 (67.5)</td>
<td>42 (71.2)</td>
<td>124 (66.3)</td>
</tr>
<tr>
<td>Influenza A H1N1</td>
<td>30 (12.2)</td>
<td>6 (10.2)</td>
<td>24 (12.8)</td>
</tr>
<tr>
<td>Influenza A H3N2</td>
<td>92 (37.4)</td>
<td>16 (27.1)</td>
<td>76 (43.8)</td>
</tr>
<tr>
<td>Influenza B</td>
<td>47 (19.1)</td>
<td>13 (22.0)</td>
<td>34 (18.2)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>200 (81.2)</td>
<td>46 (78.0)</td>
<td>154 (82.4)</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>154 (62.6)</td>
<td>33 (55.9)</td>
<td>121 (64.7)</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>24 (9.8)</td>
<td>6 (10.2)</td>
<td>18 (9.6)</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>96 (39.0)</td>
<td>27 (45.8)</td>
<td>69 (36.9)</td>
</tr>
<tr>
<td>Varicella zoster virus</td>
<td>45 (18.3)</td>
<td>8 (13.6)</td>
<td>37 (19.8)</td>
</tr>
<tr>
<td>Epstein-Barr virus</td>
<td>64 (26.0)</td>
<td>8 (13.6)</td>
<td>56 (29.9)</td>
</tr>
<tr>
<td>Human herpes virus 6</td>
<td>207 (84.2)</td>
<td>47 (79.7)</td>
<td>160 (85.6)</td>
</tr>
</tbody>
</table>

To study if IgE sensitisation was associated with the number of viruses, to which the children were seropositive against, the children were divided into 4 groups (based on number of seropositive results) defined by quartiles. There was a tendency that the IgE-sensitised children more often were seropositive to fewer than to many viruses (Fig. 9).
Seropositivity and IgE sensitisation

Fig. 9. The number of viruses to which children were seropositive against divided into 4 groups and IgE sensitisation. The Y-axis shows the percentage of children in the different groups.

Children seropositive against EBV were statistically significantly less likely to be IgE sensitised compared with children who were seronegative against EBV (adjusted odds ratio (OR_{adj}) = 0.34; 95% confidence interval (CI) 0.14 – 0.86) (Fig 10). Seropositivity against both EBV and CMV gave a further decrease in the risk of being sensitised and the calculation showed an interaction between those viruses (OR_{adj} for the interaction = 0.10; 95% CI 0.01 – 0.92) (Fig 10). There were no statistically significant associations between IgE sensitisation and the other viruses. The analyses were adjusted for sex, parental allergy (none, maternal or 2 parents with allergy), maternal age, parental smoking, furred pets at home, month of birth, older siblings, duration of breast feeding, socioeconomic status, parentally reported infections and seropositivity against the viruses.
Fig. 10. Associations with IgE sensitisation for seropositivity against CMV and EBV among children at 2 years of age presented as adjusted odd ratios and 95 % confidence interval. Seronegative children are the reference group.

5.4 CYTOKINES AT 2 YEARS OF AGE (PAPER IV)

Following PHA stimulation, associations were assessed for cytokine-profiles of peripheral mononuclear blood cells (PBMC) with serostatus against CMV and EBV and IgE sensitisation among 75 children at 2 years of age.

Fifteen (20.0%) children were classified as IgE sensitised at two years of age and 29 (38.7%) children were seropositive against CMV. Boys predominated among those seropositive against CMV. Some 22 children (29.3 %) were seropositive against EBV. The cytokine producing cells were not normally distributed so they were divided into thirds (low, medium and high numbers of cytokine producing cells) with 25 children in each group. Dividing the distribution into thirds facilitates the comparison of associations and has the additional advantage that no assumptions are made about the linearity of associations.

