Influence of maternal allergy on the *intra uterine* environment and on immune functions of the neonate

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Stockholm 2005
To my family

"It would be possible to describe everything scientifically, but it would make no sense; it would be without meaning, as if you described a Beethoven symphony as a variation of wave pressure." — Albert Einstein
ABSTRACT

The prevalence of IgE mediated allergic diseases has drastically increased during the past few decades and there is no satisfactory explanation. A family history of allergic disease is clearly a strong risk factor, but in view of the rapidity of the increase in allergy prevalence environmental factors are likely to have played a crucial role. This thesis aimed at elucidating the impact of maternal allergy on the in utero environment and on innate immune functions of the newborn baby.

Allergic symptoms usually appear early in life. This implies an early priming for allergic disease, possibly even at the fetal level. We therefore compared the presence and production of IgE in placenta from allergic and non-allergic women. Surprisingly, numerous IgE+ cells, located primarily in the fetal villous stroma, were detected immunohistochemically in a majority of the investigated placentas irrespective of maternal allergy, maternal or fetal total serum IgE levels. The placental IgE could not be demonstrated to be bound to IgE receptors, but was shown to be bound to fetal macrophages, possibly via FcyRI. No evidence was found for local fetal IgE production. The novel finding of numerous IgE+ cells in the placenta irrespective of maternal allergy could indicate a physiological role of IgE in utero during pregnancy.

The placenta has been suggested to be a pregnancy-specific component of the innate immune system. We describe the novel findings of expression of Toll-like receptors 2 and 4 (TLR2 and TLR4) on villous trophoblast, whereas no immune reactivity was present in the villous core. The intermediate trophoblast was also found to express both TLR2 and TLR4. Regulation of TLR2 and TLR4 was investigated by stimulating placental explants with LPS and zymosan. Stimulation readily induced a release of IL-6 and IL-8 in the placental cultures, whereas TLR2 and TLR4 mRNA and protein expression remained at the same high level as in unstimulated explants. We believe that the TLRs and the trophoblast are important components of the innate immune system with a crucial role in the defense against placental infection, but they might also influence the cytokine environment in utero, something that could have impact on the baby’s immune system.

The newborn child’s ability to respond to microbial stimuli in the environment is believed to be important for maturation of the neonatal immune system. We investigated how cord blood mononuclear blood cells (CBMC) from children of allergic and non-allergic women respond to microbial stimuli. Cord blood (CB) monocytes from children with allergic mothers had significantly lower expression of TLR2 and TLR4 compared to maternal peripheral blood mononuclear cells (PBMC) both before and after microbial stimulation, in contrast to CB monocytes from children with non-allergic mothers. Further, CBMCs from children with allergic mothers had a lower (p<0.03) IL-6 response after microbial stimulation than CBMCs from children with non-allergic mothers. Our results imply that CB monocytes and CBMC immune responses are influenced by maternal allergy. Based on these findings we speculate that monocytes from children with allergic mothers have a reduced capacity to respond to microbial stimuli.

The soluble form of the endotoxin receptor CD14 has been shown to be negatively associated with total serum IgE levels. We investigated the levels of soluble (s) and membrane bound (m) CD14 in cord blood and at two years of age from children with allergic or non-allergic mothers and related these parameters with allergy development at two years of age. Children with allergic mothers had significantly higher sCD14 levels in CB compared to children with non-allergic mothers. At two years of age no significant differences in sCD14 levels were observed between the two groups of children and no association between sCD14 and allergic disease was found. Further, we observed large differences in sCD14 and mCD14 with respect to age. These findings highlight the complexity of the interaction between innate and adaptive immune responses. CD14 might be involved in the regulation of IgE production, but we suggest that CD14 could also be important for the maturation and development of the neonatal immune system.

In conclusion, the work presented in this thesis has increased our knowledge on the in utero environment in allergic and non-allergic mothers and suggests that maternal allergy influences innate immune functions of the newborn baby.
Hur miljön i livmodern under graviditeten påverkas av moderns allergi samt dess betydelse för det nyfödda barnets immunsvaret.

Allergier hos barn har ökat kraftigt de senaste åren, framför allt i de industrialiserade länderna, och mer än 30% av alla barn visar tecken på allergi såsom astma, hörselvärk och eksem. Allergier av denna typ (atopisk allergi) beror på att barnet har bildat allergiantikroppar (IgE) mot ett eller flera kroppsfrämmande ämne (allergen). Exempel på allergen är pollen, djurepitel och födömnens. IgE sitter sedan bundet på speciella celler. När barnet på nytt utsätts för allergenet binder detta till IgE antikropparna. Då frisätts en rad olika ämnen som ger de allergiska symptomen. Atopisk allergi är ärligt. Den senaste tidens snabba ökning av allergiska sjukdomar tyder dock på att faktorer i vår miljö spelar en viktig roll. I många fall debiterar atopisk allergi tidigt i livet. Ett stort intresse inom allergiforskningen riktar nu mot miljön som omger fostret, då interaktionen mellan foster och mor kan ha betydelse för utvecklingen av allergi.

I den här avhandlingen har jag studerat hur allergi hos kvinnan påverkar miljön i livmodern under graviditeten och hur detta kan påverka fostret så att allergisk sjukdom eventuellt utvecklas hos barnet. Vår hypotes är att den intrauterina miljön skiljer sig åt med avseende på immunologiska faktorer hos allergiska och friska kvinnor och att den påverkar barns framtidallergiutveckling. Skillnader i denna miljö kan ha betydelse för fostret under graviditeten på så sätt att barnets immunförsvar mognar olika och därmed benägenheten att utveckla allergiska symptomer påverkas.


I min första studie har vi visat att det finns rikligt med allergiantikroppar, IgE, på celler i moderkakan oavsett om mamman är allergisk eller inte. De IgE positiva cellerna finns framförallt i barnets del av moderkakan, vilket kan tyda på att det är barnets IgE. Produktion av IgE verkar inte ske i moderkakan utan det kan vara IgE från barnets blodbana. Om IgE i placentan inte är barnets kan det komma från mamman genom transport över moderkakan tillsammans med IgG. IgG är den antikropp som kan passera från mamman till fostret. Vårt spännande fynd kan tyda på att IgE spelar en viktig roll under graviditeten.

I min andra och tredje studie har vi tittat på signalmolekyler inblandade i det tidiga, ospecifika immunsvar (s.k. Toll-like receptor) (TLR) som även visats kunna påverka specifika immunvar och experimentella allergiska reaktioner. Dessa molekyler är viktiga för vårt försvar mot bakterier och virus. De signalerar till kroppen att nu är det tåra att försvare sig och hjälper till så att kroppen enklare kan bekämpa en infektion genom att aktivera det specifika immunsvar. I min andra studie har vi funnit ett högt uttryck av dessa signalmolekyler (TLR2 och TLR4) på barnet och mammans celler i moderkakan. Det intressanta är att dessa molekyler uttrycks på barnets celler som är i närmast kontakt med mammans blod och på mammans celler i den del av
moderkakan som fäster i livmoderväggen. Om en infektion skulle uppstå i mammans blod eller i livmodern finns dessa signalmolekyler där och signalerar efter hjälp att försvara moderkakan och fostret. Genom att stimulera vävnad från moderkakan med mikrobiella substanser kunde vi även visa att dessa signalmolekyler inte bara finns där utan även fungerar som signalmolekyler.

I mitt tredje projekt har vi studerat dessa signalmolekylers (TLR2 och TLR4) uttryck och reglering på celler i blodbanan hos nyfödda och deras mödrar och jämfört barn med allergiska eller icke-allergiska mammor. Vi kunde se att barn till allergiska mammor uttryckte lägre nivåer av dessa signalmolekyler jämfört med vad deras mammor gjorde. Blodceller från barnen till allergiska mammor hade även en sämre förmåga att svara på stimulering med mikrobiella substanser jämfört med blodceller från barn till icke-allergiska mammor. Detta kan ha betydelse för framtidens allergiutveckling hos dessa barn. Tidigare studier har nämligen visat att om blodceller från ett barn har en sämre förmåga att svara på mikrobiella stimuli vid födseln finns en ökad risk att barnet blir allergiskt senare i livet.

I min fjärde studie har vi titlat på signalmolekylen CD14 som uttrycks på vissa blodceller. Den här signalmolekylen finns även lösligt i blodet (sCD14) och är viktig i försvaret mot infektioner. Tidigare studier har visat att låga nivåer av sCD14 i blodet ökar risken för att bli allergisk. Vi undersökte därför detta i barn med allergiska eller icke-allergiska mammor, både vid födseln och vid två års ålder. Barnen hade mycket lägre nivåer av sCD14 jämfört med sina mammor och vi kunde se att nyfödda barn med allergiska mammor hade en högre nivå av sCD14 i blodet jämfört med barn till icke-allergiska mammor. Intressant var att vi inte kunde se någon skillnad mellan barnen som blivit allergiska vid två års ålder och de som förblev friska. Nivåerna av sCD14 vid födseln verkar därför inte vara ett bra mått på risken för ett barn att bli allergiskt.