Among the CMV seropositive children, 18 (62 %) had IFN-γ producing cells in the upper third and 3 (10 %) in the lower third of the distribution. Compared with the lower third, the upper third of the IFN-γ producing cell distribution was positively and statistically significantly associated with seropositivity against CMV with an OR of 18.85; (95 % CI 4.25 - 83.59) (Table IV). Adjustment for the other cytokines notably
altered the association and this was almost entirely due to adjustment for IL-4 alone. When comparing the numbers of IL-4 producing cells and seropositivity against CMV there was a non-statistically significant tendency for a negative association, as children who were CMV seropositive tended to produce lower numbers of IL-4 producing cells (Table IV). After adjustment for IFN-γ, IL-10 and IL-12 the odds ratio became statistically significant: OR_{adj} 0.05 (95 % CI 0.01 - 0.46). The statistical significance of our results was enhanced after logarithmic transformation of the distributions of the cytokine-producing cells and modelling of these transformed measures as continuous variables. Using the continuous transformed measures, there was still a statistically significantly association of CMV seropositivity with the numbers of IFN-γ-producing cells and IL-4-producing cells.

EBV seropositive children had higher numbers of IFN-γ, IL-4 and IL-10 producing cells, than EBV seronegative children but none of these associations were statistically significant.

IgE sensitised children had statistically significantly higher numbers of IL-4-producing cells compared with non-sensitised children (OR = 13.50; 95% CI 1.56 - 117.13).

<table>
<thead>
<tr>
<th>Number of cytokine producing cells; min-max (median)</th>
<th>CMV sero-</th>
<th>CMV sero-</th>
<th>OR; (95 % CI)</th>
<th>ORadj*; (95 % CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFN-γ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low 5.33-32.67 (21.00)</td>
<td>3 (10.3)</td>
<td>22 (47.8)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Medium 33.33-125.67 (70.67)</td>
<td>8 (27.6)</td>
<td>17 (37.0)</td>
<td>3.45; (0.79 - 15.01)</td>
<td>4.25; (0.78 - 23.15)</td>
</tr>
<tr>
<td>High 128.00-405.67 (212.67)</td>
<td>18 (62.1)</td>
<td>7 (15.2)</td>
<td>18.85; (4.25 - 83.59)</td>
<td>37.48; (5.71 - 246.15)</td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low 1.33-130.33 (59.67)</td>
<td>12 (41.4)</td>
<td>13 (28.3)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Medium 132.67-225.00 (176.33)</td>
<td>9 (31.0)</td>
<td>16 (34.8)</td>
<td>0.61; (0.20 - 1.89)</td>
<td>0.16; (0.03 - 0.87)</td>
</tr>
<tr>
<td>High 226.00-510.67 (320.33)</td>
<td>8 (27.6)</td>
<td>17 (37.0)</td>
<td>0.51; (0.16 - 1.61)</td>
<td>0.05; (0.01 - 0.46)</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low 4.67-57.00 (37.00)</td>
<td>8 (27.6)</td>
<td>17 (37.0)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Medium 58.33-138.67 (101.70)</td>
<td>10 (34.5)</td>
<td>15 (32.6)</td>
<td>1.42; (0.44 - 4.52)</td>
<td>1.61; (0.34 - 7.64)</td>
</tr>
<tr>
<td>High 146.00-610.67 (277.33)</td>
<td>11 (47.9)</td>
<td>14 (30.4)</td>
<td>1.67; (0.53 - 5.29)</td>
<td>3.79; (0.51 - 28.00)</td>
</tr>
<tr>
<td><strong>IL-12</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low 1.00-36.00 (20.17)</td>
<td>8 (27.6)</td>
<td>16 (34.8)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Medium 36.33-75.66 (51.17)</td>
<td>9 (31.0)</td>
<td>17 (37.0)</td>
<td>1.06; (0.33 - 3.42)</td>
<td>1.33; (0.26 - 6.95)</td>
</tr>
<tr>
<td>High 80.00-289.00 (131.67)</td>
<td>12 (41.4)</td>
<td>13 (28.3)</td>
<td>1.85; (0.58 - 5.86)</td>
<td>1.40; (0.22 - 8.91)</td>
</tr>
</tbody>
</table>
6 DISCUSSION

This thesis focuses on the associations between cytokine profiles, viral infections and IgE sensitisation during infancy.