Den här avhandlingen har ökat vår kunskap om hur miljön i livmodern under graviditeten hos allergiska och icke-allergiska mammor påverkar immunförsvaret hos deras nyfödda barn.
LIST OF PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by their Roman numerals:


*These authors contributed equally to the work.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
</tr>
<tr>
<td>CB</td>
<td>Cord blood</td>
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<tr>
<td>CBMC</td>
<td>Cord blood mononuclear cells</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>Fas-L</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipid binding protein</td>
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<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>mCD14</td>
<td>Membrane CD14</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>sCD14</td>
<td>Soluble CD14</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>T1f</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>uNK cell</td>
<td>Uterine NK cell</td>
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1 INTRODUCTION

1.1 INNATE AND ADAPTIVE IMMUNITY

The immune system has evolved to protect us from invading pathogens. It can be divided into two parts: innate and adaptive immunity. The innate immune system, which is the more ancient part, is the first line of defense against invading pathogens that get past the anatomical barriers that prevent the entry of microorganisms into the body (skin and mucus). If the pathogens manage to penetrate these mechanical barriers, there are physiological barriers (like body temperature, pH and soluble factors), endocytic and phagocytic defences and potent inflammatory responses to combat the pathogen, including antimicrobial proteins and peptides (reviewed in 3). We are born with a well functioning innate immune system, ready to protect us against invading pathogens. The innate immune system is involved in initiating the more specific adaptive immune system, which acts via T cells and B cells expressing highly diverse antigen receptors, and is therefore able to respond to a wide range of antigens. The adaptive immune system is continuously developing during a lifetime. However, with age it starts to weaken, making elderly people more susceptible to infections.

1.2 HEMATOPOIESIS

The cells of the immune system all originate from hematopoietic stem cells in the bone marrow which can give rise to all the different blood cells. The lymphoid progenitor gives rise to the lymphocytes (T cells and B cells) and to natural killer cells (NK cells), while the myeloid progenitor gives rise to granulocytes (neutrophils, eosinophils, basophils), monocytes/macrophages, dendritic cells (DC) and mast cells. However, the DCs have been shown to originate from both the lymphoid and the myeloid precursors. The DCs are also important as a link between innate and adaptive immunity. 4

1.3 PATTERN RECOGNITION RECEPTORS

Recognition of a microbial infection and the initiation of an immune response are controlled by multiple mechanisms. One mechanism involves pattern recognition receptors (PRRs). PRRs recognize pathogen-associated molecular patterns (PAMPs) and signal to the host the presence of potential pathogens. PRRs discriminate between self and microbial non-self5 and recognize patterns essential for the survival of the pathogen, like components of the cell membrane of Gram positive and Gram negative bacteria or the flagella of flagellated bacterial species.

1.3.1 Toll-like receptors

One key component in innate immunity is the toll-like receptors (TLRs), which are the best characterized PRRs in mammalian species. Toll was first discovered in Drosophila (dToll) where it has a function in development of dorso-ventral polarity during embryogenesis. 4 Adult Drosophila flies lack an adaptive immune response but are resistant to fungal infection through the action of Toll. Upon infection, the Toll ligand Spätzle is processed into a biologically active form and by binding to Toll induces a
signaling cascade, resulting in the production of the anti-microbial peptide Drosomycin.6,5

The first human homologue to dToll was discovered by Medzithov et al7 and was later named TLR4. Mutations in the Tlr4 gene were shown to be responsible for the hypo-responsiveness seen in C3H/HeJ and C57BL10/scCr mice8,9 and by creating TLR4-deficient mice it was confirmed that TLR4 is responsible for the recognition of LPS.10 TLR4 mutations have also been associated to endotoxin hypo-responsiveness in humans.11

All TLRs belong to the interleukin-1 receptor (IL-1R)/TLR superfamily, which has a conserved region in the cytosolic domain called a Toll/IL-1R (TIR) domain. It is likely that most mammalian species have 10 to 15 TLRs. The ligands for TLR1-9 and 11 are known (see table 1). The TLRs seem to be divided into two classes: those that recognize bacterial PAMPs (TLR 1, 2, 4, 5, 6, 11) and are mainly located on the surface of the cell, and those that recognize viral PAMPs (TLR 3, 7, 8, 9) and are localized to intracellular compartments. In response to receptor engagement the TLRs initiate a cascade of steps in the inflammatory reaction to help to eliminate the pathogen and initiate an adaptive immune response (simplified signaling cascade Fig. 1). The TLRs are also important for DC function and maturation, and consequently important for the initiation of an adaptive immune response.12 Further, Toll-like receptors have been shown to be important for activation of antigen-specific T

1

The LPS-CD14 binding is markedly enhanced by presence of LPS-binding protein (LBP).22,23 CD14 also exists in a soluble form (sCD14), generated through various pathways (membrane cleavage or secretion).20 Importantly, cells that lack membrane bound (m) CD14 become responsive to PAMPs in the presence of sCD14.24,25 The gene encoding CD14 is located in the chromosomal region 5q31-33, a region which has been linked to total IgE levels as well as allergic disease.26,27 The levels of sCD14 have been implicated as
Table 1. Toll-like receptors and their ligands.

<table>
<thead>
<tr>
<th>Toll-like receptors</th>
<th>Ligands</th>
</tr>
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<tbody>
<tr>
<td>TLR1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Triacyl lipopeptides, soluble factors</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipoprotein/lipopeptides, lipoteichoic acid, lipoarabinomannan, phenol-soluble modulin, glycolipids, porins, zymosan</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double-stranded viral RNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>Lipopolysaccharide, fusion protein (respiratory syncytial virus)</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacterial flagellin</td>
</tr>
<tr>
<td>TLR6&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Diacyl lipopeptides, lipoteichoic acid, zymosan</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded viral RNA</td>
</tr>
<tr>
<td>TLR8</td>
<td>Single-stranded viral RNA</td>
</tr>
<tr>
<td>TLR9</td>
<td>Unmethylated CpG DNA from bacteria and viruses</td>
</tr>
<tr>
<td>TLR10</td>
<td>Not determined</td>
</tr>
<tr>
<td>TLR11</td>
<td>Uropathogenic bacteria</td>
</tr>
</tbody>
</table>

<sup>1</sup>Reviewed by Akira and Takeda 2004<sup>11</sup>

<sup>2</sup>Cooperate with TLR2

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**Figure 1.** Simplified signaling pathway for TLRs in mammals (Modified from Akira and Takeda 2004<sup>11</sup>)

- **MyD88** - Myeloid differentiation primary-response protein 88
- **IRAK** - IL-1R-associated kinase
- **TRAF6** - TNF receptor-associated factor 6
- **IκB** - inhibitor of nuclear factor-κB kinase
- **NF-κB** - Nuclear factor κB

**Cell membrane**

**Cytoplasm**

**Nucleus**

Transcription resulting in a pro-inflammatory response
being important for maturation of the neonatal immune system, and low levels of sCD14 in serum,\textsuperscript{28} amniotic fluid and breast milk\textsuperscript{29} have been proposed to be associated with increased susceptibility to allergic disease. Further, polymorphisms in the promoter region of the CD14 gene have been associated with increased total serum IgE levels and decreased levels of sCD14 in allergic individuals.\textsuperscript{30} However, although several groups have investigated this further,\textsuperscript{31-34} the results are contradictory and therefore a lot remains to be clarified.

1.4 IMMUNE CELLS AND IMMUNE MEDIATORS

1.4.1 Monocytes and macrophages

Monocytes circulate in the blood and differentiate into macrophages upon migration into the tissue. The two cell types share many characteristics, but the macrophage acquires new functions when it becomes a resting cell in connective tissue. Macrophages express several receptors for bacterial components (mannose and glucan receptors, TLRs, CD14 and scavenger receptors) making these cells an important component of the innate immune system. Macrophages are phagocytes and are widely distributed in tissue. Phagocytosis by macrophages is mediated through receptor recognition and internalization of the pathogen into intracellular vesicles, so called phagosomes. The phagosomes become acidified, killing most pathogens. They can also fuse with lysosomes, creating a phagolysosome containing enzymes, proteins and peptides with antimicrobial activity. Signaling through some of the receptors (e.g. TLRs) induces secretion of pro-inflammatory cytokines. The macrophages are also involved in the effector phase of an adaptive immune response.\textsuperscript{35}

1.4.2 T cells

T cells originate in the bone marrow but their development takes place in the thymus. In the thymus the T cell precursors pass through a series of distinct phases characterized by changes in the T cell receptor (TCR) genes (VDJ recombination), expression of the TCR and cell-surface proteins. The T cells further go through both positive and negative selection and only T cells that recognize self-MHC peptide complexes properly are allowed to leave the thymus as mature T cells. About 98% of all T cells undergo apoptosis within the thymus.\textsuperscript{35}

The αβ T cells develop into either of two subclasses: CD4 or CD8. CD8 T cells, also referred to as cytotoxic T cells, recognize peptide bound to MHC class I molecules on antigen presenting cells (APCs) and kill their target cells by releasing cytotoxic effector molecules or by binding through Fas ligand to Fas on the target cell, inducing apoptosis. CD4 T cells, also named T helper (Th) cells, recognize peptides bound to MHC class II, and their primary function is to help activate other cells (e.g. B cells and macrophages). There are two functional types of Th cells: Th1 and Th2. Their function is determined by the environment surrounding the T cell during early activation (see Fig. 2) as well as by the nature and amount of ligand presented to the T cell by DCs. Low dose of antigen favors Th1 reactivity due to reduced ligation of TCR by MHC-antigen-peptide complexes and subsequently limited phosphorylation of
intracytoplasmic kinases and calcium mobilization, resulting in IL-4 production.\textsuperscript{36,37} \n
T_{H1} cells activate macrophages that are infected with or have ingested a pathogen. T_{H2} cells are specialized at helping B cells; they secrete B cell growth factors (e.g. IL-4, IL-5, IL-9, IL-13) and activate the B cells by CD40-ligand binding to CD40 on the B cell, inducing proliferation and isotype switching. However, there is an intermediate phenotype of T_{H} cells namely the T_{i}0, which produces cytokines belonging to both T_{H1} and T_{H2}.\textsuperscript{35} 

During the past few years the CD4 regulatory/suppressor T cells (T_{H}3, T_{H}1, T_{R}, and natural killer (NK) T cells) which suppress the function of other cells by cell to cell contact and/or by secreting cytokines like IL-10 and/or TGF-β have attracted a lot of interest not only in the context of autoimmune disorders, but in allergy research as well. An impaired regulation of the regulatory T cells caused by decreased exposure to microbial agents offers a possible explanation for the increase in allergy frequency\textsuperscript{38} (see the hygiene hypothesis.)

Figure 2. The development of T_{H1} and T_{H2} cells is dependent on the nature and amount of antigen presented by DCs in combination with the cytokine environment.
1.4.3 Soluble mediators

Cytokines are effector molecules secreted by cells to communicate with each other. They act via cytokine receptors on their target cells, and this could modulate the type of immune response elicited. Some cytokines act as chemokines, attracting cells that express chemokine receptors (such as macrophages and neutrophils) from the blood stream to the site of inflammation. Cytokines can also act in an autocrine manner on the producing cell itself, e.g. increase proliferation. Cytokines are mainly divided into TH1 cytokines such as IFN-γ among others and TH2 cytokines such as IL-4, IL-5 and IL-13. TH1 and TH2 cytokines cross-regulate each other: IL-4 inhibits the production of IFN-γ and IFN-γ inhibits TH2 cytokine production. IFN-γ can also be produced by NK cells36 and IL-4 and IL-13 by mast cells and basophils40-42. Further, the CD4 regulatory/suppressor T cells (TH3, TR1, TR, and natural killer (NK) T cells) suppress the function of other cells by cell to cell contact and/or by secreting cytokines like IL-10 and/or TGF-β.38

In this thesis I have mainly measured the release of pro-inflammatory cytokines and chemokines produced after engagement of PRRs such as CD14, TLR2 and TLR4. These include IL-1β, IL-6, IL-8 and TNFα. I have also measured the cytokine IL-10 which in humans is mainly a regulatory function.

1.4.4 B cells

The B cells originate and develop in the bone marrow. They passes through several developmental stages: pro-B cells, pre-B cells to immature B cells. This development involves the expression and regulation of various surface molecules as well as B cell receptor (BCR) gene rearrangement (VDJ recombination), creating a highly diverse BCR that recognizes certain epitopes on soluble antigen. The immature B cells express IgD as well as IgM on the surface.

Immunoglobulins (Ig) exist in a membrane-bound form as the BCR and in a secreted form created through differential splicing, also referred to as antibodies. The effector function of an antibody is determined by its heavy-chain C-region or Fc part, which can be changed through the process of isotype switching. The isotype classes in humans are: IgM, IgD, IgG (IgG1-IgG3), IgA (IgA1 and IgA2) and IgE. Most of them exist as monomers while IgM and IgA can exist in multimeric forms. Isotype switching is directed by cytokines, especially from armed effector CD4 TH1 cells expressing CD40 ligands.35

1.5 Allergic diseases

Atopic allergy is a common inflammatory disorder influenced by genetic and environmental factors. The term atopy describes a genetic predisposition to become IgE-sensitized to allergens in the environment. However, the term should only be used when IgE sensitization has been documented. Allergy is a hypersensitivity reaction mediated by IgE antibodies against allergens.43

The prevalence of atopic conditions such as allergic asthma, rhinoconjunctivitis and eczema has increased dramatically in recent decades resulting in a substantial
increase in morbidity, mortality and medical economic burdens. However, recent studies indicate that the prevalence rates are stabilizing. Still, the high prevalence of allergic disease underscores the importance of defining the mechanisms responsible for atopic susceptibility.

1.5.1 IgE

IgE was found to be the effector antibody in allergic reactions in the late 1960s. It is the least abundant antibody class in serum, with a normal concentration of 50-200 ng/ml in non-allergic individuals. In certain parasitic diseases and in allergic adults the IgE concentrations can reach three times higher values. Most of the IgE is bound to its high affinity receptor (FceRI) present on mast cells, basophils and activated eosinophils. Mast cells continuously express FceRI, while the low affinity IgE receptor CD23 (FceRII) is expressed on several cell types including B cells activated T cells, monocytes, eosinophils, platelets, follicular dendritic cells and some thymic epithelial cells. CD23 is involved in the regulation of IgE levels, but studies in mice showed that it is also involved in enhancing the IgE response by stimulating proliferation of IgE-committed plasmablasts.

Helminth infections are associated with responses stimulated by T\textsubscript{h}2 type cytokines, high IgE levels, eosinophilia and mastocytosis. The normal physiological role for IgE seems to be in defense against parasitic infections. Experiments \textit{in vitro} have shown that eosinophils can kill IgE-coated \textit{S. mansoni} by binding via their FceRI and killing the parasite through exocytosis. It has been assumed that the low prevalence of parasitic infections in the industrialized world may account for the high prevalence of allergic diseases.

Isotype switching to IgE is dependent on secretion of T\textsubscript{h}2 cytokines like IL-4 and IL-13 that will induce production of so called e-germ-line transcripts; however, a second signal through CD40, CD40 ligand interaction is required for complete switching. Other cytokines that are known to enhance IgE production include IL-2, IL-5, IL-6, IL-9 and TNF-\textalpha.. Soluble factors known to down regulate IgE production in mice includes IL-18 which induces the T\textsubscript{h}1 cytokine IFN-\gamma in the presence of IL-12 counteracting the T\textsubscript{h}2 response. Further, IL-10 is known to down-regulate IgE production in humans.

1.5.2 Mechanism of an IgE-mediated allergic reaction

Both allergic and non-allergic individuals can mount a response against allergens: the difference lies in the isotype produced. Non-allergic individuals mount an IgG response, whereas allergic individuals have IgE as the predominating isotype. The allergen specific IgE produced by plasma cells with the help of allergen specific T\textsubscript{h}2 cells, in allergic individuals, will be bound to FceRI on mast cells which are located in mucosal and epithelial tissues in the vicinity of small blood vessels. This is also known as the sensitization phase. In a subsequent encounter with the same allergen the allergen will bind to IgE on the mast cells, cross-linking the receptors (FceRI) and triggering mast cell activation. Mast cell degranulation takes only seconds and the preformed
inflammatory mediators stored in the granules will be released. Among the mediators is histamine, which causes immediate increase in local blood flow and vessel permeability, and enzymes such as mast-cell chymase, tryptase and serine esterases. These enzymes activate metalloproteinase which causes tissue destruction. On activation the mast cell also synthesizes and releases chemokines, lipid mediators and cytokines such as IL-4 and IL-13 which perpetuate the T\textsubscript{h}2 response. The production of IL-5, mainly by T\textsubscript{h}2 cells, increases the production of eosinophils in the bone marrow, which are then recruited to the site of the allergic reaction. Upon activation the eosinophils start to express FceRI and participate in the allergic reaction by release of their granule contents, causing tissue injury (Fig. 3).\textsuperscript{50}
1.5.3 Different types of IgE-mediated allergic reactions

The dose and route of allergen uptake determines the type of IgE-mediated allergic reaction. Mast cells are mainly distributed at two different anatomical sites: in vascularized connective tissues, and in submucosal layers of the gut and respiratory tract. In an allergic individual all of these mast cells bind allergen specific IgE on their FceRI receptors. Which mast cell becomes activated depends on the route of allergen administration. Allergen in the blood stream will result in an activation of mast cells in connective tissue throughout the body, causing a systemic release of histamine and other mediators and resulting in life threatening systemic anaphylaxis. Subcutaneous administration of allergen activates connective tissue mast cells resulting in a local response. When the allergen is inhaled and crosses through epithelia the mucosal mast cells are activated, causing smooth muscle contraction in the lower airways, with difficulty in breathing as a result. There will also be an increase in local secretion of mucus by epithelial cells, which will cause irritation. Ingested allergens also activate mucosal mast cells that penetrate across the gut epithelium, causing vomiting and diarrhea due to an increase in the outflow of fluid across the epithelium. Urticaria can also be the result of food allergy, causing hives in the skin when the allergen disseminated in the blood stream reaches the skin.50