Since the 1990s there has been a general acceptance of the concept that having a mother with allergic disease is a greater risk for allergy than having a father with allergic disease (21, 26, 162). In Paper I our aim was to elucidate the mechanisms underlying this hypothesis. This was also our reason for selecting three study groups based on parental allergy. Our findings revealed that cord-blood cells obtained from children having two parents with allergic diseases had the highest PHA induced IL-4/IFN-γ ratio indicating a Th2-skewed cytokine profile in cord blood. Surprisingly, children who had a mother but not a father with allergic disease had a low IL-4/IFN-γ ratio, almost comparable to that seen in the children without allergic disease in either parent. In a comparable study conducted by others, the influence of a family history of allergy on cytokine production in CBMC showed that after stimulation with cow’s milk, the IL-4 responses were higher in children with a family history of allergy compared to children without any family history of allergy (163). However, in that study only the mothers were allergic and there was no investigation of a group where both parents had an allergic disease. Their results are consistent with previous studies where mononuclear cells in cord blood from infants, with at least 1 allergic parent, showed a diminished IFN-γ-production (164, 165). Taken together, all these data suggest a strong genetic influence on the cytokine pattern in CBMC and that a father with allergic disease has at least as much influence on the CBMC cytokine profile as an allergic mother. Unfortunately our study did not include a group of children with allergic disease only in the father, so we could not investigate how this may influence the development of allergy in offspring.

One important finding in this study (Paper I) was the presence of high numbers of IL-12-producing CBMC following allergen stimulation. Interestingly, the numbers of IL-12-producing CBMC were highest in the children with parents who did not have allergic disease and lower among the children with allergic parents, especially where their mothers had allergic diseases. This indicates an important role for IL-12 in the risk of developing allergy in early life. Others (143) have also suggested that low levels of IL-12 in children indicate a risk for developing allergic disease. A reduced
capability to produce IL-12 in cord blood was associated with reduced T-cell activation, stronger Th2 responses and weaker Th1 responses to allergen at 24 months of age (143). A clinical study from Spain reported that infants who developed severe bronchiolitis after infection with respiratory syncytial virus (RSV) had lower levels of circulating IL-12 in cord blood (166). This is of interest since RSV is known to induce synthesis of IgE and has been putatively linked with allergy (70, 74).

The observed differences in the IL-12 cytokine profile in CBMC after stimulation with allergens/mitogens suggested the relevance of investigating associations of cytokine profiles in cord blood with IgE sensitisation and development of various allergic phenotypes among children at 2 years of age (Paper II). A lower number of IL-12-producing CBMC following allergen stimulation was found to be associated with an increased risk of IgE sensitisation in two-year old children. However, the induction of IL-12-production was not associated with the development of clinical manifestations such as atopic eczema or wheezing/asthma during the observation period. There was a positive correlation between the numbers of IL-12 and IFN-\(\gamma\)-producing cells, indicating close co-regulation of the two cytokines.

Reduced levels of cord blood IFN-\(\gamma\), in response to allergens and mitogens, in children developing, or at risk of developing, different allergic diseases have been described by others (137, 139). Interestingly, the majority of children in these studies developed atopic eczema as the predominant clinical manifestation of allergic disease and this is consistent with our findings. Our observation that the number of IFN-\(\gamma\)-producing CBMC is decreased in children with atopic eczema and that the number of IL-12-producing CBMC is decreased in IgE-sensitised children might indicate that different cytokine profiles in cord blood are associated with development of different allergic phenotypes.

There are several possible explanations for the importance of IL-12 in the development of allergy. IL-12 is a pro-inflammatory cytokine that has the ability to stimulate both the innate and the adaptive components of the immune system. However, so far, little is known about IL-12 and its influence on morbidity among children. It has been proposed that the IL-12-producing capability in childhood matures gradually until 12 years of age (142). IL-12 is mainly produced by APC such as dendritic cells and macrophages, in response to microbial products, cytokines produced
by T-cells or after antigen presentation and CD40 ligation (167). Thus, the fact that APC from children that develop early allergy produce less amount of IL-12 indicate that defects at the APC level might be involved in the development of allergy (143).