1.5.4 Genetics

Studies on atopic families have identified certain chromosomal regions that may be connected to allergic diseases. One candidate gene is on chromosome 11q13 encoding the β subunit of the high affinity IgE receptor (FceRI) and a polymorphism in the β subunit of FceRI has been associated with elevated SPT responses to grass and house dust mite.58 On chromosome 5q31-34 there is a cluster of genes encoding the Th2 promoting cytokines IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF)50,59; and the gene for CD14 is also located in this chromosomal region (reviewed in61). Further, polymorphisms in the promoter region of the IL-426,61 and IL-1362 have been associated with higher IgE levels in atopic individuals. Other candidate genes that have been proposed to be important in the development of allergic disease are those encoding HLA class II and TNF-α on chromosome 6, IFN-γ and mast cell growth factor (MGF-1) among others on chromosome 12q, the gene for TCRα on chromosome 14 and the gene for IL-4R on chromosome 16.50 Recently, four novel genes for asthma susceptibility were identified: DPP10 (dipeptidyl peptidase 10) on chromosome 2q14,63 GPRA (G-protein-coupled receptor for asthma susceptibility) on chromosome 7p14,64 PHF11 (plant homeodomain finger protein 11) on chromosome 13q14,65 and ADAM33 (a disintegrin and metalloproteinase 33) on chromosome 20p.66 However, recent genome-wide screens, based primarily upon clinically defined phenotypes, confirm that atopy does not conform to a simple one-gene one-disease model of inheritance.67
1.5.5 Risk and protective factors for allergy

Environmental pollution\textsuperscript{68} has been proposed as one explanation for the increase in allergy prevalence. However, since highly polluted areas in Eastern Europe have a lower frequency of allergic individuals this is unlikely. Tobacco smoke can also be regarded as a kind of environmental pollution. Today it is generally believed that pre- and post-natal exposure to tobacco smoke adversely affects pulmonary function, and predisposes to asthma symptoms and possibly bronchial hyper-responsiveness in childhood but plays little or no role in allergy development.\textsuperscript{69} Severe respiratory syncytial virus (RSV) infection in infancy also increases the risk of asthma, but in contrast to pollution an association with increased sensitization to inhalant allergens has been observed,\textsuperscript{70} although this could be explained by a genetic predisposition, since an atopic genotype may increase the susceptibility to RSV infections as well.

Breast feeding has long been considered to protect against allergy development. Sears \textit{et al}\textsuperscript{71} reported in a long-term study from New Zealand that breast feeding does not protect against allergy development and may even increase the risk. However, other studies that report beneficial effects of breast feeding on allergic disease indicate that both the duration\textsuperscript{72,73} and exclusivity\textsuperscript{74} are of importance. Other studies have shown that there are additional benefits of breast feeding for neural development\textsuperscript{75} and against chronic diseases such as overweight\textsuperscript{76} and hypertension.\textsuperscript{77} The general view today seems to be that breastfeeding should be recommended both to protect against allergy development and for all its other beneficial effects (reviewed in\textsuperscript{78}).

An other environmental factor is that the diet has changed in the industrialized world\textsuperscript{79} and we consume more omega-6 fatty acids and less of the omega-3 fatty acids found in fish oil.\textsuperscript{80} Omega-6 fatty acids are involved in the synthesis of arachidonic acids, e.g. prostaglandins. This could affect the monocytes and/or lymphocytes and promote allergic disease.\textsuperscript{81}

1.5.5.1 The hygiene hypothesis

A theory that has attracted a lot of interest lately is the hygiene hypothesis.\textsuperscript{16,17,82,83} The initial interpretation of the hygiene hypothesis was that exposure to specific infections during early life drives the maturation of the immune system towards the Th1 phenotype and away from the Th2 phenotype associated with allergic disease.\textsuperscript{84} Today it is believed that not only infections but also an early exposure to non-pathogenic microbes in our environment is involved in development of the immune system.

Lately, interest has focused on the CD4 regulatory/suppressor T cells (Th3, TR1, Tr, and natural killer (NK) T cells) which suppress the function of other cells by cell to cell contact and/or by secreting cytokines like IL-10 and/or TGF-β.\textsuperscript{85} An impaired regulation of the regulatory T cells caused by decreased exposure to microbial agents offers an alternative explanation for the increase in allergy frequency.\textsuperscript{85}

A newborn child is believed to have a Th2 skewed immune system (reviewed in\textsuperscript{85}), which matures over time by encountering pathogenic and non-pathogenic microbes in our environment, balancing the child’s Th1 and Th2 immune responses. Having older siblings\textsuperscript{86-89} and attending day care centers\textsuperscript{90,91} have been shown to have
a protective effect against allergy development. The probable mechanism may be a
greater exposure to infections and non-pathogenic microbes brought home by the older
children or by interacting with other children at the day care centers. Higher IL-4, IL-5,
and IL-13 levels at six and twelve months of age have been observed in high-risk
children who developed allergic disease. The allergic children also had lower IL-10
levels compared to non-allergic children, which might aid in the development of
allergic disease by a decreased down regulation of immune responses.\textsuperscript{91} Further,
wheezing in the first year of life has been associated with skewing towards a T_{H}2
profile with an increased serum IL-10/IL-12 ratio.\textsuperscript{92} van der Velden \textit{et al.}\textsuperscript{91} also
proposed that the first six months of life are critical for the initiation of immunological
changes that might result in allergic disease. Indeed, in children with high risk of
developing allergic disease it might be even more important to achieve an early
maturation of the immune system towards a T_{H}1 phenotype. However, in a recent study
by Holt \textit{et al}\textsuperscript{93} they show that atopy to inhalant allergens is associated with a mixed
T_{H}1/T_{H}2 immune response in children at the age of 7-13 years. Previous studies have
demonstrated that an attenuated production of IFN-γ at birth is associated with an
increased risk for atopic sensitization,\textsuperscript{94} whereas children sensitized later in childhood
have mixed T_{H}1/T_{H}2 responses.\textsuperscript{95-97}

Further, allergen exposure early in life was long considered a risk factor for
allergy development, but studies have now indicated that a cat or a dog may even
protect against allergy development.\textsuperscript{98,99} Exposure to allergens early in life may
enhance the induction of tolerance and protect against allergy development later in
life.\textsuperscript{100-102} However, having a pet may also expose the child to more microbial agents
and fit with the hygiene hypothesis. Indeed, growing up on a farm, where one is
constantly exposed to the microbial products present in an environment with livestock,
have been shown to be associated with protection against allergy development later in
life.\textsuperscript{103} However, Eder \textit{et al}\textsuperscript{104} demonstrated that genetic variations in the TLR2 gene are
important for the susceptibility of farmers’ children to allergic disease. There have been
speculations regarding the possibility that the ratio between exposure to allergens and
to microbial products like endotoxin is more important than their individual amounts.
This is in agreement with the studies of farmers’ children since they are heavily
exposed to both endotoxin and house dust mite (reviewed in\textsuperscript{104}).

Further, a study by Alm \textit{et al}\textsuperscript{105} has shown that factors connected to an
anthroposophic life style, e.g. a diet including vegetables spontaneously fermented by
lactobacilli, restrictive use of antibiotics, anti-pyretics and vaccinations, are associated
with reduced allergy prevalence among school children. However, they could not
distinguish a single factor responsible for this protective effect. Further, it has now been
more or less concluded that BCG and other vaccinations neither increases the risk of
nor give a protection against allergic diseases.\textsuperscript{106,107} It has been postulated that the
normal intestinal microflora is the principal environmental signal for postnatal
maturation of T cell (T_{H}1 cells) function, and that this effect is mediated through TLRs
expressed by innate immune cells.\textsuperscript{108} Studies made regarding the significance of the
intestinal microflora have observed higher counts of \textit{Staphylococcus aureus} and lower
counts of \textit{Bifidobacteria} in allergic 2-year-olds (reviewed in\textsuperscript{109}) indicating important
differences between allergic and non-allergic children’s intestinal microflora, with potential implications for maturation of neonatal immune responses.