It has been suggested that the low levels of IL-12 and IFN-γ seen in allergic children or children at risk of developing allergic disease may lead to a dysregulation in the cytokine balance with elevated quantity of Th2-type cytokines (137, 139). Genes encoding these cytokines are prime candidates for genetic analysis in allergic diseases. We therefore recently completed a genetic analysis of the association between IgE sensitisation and various allergic symptoms with the genetic polymorphism influencing IL-12 production among our study children (A-K Larsson et al., in manuscript). Three different IL-12-related single nucleotide polymorphisms (SNPs) were investigated. The data showed that atopic eczema, wheezing/asthma and a positive skin prick test at two years of age were more common among children homozygous for the IL-12B SNP (1188 A to C transition) present in the gene encoding the IL-12 p40 protein. Based on these findings we speculate that impaired IL-12 production is inherited. This impaired production may be mediated through a decreased number of IL-12-producing cells which in turn might lead to less IFN-γ production and thus immune deviation towards IgE sensitisation/allergic disease in young children. An association between genetic polymorphisms in the IL-12 gene with allergic diseases has also been suggested in several recent publications (168, 169).

Besides family history of allergy, exposure to various environmental factors has been proposed as important in the development of allergic diseases during childhood. Therefore, another aim of the studies described by this thesis was to evaluate the associations of common viral infections, respiratory infections as well as herpes infections, with IgE sensitisation in infancy. In paper III we provided some limited evidence that that the number of serologically verified viral infections was inversely associated with IgE sensitisation.

However, IgE sensitisation was statistically significantly less prevalent among those children who were seropositive against EBV compared with children who were seronegative against this virus. We also noted an interaction between CMV and EBV where children seropositive against both viral infections showed a further reduction in the risk for IgE sensitisation. A recent study of EBV serostatus and sensitisation in
four-year old children (170) does not corroborate our findings. The explanation for this discrepancy could be that we studied children at 2 years of age while in the study by Sidorchuk et al. the children studied were 4 years old. This might indicate an age-dependent role of EBV in relation to IgE sensitisation. This is in consistent with what Calvani et al. have reported (86). The discrepancies between the results from our study and the one by Sidorchuk et al. cannot be explained by unusually low or high prevalence of seropositivity against EBV and CMV among our children, as our prevalence data are comparable with those described for other industrialized countries (87, 171).

CMV and EBV are persistent viral infections and may therefore influence the immune system with respect to the development of allergy. One explanation for the protective effect of EBV could be that EBV infection in young children drives more rapid maturation of B cells; and rapid maturation transforms the B cells so that they produce IgG 4 rather than IgE. IgG4 would then act as a blocking antibody preventing the cross-linking of the IgE-sensitised mast cells (172). We did not observe any association between seropositivity against CMV and IgE sensitisation in our study children. This is in consistent with the few studies published on the relation between CMV and allergic disease (89, 173). In the Swedish BAMSE study no association was found between CMV seropositivity and IgE sensitisation in 4-years old children. However, among children with both seropositivity against CMV and seronegativity against EBV, there was a positive association with IgE sensitisation against air-born and food allergens (89).

A statistically significantly association between the number of parentally-reported infections and IgE sensitisation was not observed (Paper III). This is in contrast to other studies, where parental report of ≥ 2 episodes of having a runny nose during the first year of life, was associated with an decreased prevalence of asthma (174, 175). However, some parents may overestimate while others underestimate the infectious status of the child, producing a very imprecise measure. Evidence for this comes for the lack of association between seropositivity against the selected viruses and parentally-reported infections in our study. Another factor that may help to account for this difference is that many viruses, e.g. rhinovirus and corona virus which are proposed to be the major causes of upper respiratory infections in infants, were not included in our analysis of serostatus (176).
There is limited knowledge about the associations between cytokine profiles and viral infections. Therefore, the cellular cytokine profiles (IL-4, IL-10, IL-12 and IFN-γ) in peripheral blood mononuclear cells (PBMC), serostatus against EBV and CMV and IgE-sensitisation were evaluated in our children at two years of age (Paper IV). High numbers of IFN-γ-producing PBMC and low numbers of IL-4-producing PBMC were found in the samples from CMV seropositive children. This is consistent with previous reports showing that CMV seems to induce Th1-type of cytokine responses (177). The important sources of IFN-γ production are T-helper cells (CD4+) and NK cells, both implicated in defence against CMV (178). Thus, one can speculate that young children may have an increased NK-cell response, in addition to specific CD4+ and CD8+ T cells, which controls herpesvirus infections (179, 180). However, in our study we did not phenotype the producing cells so we do not know the cellular source of IFN-γ.