1.5.5.2 Epigenetic inheritance of fetal genes in allergy

Epigenetics denotes the chain of processes linking genotype and phenotype, other than the initial gene action. Bousquet et al.\textsuperscript{110} recently proposed that many of the genes involved in IgE synthesis and airway remodeling in asthma are persistent fetal genes that have not been silenced during late pregnancy or early life. Newborn babies are believed to have a Th2 skewed immune system mirroring the milieu \textit{in utero} (though this may be an oversimplification see the section “The Th1/Th2 paradigm of pregnancy”). In some of the infants who later will develop allergies, the Th2-mediated cytokine response seems to be preserved in infancy.\textsuperscript{111,112} This might indicate that the fetal stage persists in the children who develop allergic disease due to a defect in Th2 dampening mechanisms. Further, several features of the developing lung are present in allergic asthma. Allergen exposure induces embryologic features such as enhanced expression of tenascin\textsuperscript{113} and the development of myofibroblasts.\textsuperscript{114} Bousquet \textit{et al} also propose that in atopic asthma, some genes expressed in the fetus may not be repressed prenatally or in early infancy under the influence of the environment. Some experiments suggest that endotoxin has a role in gene silencing, since LPS is a potent inflammagen which binds to histones\textsuperscript{115,116} and this would indeed fit with the hygiene hypothesis (see “Hygiene hypothesis”).\textsuperscript{14,103,117}

1.6 IMMUNOLOGY OF PREGNANCY

1.6.1 The placenta

Pregnancy is an immunologically interesting state, where the mother carries the fetus—essentially a non-self invader—for an extended period. The barrier between the two entities is the placenta. The fetal part of the placenta consists of the two membranes, amnion and chorion, the umbilical cord and the chorionic villi covered by the trophoblast. The maternal part consists of the decidua and the intervillous space (Fig. 4). Implantation in humans occurs in the uterus between postovulatory days 5 and 7 of a normal 28-day menstrual cycle. First the blastocyst attaches to the endometrium after which the trophoblasts start to invade the uterine tissue and maternal spiral arteries. This results in decidualization and vascular remodeling and interaction between the trophoblast and the maternal immune system. Further, there seems to be a “cross talk” between the fetus and the reproductive tissue, mediated by secreted molecules.\textsuperscript{118}

Maternal blood is brought by uterine arteries to placenta and the endothelial walls of these vessels end at the intervillous space. Each villus is supported with blood from fetal umbilical arteries and is connected through a capillary network to the umbilical veins that return the blood to the fetus. During the third trimester only two placental cell layers separate the maternal and fetal circulation: the syncytiotrophoblast and the endothelium of the fetal blood vessels (reviewed in\textsuperscript{119}).
1.6.2 Cells in the decidua

In the decidua there are numerous natural killer (NK) cells specific to the uterus, the so-called uterine NK (uNK) cells with NK cell-like functions. These uNK cells increase markedly during early pregnancy and comprise about 70% of the leukocytes in decidua. The uNK cell expresses inhibitory receptors that bind to HLA-G on extra-villous cytotrophoblast and non-villous cytotrophoblast and HLA-G has been proposed to confer resistance to uNK cell lysis. The uNK cells have been suggested to be important for protection against infection or in the regulation of immunity, but they may also be important for implantation and placentation. However, there seems to be a fine balance between the number of uNK cells that are beneficial for pregnancy and the number that is deleterious, since an increased number of uNK cells has been associated with recurrent spontaneous abortion (reviewed in\textsuperscript{126}).

![Figure 4. Schematic picture of the placenta. The placenta consists of a fetal and a maternal part. The fetal part consists of the umbilical cord, the fetal membranes: amnion and chorion and the chorion villi. The maternal part consists of the decidua and the intervillous space.](image)
Decidual T cells are in close contact with the trophoblast cells, but they do not kill them because the villous syncytiotrophoblast cells lacks MHC class II antigen. Further, the T lymphocytes are present in a small number in the decidua and endometrium during early pregnancy. Decidual T cells as cytokine producers are considered important for a successful pregnancy. Macrophages are present in the decidua and it has been suggested that their number may be affected by estrogens, since these hormones stimulate the influx of leukocytes. However, there must be a tight regulation since an excess of macrophages in the pre-implantation myometrium has been associated with recurrent miscarriage. The macrophages are also believed to be important in the non-specific host defence in the placenta and also as phagocytes to remove tissue debris associated with trophoblast invasion. Further, the macrophages have a function in placentation by producing immunosuppressive prostaglandins.

1.6.3 Trophoblast cells

The trophoblast cells are the most important fetal cells that are in close contact with maternal cells (reviewed in). There are different populations of trophoblasts cells; the first population is the villous cytotrophoblast which is an actively dividing trophoblast cell that remains in the villi. The second population covers the villous cytotrophoblast and is called syncytiotrophoblast. It is bathed in maternal blood. The third trophoblast population is the non-villous cytotrophoblast which is a proliferating precursor trophoblast that migrates into the decidua and myometrium. Early in pregnancy the cytotrophoblast forms a continuous layer between the syncytiotrophoblast and the stroma of chorion villi, but it will later be discontinuous as the chorion villi expand. The surface of the total trophoblast cells is about 15 m² during the 20th week of human pregnancy (reviewed in).

The trophoblast has several mechanisms for protecting itself against attack by maternal immune cells. The extra-villous cytotrophoblast and non-villous cytotrophoblast strongly express the HLA-Ib molecule HLA-G, but no expression has been found on villous cyto- or syncytiotrophoblast. HLA-C and HLA-E has also been shown to be expressed by the non-villous trophoblast. The two apoptosis inducing pathways Fas Fas-ligand and TRAIL seem to be functioning in the placenta. Both Fas-ligand and TRAIL are expressed on syncytiotrophoblast cells which are in direct contact with maternal peripheral blood cells. Further, villous syncytiotrophoblast cells express indoleamine diogenase (IDO), which is an enzyme active in the catabolism of tryptophan and indirectly suppresses maternal T cell activity by tryptophan deprivation.

1.6.4 Hofbauer cells

The core of the chorionic villi is filled with stromal cells and numerous fetal tissue macrophages called Hofbauer cells. The Hofbauer cells may have different origins throughout gestation. It has been proposed that they originate from stromal cells of the villous core but later also from fetal bone-marrow derived monocytes.
Since the placenta lacks a lymphatic system, Hofbauer cells have been considered to be involved in the reduction of fetal serum proteins in the villous stroma and in the fluid balance of early pregnancy. Indeed, stromal channels that may function as a substitute for a lymphatic system have been detected in placenta and would allow the Hofbauer cells to move around and perform functions such as host defense and remodeling of the villous core. Further, Hofbauer cells have been postulated to exert a protective effect by binding maternal antifetal-antibody complexes. They express FcRI, FcRII, FcRIII which bind immune complexes and are involved in the clearance of immune complexes by phagocytosis, favoring clearance of complexes containing the subclasses IgG1 or IgG3.

1.6.5 Maternal peripheral immune responses

During pregnancy the mother’s white blood cell count increases as does the expression of activation-associated adhesion molecules on granulocytes and monocytes. The innate immune cells are also functionally activated during pregnancy, and taken together all these changes resemble what can be seen in patients with sepsis.

Various mechanisms can be responsible for these changes. Pregnancy hormones have been suggested to be responsible for the activation of innate immunity. Estrogen and progesterone may increase monocyte cytokine production and it has also been suggested that the placenta is involved in this activation. Several soluble placental products released into the maternal circulation can activate monocytes and the elimination of fetal cells or particles in the maternal circulation by phagocytes may also result in their activation. However, the number of peripheral NK cells is decreased in pregnant women compared to non-pregnant women and the cells also produce less IFN-γ. The NK cells in pregnant women are embryotoxic and it has been shown in an in vitro fertilization population that no live infants were born if the maternal peripheral NK cells constituted more than 18% of the peripheral blood lymphocytes. Further, a history of recurrent spontaneous abortions has been associated with embryotoxic NK cells and T cells in vitro.

Since there seems to be no general suppression of maternal peripheral immune responses during pregnancy the suppression of lymphocyte function (cell-mediated immunology) might be compensated by an activation of the innate immune system. This might be crucial to ensure the mother’s immune integrity (reviewed in).

1.6.6 The T_{H}1/T_{H}2 paradigm in pregnancy

Wegman was the first to propose the concept of pregnancy as a T_{H}2 phenomenon. The general view has been that T_{H}2 cytokines promote successful pregnancy, while T_{H}1 cytokines (e.g. IFN-γ and TNF-α) are deleterious for pregnancy (reviewed in). A shift towards a T_{H}1 cytokine profile has in some studies been associated with recurrent spontaneous abortions, while other results do not fit at all with this hypothesis.
Today there is evidence that implantation is an “inflammation-like process” associated with increased expression of adhesion molecules (integrins and adhesins) that are important if trophoectoderm cells are to adhere to the uterine stroma, and of a series of enzymes that are important for the invasion stage.\textsuperscript{155,157} Further, there is no report of implantation defects in T_{H2} KO mice whereas T_{H1} cytokines seem to be more important.\textsuperscript{158,159} IL-1, for example, seems to have an important role in implantation. Human pre-implantation embryos secrete IL-1β, inducing other cytokines (leukemia inhibitory factor (LIF), IL-1 etc). Binding of IL-1 to IL-1 receptor present in the luminal epithelium \textit{in vitro} induces increased secretion of prostaglandin E\textsubscript{2} and LIF from the endometrium.\textsuperscript{160,161} and indeed, LIF has been shown to be crucial for implantation.\textsuperscript{159,162-164} It has recently been shown that uNK cells and the cytokines IL-12, IL-15, IL-18 and IFN-γ are important for local angiogenesis and tissue remodelling and subsequently for implantation and pregnancy success in mice.\textsuperscript{165-167} However, although IL-12 and IL-18 are needed for a successful pregnancy, excessive levels of one or both may lead to spontaneous abortion or preeclamptic syndrome.\textsuperscript{168}

Further, there is also evidence of a decreased ratio of T_{H1}/T_{H2} cytokine production by peripheral T lymphocytes especially in the third trimester of human pregnancy.\textsuperscript{148,169,171} This might either be due to a decreased production of T_{H1} cytokines\textsuperscript{148,171} or an increase in T_{H2} cytokine production.\textsuperscript{169} Further, it has been shown that cultured third trimester decidua lymphocytes spontaneously produce less T_{H1} cytokines and—after mitogen stimulation—more T_{H2} cytokines than peripheral blood lymphocytes.\textsuperscript{172} A constitutive expression of both IL-4 and IL-4 receptor in human term placenta has also been reported, suggesting an immunobiological role for IL-4 in pregnancy.\textsuperscript{173} However, comparison of IL-6 production in early and late gestation showed that near the time of parturition (elective caesarian sections) the levels of IL-6 mRNA in placenta were four fold higher at term compared to first trimester\textsuperscript{174} suggesting that IL-6 is important in induction of labor.