For the children seropositive against EBV there was an indication (although not statistically significant) of higher numbers of IFN-γ, IL-4 and IL-10-producing cells compared to EBV seronegative children. Our findings are consistent with results from studies investigating the cytokine pattern in young adults with infectious mononucleosis (IM) (135). We do not know when the seropositive children in our study were infected since primary EBV-infections are normally asymptomatic in infants. However, the suggestion of a similar cytokine pattern in EBV seropositive children as observed in IM is intriguing. These data support the idea that persistent viral infections may affect the immune system for a considerable period of time. EBV and CMV have much in common and the successful coexistence of the viruses with their host requires a variety of mechanisms for evasion of anti-viral immunity. For example, IL-10 homologues present in the viruses might down-regulate the antigen processing/presentation capability of dendritic cells/macrophages and thereby switch off the host T cell system, similar to that observed for Treg cells (181, 182). Alternatively, both EBV and CMV can polyclonally activate B-cells to produce antibodies with many different specificities and thereby hinder the capacity of allergens to cross-link the B-cell receptor as seen for helminthic infections (172).

As expected, the numbers of IL-4 producing PBMC were found to be higher after PHA stimulation in the two-year old IgE-sensitised children compared with the non-sensitised children. IL-4 is one of the cytokines responsible for the B-cells switching from IgM/IgG to IgE (113) and for polarization of Th2 cells into Th2-type cytokine
production (103). Our findings are consistent with previous studies, e.g. from Koning et al., that reported children with allergic asthma had high IL-4 production compared with their non-sensitised counterparts (141). However, the sensitised children in our study tended to have higher numbers of cytokine producing cells for all cytokines studied (IL-4, IL-10, IL-12 and IFN-γ) indicating that allergic individuals can have both Th1- and Th2-type cytokine profiles. These findings are in agreement with what has been reported in adults with IgE-mediated allergy (183) and among children in another of our studies (184).

We recognise some limitations and strengths of the studies in this thesis. The study population was selected on the basis of family history of allergy and was not general population based. The selection of children (Paper I, II, IV) was performed randomly among those with a sufficient amount of CBMC in cord blood samples for the ELISpot method. Besides, selection was designed to produce an even distribution in relation to allergic heredity. Therefore, it seems unlikely that our results are an artefact of the sub-samples used or the original inclusion criteria for the study (Paper I, II, IV). In the analysis for paper III, where the proportion of children with allergic heredity was increased, we adjusted for family history of allergy and other potential confounding factors using multivariate techniques to reduce the risk that our results are due to bias.

A strength of our study is the careful characterization of parental allergic status. Another strength is that the children were followed prospectively by one paediatrician (CN). The clinical allergic status of each child was evaluated repeatedly and objectively using predefined disease definitions. IgE sensitisation was studied both with SPT and analysis of specific IgE antibodies in plasma; while viral infections were evaluated using objective serological measurements. The number of subjects lost to follow-up between birth and 2 years of age was 17 (6 %) children. Thus, 264 children came for the evaluation at 2 years of age among whom complete data (clinical evaluation, SPT and blood sample) were available among 246 (88 %) children (Table I). The children that were lost to follow-up (n = 17) more often had a father who smoked, but otherwise they did not differ significantly, with respect to demographic data, from the rest of the participating children in our studies.

In summary, our findings illustrate the complexity of mechanisms and risks relevant to allergic sensitisation in infancy. The associations between clinical phenotype, viral
identification, specific cytokine responses and genetic variation are likely to provide significant insights into the immunopathogenesis of childhood allergic diseases.
7 CONCLUSIONS

Based on the presented studies, the following conclusion can be drawn.

✓ Children with parents who both had allergic diseases displayed a Th2-type cytokine profile in their cord blood, in contrast with children among whom only their mother or neither parent had allergic disease. These findings suggest a strong genetic influence on the cytokine pattern in CBMC, where having a father with allergic disease has at least as much influence as a mother with allergy.

✓ Children with a high IL-4/IFN-γ ratio in cord blood were not more likely to develop allergy by the age of two years.

✓ Children IgE sensitised at two years of age had a cord blood cytokine profile with a low number of IL-12-producing CBMC and children with atopic eczema displayed a low number of IFN-γ-producing CBMC. These results might indicate that different cytokine profiles in cord blood are associated with different allergic phenotypes.

✓ IgE sensitised children at two years of age had a high number of IL-4-producing PBMC.

✓ Seropositivity against EBV at two years of age may be associated with a decreased prevalence of IgE sensitisation at this age and the protective effect against IgE sensitisation might be enhanced if EBV seropositivity is combined with seropositivity against CMV.

✓ CMV but not EBV seropositivity seems to be associated with reduced numbers of IL-4-producing cells, suggesting that a negative association between EBV seropositivity and IgE sensitisation does not operate through down-regulation of this cytokine.
8 SVENSK SAMMANFATTNING

En ökande allergiförekomst, framför allt hos barn, ses i hela den industrialiserade världen. Orsaken till utvecklingen av IgE-medierad allergi är sannolikt multifaktoriell där ärftlighet och olika miljöfaktorer samverkar. Allergiker anses ha en förskjutning i immunsvaret med en övervikt av signalsubstanser (cytokiner) som tillverkas av T hjälp-celler typ 2 (Th2). Friska individer verkar istället ha en balans mellan signalsubstanser tillverkade av Th2 respektive T hjälp-celler typ 1 (Th1). Tidigare studier har visat att barn med fler äldre syskon och barn som börjar tidigt på dagis hade en lägre förekomst av allergi och astma. Detta fenomen har tolkats bero på en ökad infektionsbörda i barndomen. Efterföljande studier av infektioner hos barn och allergiutveckling har dock varit motsägelsefulla.

Tidigare rapporter har visat att barn som har en allergisk mamma löper större risk att själva bli allergiska jämfört med där bara pappan är allergisk. Detta skulle kunna tala för att graviditeten i sig och inte bara det genetiska arvet kan påverka barnets benägenhet att utveckla allergi. Flera studier har försökt påvisa skillnader i signalsubstanser i nyfödda barns navelsträngsblod beroende på om de senare utvecklar allergi eller inte. Barn som utvecklar allergier, under förskoleåldern, ser ut att ha en lägre mängd av interferon-gamma (IFN-γ) (signalsubstans av Th1 typ) i navelsträngs blod än barn utan allergiska besvär.

I den här avhandlingen har jag studerat om det finns något samband mellan allergisk ärftlighet, miljöfaktorer så som virusinfektioner, signalsubstansprofilen i navelsträngsblod och allergiutveckling (astma, eksem och/eller förekomst av allergiantikroppar) vid 2 års ålder. I de fyra delarbetena i avhandlingen ingår 281 barn med antingen två allergiska föräldrar, inga allergiska föräldrar eller en allergisk mamma men ingen allergisk pappa. Föräldrarnas allergi, kartlades före förlossningen med sjukhistoria och pricktest. Barnen följes från födelsen till 2 års ålder med läkarundersökningar och pricktester. Vid födelsen och vid 2 års ålder togs blodprov för analys av er. Förekomst av allergiantikroppar (IgE-antikroppar antikroppar) mot födoämnen, pälsdjur och pollen samt serostatus (tecken på genomgången virusinfektion) för utvalda virus analyserades vid 2 års ålder.