Taken together, this suggests that pregnancy might not be solely a T_{H2} phenomenon, but that different cytokines belonging to the T_{H1} and T_{H2} group may have important functions during different stages of pregnancy and in different local sites in the placenta during pregnancy. The scenario could be that implantation is a T_{H1} phenomenon while the continuation of the pregnancy through second and third trimester is more T_{H2} biased. However, near parturition T_{H1} cytokines seem to have a role in inducing labor.

\subsection*{1.6.7 The in utero environment in relation to allergy development}

Allergies often begin early in life, indicating that the child is subjected to atopic influence already at the fetal level. The human fetus has the potential to produce IgE as early as week 11 of pregnancy, as demonstrated by culturing of fetal tissues.\textsuperscript{175} Moreover, evidence for endogenous IgE production at 20 weeks of gestation was also provided by RT-PCR and by the presence of sterile Ig transcripts in fetal samples, including the spleen, as early as at 8 weeks of gestation, indicating that the fetus contains B cells primed and ready for IgE class switching already at the beginning of pregnancy.\textsuperscript{176} Indeed it has been demonstrated that the fetus is capable of mounting antigen-specific IgE responses both before birth and after birth. It has been demonstrated that cord blood B cells are mature in their capacity to switch to IgE-
producing cells in response to interleukin-4 in vitro and allergen specific reactivity at birth to a range of common allergens, both dietary and inhalant allergens, further supports the theory of atopic influence already in utero. However, no studies had previously been performed on the presence of IgE in the placenta from allergic and non-allergic mothers. The risk of allergy development has been shown to be greater among children with an allergic mother than with an allergic father, suggesting that the pregnancy might be important in determining disease development maybe through fetal programming. However, this could be explained by genetic factors as well.

The fetus is probably exposed to low doses of allergen and it has been shown that low antigen doses favor a T<sub>H</sub>2 immune response (Fig. 4). The cytokine environment in which antigen presentation takes place might also influence whether a T<sub>H</sub>1 or a T<sub>H</sub>2 immune response will develop. Both IL-4 and PGE<sub>2</sub> favor T<sub>H</sub>2 reactivity and IgE class switching and both are expressed in utero. There is a general skewing towards T<sub>H</sub>2 reactivity at birth, but the response is not enhanced in children who later develop allergic disease; instead the response is dampened. Down regulation of immunological functions such as opsonization and also of IgA levels has been seen in children with a family history of allergic disease. This could be due to either down regulation or delayed maturation.

Transport of maternal IgG across the placental syncytiotrophoblast is believed to be dependent on the neonatal Fc receptor (FcRn). Early in gestation only small amounts of IgG cross the placenta, probably due to an intact cytrophoblast cell layer, but the levels increase rapidly from 20 weeks of gestation and becomes maximal at 32 weeks until birth. Since allergen reactivity can be demonstrated as early as at 22 weeks of gestation it is unlikely that allergen exposure occurs via IgG complexes crossing the placenta. However, the nutritive allergens ovalbumin and β-lactoglobulin have been shown to be released from placental tissue in an in vitro placental perfusion model indicating placental allergen transfer. Other pathways suggested for fetal allergen exposure are through the fetal membranes, which are in close contact with the maternal decidua (reviewed in) and decidually derived prolactin has been detected in amniotic fluid. IgE can be detected in amniotic fluid at 16-17 weeks of gestation as well as at term and the levels can be correlated to maternal IgE levels. This suggests that IgE passes from the vascularized decidual to the amniotic fluid. Further, the fetal gastrointestinal tract is exposed to amniotic fluid by fetal swallowing and to some degree also the fetal lungs are exposed due to fetal respiratory movements. Peyer’s patches are structurally mature in the fetus at 19 weeks of gestation and T cells and CD83+ DCs have been detected in this tissue already at 13 and 17 weeks of gestation respectively. Further, CD45 RO+/RA- “memory cells” are abundant in fetal blood and spleen at 14-21 weeks of gestation, although they are seldom detected in neonatal blood and spleen. CD40L expression induced by phytohaemagglutinin (PHA) stimulation at 21 weeks of gestation is similar to the expression in adults, though this up regulation declines towards term. This could suggest that there is a time during fetal life where the fetus is especially sensitive to initiation of antigen-specific reactivity. Indeed, the fetus is mature enough to be able to survive outside the womb at approximately 24 weeks of
gestation. An intense activity of the immune system to achieve this maturation might precede this time point and explain this up regulation in immunological markers and number of immune cells.
THE AIMS OF THE THESIS

The overall aim of this thesis was to investigate the influence of maternal allergy on the \emph{in utero} environment and on innate immune functions of the newborn baby. The more specific aims were to investigate:

**I:** The presence and production of IgE in placenta from allergic and non-allergic women.

**II:** The presence and regulation of TLR2 and TLR4 in the human placenta.

**III:** How cord blood mononuclear blood cells (CBMC) from children of allergic and non-allergic women respond to microbial stimuli.

**IV:** The levels of soluble (s) and membrane (m) bound CD14 in cord blood and at two years of age from children with allergic or non-allergic mothers and relate these parameters with allergy development at two years of age.
3 METHODOLOGY

Methods used for paper I-IV are described in detail in the respective “Material and methods” sections. The following methods were used in this thesis:

- Clinical evaluation (paper I and IV)
- Phadiatop (paper I-III)
- Skin prick test (SPT) (paper I and IV)
- Questionnaire (paper I-IV)
- Processing and immunohistochemical staining of placental tissue (paper I and II)
- Double immunofluorescence staining (paper I)
- In situ hybridization (paper I)
- Tissue explant processing and stimulation (paper II)
- ELISA (paper I-IV)
- RNA preparation and real time RT-PCR (paper II)
- Separation of CBMC and PBMC (paper III and IV)
- In vitro activation of blood cells (paper III)
- Cytometric Bead Array for cytokine and chemokine analysis (paper III)
- Flow cytometric analysis (paper III and IV)
- Statistical analysis (paper I, III and IV)

The studies were approved by the Ethics Committees of the Karolinska University Hospital and Stockholm Söder Hospital, Stockholm, Sweden. All families gave their informed consent.
4 RESULTS AND DISCUSSION

4.1 HIGH EXPRESSION OF IgE IN THE HUMAN PLACENTA IRRESPECTIVE OF MATERNAL ALLERGY (PAPER I)

Several different findings regarding the in utero environment indicate that there could be a maternal influence on this milieu. IgE is present in amniotic fluid at 16-17 weeks of gestation and at term, and the levels have been observed to correlate with maternal total serum IgE levels. In relation to this finding, it is interesting to note that the placenta has the capacity to bind IgE through mast cells. Together with reports on a maternal influence on the fetus in utero, the observation of allergen specific reactivity in the fetal circulation already at 22 weeks of gestation as well as at birth indicates that the fetus is subject to a maternal influence which could promote allergic disease already in utero. However, at the time of our study nothing was known regarding IgE in the placenta in relation to maternal allergy. This prompted us to investigate the presence and production of IgE in placentas from allergic and non-allergic women.

Interestingly, we observed numerous IgE+ cells in the placenta primarily located in the fetal villous stroma (Fig. 5) and sparse, scattered IgE+ cells in the vessel walls as well as in the mesenchyme of the umbilical cord. We verified the presence of IgE+ cells using five different mouse monoclonal antibodies (mAbs), all of which gave a similar staining patterns and no immunoreactivity was observed with an isotype matched negative control. IgE+ cells were detected in all 86 placentas but differed in amount from a few positive cells to many positive cells. Interestingly, no statistically significant difference (p= 0.7) between the allergic and non-allergic groups was found and no correlation between the amount of IgE+ cells in the placenta and maternal or fetal total serum IgE levels was seen. Our finding of IgE+ cells primarily in the fetal villous stroma is not in agreement with a previous study by Maeno et al who detected IgE immune complexes on the syncytiotrophoblast cells and in fetal blood vessels in placenta from women infected with Plasmodium falciparum. The amount of deposition of IgE in the placenta was inversely correlated with the degree of parasitemia at that site. They used a rabbit anti human IgE serum, which had not been absorbed against human serum and as a negative control they used normal rabbit serum. There might be differences in antibody (Ab) specificity between our five mouse mAbs and the rabbit serum used by this group. However, our own experience shows that when one stains placental tissue with rabbit antiserum that has not been absorbed against human serum, background staining is quite high. This could make the results of Maeno et al difficult to interpret.