I den första studien undersökte vi om det fanns något samband mellan produktionen av signalsubstanser i navelsträngsblod och olika grad av allergisk ärftlighet hos 57 barn. Barn med 2 allergiska föräldrar hade en cytokinprofil av Th2 typ (d.v.s. ökad produktion av interleukin-4 (IL-4, signalsubstans av Th2 typ) och låg produktion av signalsubstansen IFN-γ) i navelsträngsblod och som skiljde sig signifikant från barnen med en allergisk mamma och barnen utan allergiska föräldrar. Interleukin-12 (IL-12, signalsubstans av Th1 typ) fanns i signifikant högre mängd hos barn utan allergiska föräldrar jämfört med barnen med en allergisk mamma eller 2 allergiska föräldrar. Våra fynd tyder på att det finns ett starkt ärfältligt inflytande på mönstret av signalsubstanser i navelsträngsblod. En allergisk pappa verkar ha minst lika stort inflytande som en allergisk mamma.

I den andra studien undersökte vi om mönstret av signalsubstanser av Th1 och Th2 typ i navelsträngsblod var associerat till uppkomsten av allergisk sjukdom hos 82 barn vid 2 års ålder. Signalsubstansen IL-12 fanns i signifikant lägre mängd hos de barn som utvecklade IgE-antikroppar antikroppar vid 2 års ålder jämfört med barnen utan IgE-antikroppar antikroppar. Barn med eksem hade lägre mängd av signalsubstansen IFN-γ i navelsträngsblod jämfört med barnen utan eksem. Resultaten talar för att
olika profiler av signalsubstanser i navelsträngsblod kan vara kopplade till uppkomsten av olika typer av allergisk sjukdom.

I den tredje studien undersökte vi om det fanns något samband mellan 13 utvalda genomgångna virusinfektioner och förekomsten av IgE-antikroppar antikroppar hos 246 barn vid 2 års ålder. 24 % av barnen hade IgE-antikroppar antikroppar vid 2 års ålder. Barnen utan IgE-antikroppar hade statistiskt signifikant oftare haft virusinfektionen Epstein-Barr virus (EBV) än de barn som hade IgE-antikroppar antikroppar. De barn som visade tecken på att ha genomgått både EBV och cytomegalovirus (CMV) hade ännu lägre risk att ha IgE-antikroppar antikroppar. Detta tyder på att genomgången EBV-infektion under de 2 första levnadsåren förefaller skydda mot utvecklingen av IgE-antikroppar antikroppar och att detta skydd accentueras om barnet också har haft en CMV-infektion.

I den fjärde studien har vi studerat associationen mellan signalsubstansprofilen i blod från 75 2-åringar och tecken till genomgången CMV- och EBV-infektion samt förekomsten av IgE-antikroppar vid 2 års ålder. Barnen med tecken på genomgången CMV- infektion vid 2 års ålder hade en signalsubstansprofil av Th1 typ (högt IFN-γ och lågt IL-4) vid 2 års ålder, jämfört med de barn som inte visade tecken på genomgången CMV. Trots att EBV-infektion förefaller skydda mot utveckling av IgE-antikroppar (delarbete III) uppvissade barnen med genomgången EBV-infektion inte någon profil av signalsubstanser av Th1 typ. Barnen med IgE-antikroppar hade dock en signalsubstansprofil av Th2 typ i blodet vid 2 års ålder. Dessa fynd stödjer hypotesen att virus påverkar vårt immunförsvar. Våra iakttagelser av cytokinprofilen hos barn med tecken på genomgångna EBV- och CMV-infektioner under de två första levnadsåren kan inte förklara hur infektionerna påverkar utvecklingen av IgE-antikroppar.

**Sammanfattning**

Våra fynd illustrerar komplexiteten vid allergier i den tidiga barndomen.

- Allergisk ärftlighet verkar ha störst betydelse för immunsvaret (signalsubstansprofilen) i navelsträngsblod.

- Olika signalsubstansprofiler i navelsträngsblod verkar vara kopplade till uppkomsten av olika typer av allergisk sjukdom.

- Miljöfaktorer som exempelvis EBV-virusinfektion, verkar vara skyddande mot utvecklingen av IgE-antikroppar antikroppar tidigt i barndomen.

- Våra fynd stödjer hypotesen att virus påverkar vårt immunförsvar.

**Ordlista**

CMV = cytomegalovirus
EBV = Epstein-Barr virus
IFN-γ = interferon-gamma, signalsubstans av Th1 typ
IL-4 = interleukin 4, signalsubstans av Th2 typ
IL-12 = interleukin 12, signalsubstans av Th1 typ
IgE-antikroppar = allergiantikroppar
Th1 = T hjälpar celler 1
Th2 = T hjälpar celler 2
ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone involved in this work, and in particular I would like to thank:

All the children and their families who took part in all the studies, without you this work would never have been possible.

My supervisor Gunnar Lilja for his endless patience and enthusiasm for research and always believing in me. For sharing his knowledge within the field of allergology and for being a model in humility towards the knowledge of others. For all our journeys together and for our discussions about everything.

My co-supervisor Marita Troye Blomberg for her professional guidance into the complicated world of immunology and for supporting me in scientific thinking.

My co-supervisor Annika Linde for her optimistic and friendly support in the interesting field of virology.

Anna Stina Ander for her fantastic and patient work with the participating families and children. Without her careful handling there would never have been so many families coming back to us time after time. Monika Nordlund for her kindness and excellent technical assistance.

My co-workers and friends at Stockholm University Anna-Karin Larsson, Eva Sverremark-Ekström, Petra Amoudruz, Anki Höglind, Susanne Gabrielsson and Yvonne Sundström for their support and explanations within the field of immunology. For all our discussions that always “picked me up”.

Scott Montgomery for his patience with me concerning epidemiological and statistic issues.

Johan Genz and Per Sandstedt the former and the present head of Sachs’ Children’s Hospital for their generosity in providing me the opportunity to try the field of research.

The staff at Sachs’ allergy unit for their support and for doing a wonderful every day job and for our adventures in the archipelago.

My co-authors Liselott Gustafsson and Per Näsman for their inspiring collaborations.

My friends and colleagues at Dept of Environmental Health Magnus Wickman for his optimistic energy and his knowledge in paediatric allergology, Inger Kull and Lotta Egmar for their kindness and for always having time for my questions.

Roland Möllby and his group at MTC for many interesting discussions.
The former “Placenta group” for interesting meetings and discussions.

Phadia AB former Pharmacia Diagnostics for supply of reagents.

My friends and colleagues Bernice Aronsson, thank you for your warm friendship and never ending support, Lotta Buxbaum who introduced our nice Tuesday evenings, and Martina Persson for our corridor chats about life and your warm laugh.

Friends and colleagues at the allergy departments in Stockholm Natalia Ballardini, Per Thunqvist, Johan Alm, Agneta Jansson Roth and Eva Östblom for a stimulating collaborative clinical work and discussions about almost everything, and Anne Kihlström for our pleasant journeys and congress stays together.

All my colleagues and co-workers at Sachs’ Children’s Hospital, all remembered no one forgotten. To share knowledge with each other and laugh together is some of the best things of working with you.

My dear friends, Lotta Tegnér with whom I have shared many experiences, thank you for being there for me, Maria Warenmark my private “GP” for many questions in life among others, how and what to plant in my garden.

All my friends in the scout movement for always thinking optimistic.

Min mamma Christine Svensson för att du har uppostrat mig med kärlek, min pappa Sten Sture Svensson vars envishet jag har ärvt.

My daughters Emelie, Malin and Linnea, I am so grateful for your support both in discussing science and life and drinking coffee. I love you so.

Finally, my beloved husband Torbjörn, thank you for standing by my side all these years and especially these last couple of months. Without your patience I would never have made it.

Funding source
This study was financially supported by the Swedish Asthma and Allergy Association, Consul Th C Berg’s Foundation, the Samariten Foundation, Mjölkdroppen, Vårdal Foundation, Heart and Lung Foundation, Glaxo Smith Kline, Brio AB and the Karolinska Institute.
10 REFERENCES