To investigate whether the placental IgE was bound to IgE receptors, we stained placental slices for high (FcεRI) and low (CD23, FcεRII) affinity IgE receptors after eluting away IgE to free the receptors for detection. No FcεRI+ cells and only scattered CD23+ cells were identified, which speaks against IgE receptor binding. However, the IgE+ cells proved to be placental macrophages, so called Hofbauer cells, as shown by morphology and double staining with IgE and macrophage markers. The placenta-specific tissue macrophages express several known Fcγ-receptors (e.g. FcRI, FcRII and FcRIII), the neonatal Fc receptor (FcRn) and other proteins known to bind immunoglobulins (reviewed in). It has also been demonstrated in mice that IgE can bind to FcγR II and III, although at low affinity. However, since the FcγRs are involved in the clearance of immune complexes deposited in the placenta, the fact that
we observed the same staining pattern for FcγRI as for IgE, indicates that IgE is bound to fetal cells via IgG/IgE immune complexes. This theory is supported by the fact that we were able to detect the presence of IgE-IgG immune complexes in cord blood (our unpublished observations). This could be a mechanism to protect the fetus from the potential hazards of allergen-specific IgE. Actually, high IgG anti-IgE serum levels at birth have been reported to be associated with reduced allergy prevalence.207

Figure 5. IgE⁺ cells in the fetal part of the placenta in the chorionic villi mesenchyme.

The finding of numerous IgE⁺ cells in the placenta made us ask questions regarding the origin of this IgE. The placental barrier is widely believed to prevent all immunoglobulin isotypes except IgG from being transported from the mother to the fetus.208 On the other hand, human IgE administered to pregnant animals has been reported to pass from mother to fetus at a fractional rate similar to that of albumin.209 This indicates that IgE might possibly be transported across the placental barrier, although not as readily as IgG. Interestingly, it is known that FcRn transports immune complexes to the gut in neonatal rats210,211 and might therefore also deliver maternal immune complexes into the stroma in human placentas. A potential risk from postpartum leakage of IgE from mother to child in our study is not considered as important, since we collected placental biopsies immediately following caesarian sections as well as up to 48 h after normal vaginal deliveries and could not correlate the amount of IgE⁺ cells in tissue biopsies with the time period between birth and freezing of the biopsies.

The IgE⁺ cells in our study were located behind the trophoblast barrier, indicating a fetal origin of these cells. In an attempt to trace the origin of the IgE detected in the placenta we stained placental sections for plasma cells (CD38⁺). CD38⁺ cells were present in placenta, although at low frequency. The cells were scattered throughout the villous stroma and located in fetal vessels inside the villi but were also present in the
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maternal part of the placenta. However, no double positive CD38/IgE cells could be detected with double immunofluorescence suggesting that the IgE detected in placenta is produced elsewhere. To further investigate the possibility of placental IgE production, we performed in situ hybridization to detect epsilon transcripts, as an indication of local IgE production. Scattered cells containing epsilon transcripts could be observed in placenta but these cells were primarily located in the decidua and in the inter-villous space, indicating a maternal origin of the cells. Therefore, if the IgE is of fetal origin it is not produced locally in the placenta; instead, placental IgE probably originates from the fetal circulation. Cord blood IgE levels were generally very low, and this might speak against this theory, but a discrepancy between IgE detected locally in tissue and serum has been described previously.\(^{112}\)

The number of IgE\(^+\) cells in the placenta might also differ with gestational age. Recently, we observed that IgE expression in the placenta is higher during second trimester compared to third trimester (Rindsjø et al to be published). If the IgE is of fetal origin this might reflect a maturation time point in the fetus during second trimester. Indeed, the fetus is mature enough to be able to survive outside the womb at approximately 24 weeks of gestation. The up regulation of IgE during the second trimester might be explained by intense activation of the immune system in order to achieve this maturation.

Several explanations for the presence of fetal IgE in the placenta can be proposed. However, since the amount of placental IgE was independent of maternal allergy and a correlation between the placental IgE and allergy development at two years of age in these children could not be found (Sverremark Ekström personal communication), the placental IgE is probably not connected to allergy. The high expression of IgE in the placenta could be a reflection of the Th2 dominance associated with pregnancy and could consequently be a marker of the baby's response to Th2 type cytokine production.\(^{118}\) It is, however, intriguing to speculate that the finding of so many IgE\(^+\) cells in the fetal part of the placenta in allergic as well as non-allergic women may indicate that the IgE is of some immunological importance to the fetus during its intrauterine life.

4.2 TOLL-LIKE RECEPTOR-2 AND TOLL-LIKE RECEPTOR-4 IN THE HUMAN PLACENTA (PAPER II)

The placenta, and in particular the trophoblast, could play an important role in the innate immune system. Defensin transcripts have been detected in placenta,\(^{213}\) which indicates that it has the capacity to battle an infection. Further, it has been demonstrated in mice that the trophoblast cells are involved in the immune response against placental infection with Listeria monocytogenes.\(^{214}\)

When we performed this study TLRs had mainly been studied in animal models and cell cultures. A few groups had started to investigate TLR protein expression in tissues like intestinal epithelium\(^{215}\) and in human bladder urothelium,\(^{216}\) and those
observations indicated that mucosal linings had the capacity to respond to microorganisms through TLRs. mRNA for TLR1-TLR10 had been reported to be present in the human placenta\textsuperscript{217-219} and the ontogeny of TLR2 and TLR4 mRNA in murine placenta as well as in fetal murine lung and liver had been studied\textsuperscript{220}. The distribution and function of TLR2 and TLR4 in human placenta during pregnancy and their importance for the fetus was still unknown. We were therefore interested in investigating the cellular distribution and expression of TLR2 and TLR4 in the human placenta, two receptors that might have crucial functions in the battle against feto-placental infection.

When we examined slices from normal full term placentas with antibodies against TLR2 and TLR4, the receptors were found to be highly and continuously expressed on trophoblast cells covering the peripheral chorionic villi, while no immunoreactive cells were present in the villous core (Fig. 6). Intermediate trophoblast cells in free cell islands, the cell columns and the decidua were found to express both TLR2 and TLR4. The trophoblast cells had a characteristic homogenous cytoplasmic staining and the 28 placentas investigated showed no striking differences in the amount and distribution of TLR2\textsuperscript{+} and TLR4\textsuperscript{+} cells.

![Figure 6. TLR2\textsuperscript{+} and TLR4\textsuperscript{+} trophoblast cells in human term placenta.](image)

Recently a group in Japan (Kumazaki et al\textsuperscript{221}) published a paper describing the distribution of TLR4 in term and preterm human placenta. Their findings did not agree with ours in that they found no TLR4 expression on villous trophoblast cells, but rather on placental macrophages (Hofbauer cells), extravillous and intermediate trophoblast cells. In their study they used the mouse mAb HTA 125 with the isotype IgG\textsubscript{2a}. We and others\textsuperscript{222} have observed that antibodies with this isotype bind unspecifically to placental macrophages, and consequently concluded that they are not suitable for immunohistochemistry on placental tissue. When we performed immunohistochemistry on placental sections using the same TLR4 mAb as Kumazaki et al we observed the same staining pattern. However, we obtained also the same staining pattern using an
isotype matched IgG₂a negative control Ab. When the mouse mAb HTA 125 was absorbed against human serum, to block unspecific binding before being added to the placental specimens, no positive staining could be detected (our unpublished observations). This control was not included in the Kumazaki study and therefore their results are not reliable.

To assess whether the TLRs expressed by the trophoblast were capable of responding to microbial stimuli, we stimulated fresh placental explants with different concentrations of zymosan and lipopolysaccharide (LPS) or incubated them with medium alone for 2, 4 and 6 h. However, neither stimulation with zymosan, nor with LPS led to a difference in TLR2 or TLR4 mRNA expression (detected by real-time RT PCR), nor protein expression (detected with immunohistochemistry) compared to unstimulated controls. Human trophoblast cells can produce both the proinflammatory cytokine IL-6 and the chemokine IL-8 after stimulation with LPS.223 To ensure that placental explants had retained their capacity to react to stimulation with zymosan and LPS we measured the release of the pro-inflammatory cytokine IL-6 and the chemokine IL-8 in the culture supernatants. LPS and zymosan did induce IL-6 and IL-8 release by placental explants, indicating that they responded to the stimuli. It is possible that the expression of TLR2 and TLR4 is normally up regulated in utero as a defense mechanism that can readily be mobilized to protect the fetus from infections during the pregnancy and labor period. This could also explain why we do not see further up regulation of these receptors following stimulation with LPS or zymosan.

Recently, Abrahams et al224 demonstrated that first trimester trophoblast are able to recognize pathogens through TLR2 and TLR4. They observed expression of TLR2 and TLR4 on human trophoblast cells by using immunohistochemistry, results that agree with our own observation of TLR4 expression in first trimester placenta (our unpublished data). Abrahams et al224 further found distinct trophoblast cell responses upon activation of TLR2 and TLR4. TLR4 promoted production of both pro- and anti-inflammatory cytokines, while TLR2 only promoted the production of IL-6 and IL-8 which was increased, unchanged or down-regulated. Interestingly, ligation of TLR2 induced apoptosis in first trimester trophoblast cells, suggesting a mechanism for a pathogen to promote elevated cell death by acting through TLR2, which is seen in many pregnancy complications.

In the Drosophila embryo a small number of genes are involved in the dorsoventral polarization,225 the signaling pathway centers on Toll.226,227 The high homology between Drosophila Toll and human TLRs might indicate a possible developmental role for these receptors in the mammalian placenta as in the Drosophila embryo. However, no pregnancy defects have been reported in KO mice. To further elucidate the importance of TLR2 and TLR4 in placenta, in vivo experiments using animal models are required.

We believe that the TLRs and the trophoblast are important components of the innate immune system with a crucial role in the defense against placental infection, but they might also influence the cytokine environment in utero, and even if this is not related to allergy it could have an impact on the baby’s immune system.
4.3 NEONATAL IMMUNE RESPONSES TO MICROBIAL STIMULI (PAPER III)

A newborn child is believed to have an immune response that is $T_{H2}$ biased (reviewed in [85]). The first year of life is of great importance for the maturation of the child’s immune system and infections and exposure to non-pathogenic microbes in our environment are believed to be important in this context. It has previously been shown that CBMC from newborn children have a reduced capacity to respond to microbial stimuli in comparison to adult PBMC, mirroring the immaturity of the neonatal immune system. If the maturation of the child’s immune system is delayed, maybe due to environmental factors influencing the innate and adaptive immune system, an error in isotype switching might occur during a normal response, resulting in a production of IgE and subsequently allergic disease.

We were interested in investigating if there is a difference in innate immune responses at birth between children with allergic mothers and children with non-allergic mothers. To assess if children with high risk of becoming allergic have a lower capacity to respond to microbial stimuli and consequently might have a slower maturation of their immune responses, we compared children with allergic ($n=9$) and children with non-allergic ($n=10$) mothers. The expression of TLR2, TLR4 and CD14 on monocytes from CB and from the mothers and the cytokine response after stimulation with peptidoglycan (PGN) and LPS was examined.

We only included women undergoing elective caesarean section to exclude possible variations in our readout system due to labor. Labor involves the release of pro-inflammatory cytokines and inflammatory mediators that affect both the child’s and the mother’s immune response. A caesarian section might be mirroring the in utero environment in a better way and it might therefore be easier to elucidate the maternal influence on the child.

TLR2 expression increased in all groups following PGN stimulation, while TLR4 and CD14 expression on monocytes decreased after LPS stimulation. Many studies have shown a down regulation of TLR4 in response to LPS, and this has been proposed as a possible mechanism for LPS tolerance. The apparent absence of significant differences in TLR2 and TLR4 expression on monocytes from women with different allergic status in our study, does not rule out the possibility of differences in the TLRs or in the signaling cascades following TLR triggering. Indeed, Poltorak et al. demonstrated that a mouse strain with a mutation of the TLR4 gene and wild type mice showed a similar down regulation of TLR4 upon LPS stimulation, suggesting that down regulation of TLR4 does not require involvement of a TLR4 dependent signaling pathway.

We further compared the cell-surface expression of TLR2, TLR4 and CD14 on stimulated monocytes from allergic and non-allergic mothers as well as between their children. No significant differences in receptor expression could be seen, between the mothers or between the children. However, monocytes from the children with allergic mothers had significantly lower expression of TLR2 and TLR4 compared to monocytes from their mothers both before ($p=0.008$) and after ($p=0.02$) stimulation. This difference was not observed between non-allergic mothers and their children.
significant difference was observed in the expression of CD14 on monocytes from any group studied.

To investigate whether the observed attenuation of TLR expression on CB monocytes from children with allergic mothers was reflected in an effector response, we measured the release of IL-1β, IL-6, IL-8, IL-10 and TNF-α in culture supernatants of PBMC and CBMC 24 h after stimulation with LPS and PGN. We saw no significant differences between secretion of any of the above cytokines from PBMC of allergic and non-allergic mothers, although the TNF-α levels tended to be higher in allergic mothers. Children from both groups had a generally lower release of cytokines and chemokines compared to their mothers both before and after stimulation with LPS and PGN. However, the allergic mothers in our study showed a tendency towards a higher surface expression of TLR2 and TLR4 on their monocytes as well as a trend towards an augmented release of pro-inflammatory cytokines (e.g. TNF-α) after PBMC stimulation in comparison with the non-allergic mothers. This is probably explained by the inflammatory nature of allergic disease, i.e. that the allergic mothers had a somewhat activated immune system even in the basal state. This could have implications for the results obtained in CB cultures and we cannot exclude the possibility that the allergic women have influenced the immune response of their unborn children, masking the child’s own immune response to microbial stimuli. The children’s “true” response to microbial stimuli might therefore be distinguishable only if analyzed later in infancy.

Interestingly, however, CBMC from children with allergic mothers had a significantly (p=0.03) lower IL-6 release in response to PGN stimulation than CBMC from children with non-allergic mothers. This observation might indicate that subtle differences at the cell surface receptor level could have implications for the signaling cascade triggered by TLRs. However, it has to be pointed out that the differences in cytokine/chemokine responses seen after PGN stimulation in our study might not only be a result of signaling through TLR2 but also through the Nod1/Nod2 pathway, since it was recently shown that PGN sensing through TLR2 is lost after removal of lipoteichoic acid (LTA) from commercial S. aureus PGN. We have used commercial S. aureus PGN and might therefore have signaling both through TLR2 due to LTA contamination and through the Nod1/Nod2 pathway due to PGN stimulation.

Reduced stimulation of TLRs might affect not only immune deviation but regulatory T cell activity as well. Regulatory T cells have been shown to express TLRs and can be activated by LPS. An impaired immune suppression caused by a reduced microbial burden might explain why the prevalence of both allergies and autoimmune diseases has increased in the past few decades. Furthermore, a study in mice by Pasare et al. showed that microbial signaling via TLRs blocked the suppressive effect of CD4+ CD25+ Treg cells by inducing IL-6 release from professional antigen presenting cells (DCs and macrophages). IL-6 seems to play a critical role in activation of T cells by overcoming the suppressive effect of Treg cells. Indeed, the reduced capacity of CBMC to produce IL-6 at birth seen among the children with allergic mothers in our study might result in a lower degree of immune deviation and a Th2 skewed immune system, increasing the risk of developing allergic disease later in life. This makes
further studies following children prospectively and monitoring their future allergy developments particularly interesting.

Since the monocytic cell-surface expression of CD14 decreased after stimulation with PGN and LPS we were also interested in investigating how stimulation affected the soluble form of the receptor. Interestingly, the sCD14 levels in PBMC/CBMC culture supernatants decreased after stimulation, which makes shedding\textsuperscript{233} of CD14 from the surface of monocytes an unlikely cause of the decreased CD14 expression seen after stimulation. The decreased sCD14 levels in culture supernatants could be due to uptake of sCD14-lipid binding protein-LPS complexes\textsuperscript{234,235} by PBMC/CBMC during stimulation. Indeed, internalization of LPS is required for activation to occur\textsuperscript{235} and LPS is taken up via CD14-mediated pathways in CD14 positive cells.

We also measured sCD14 levels in plasma from the mothers and their children. No significant differences between allergic and non-allergic mothers or between their children could be observed. However, a tendency towards higher sCD14 levels in allergic mothers and their children was seen, which is in agreement with our previous study.\textsuperscript{236} In a recent publication by Zdolsek \textit{et al}\textsuperscript{238} it was shown that 7-year-old allergic children had lower sCD14 levels compared to non-allergic children of the same age. However, no significant difference was found between sCD14 levels in CB from the same children.

Our results imply that CB monocytes and CBMC immune responses are influenced by maternal allergy. Based on these findings we speculate that monocytes from children with allergic mothers could have a reduced capacity to respond to microbial stimuli.

\subsection{4.4 CD14 AND ALLERGIC DISEASE (PAPER IV)}

Baldini \textit{et al}\textsuperscript{30} identified a single nucleotide polymorphism (SNP) in the promoter region of CD14 (CD14-159) associated with increased sCD14 levels and decreased IgE levels in allergic individuals carrying this genotype. Since then a lot of studies have been performed to elucidate the connection between CD14 and allergic disease.

We investigated how sCD14 in CB was related to maternal allergy and subsequent allergy development at two years of age by measuring sCD14 levels in cord blood from 73 neonates and in peripheral blood from their mothers. Soluble CD14 levels in CB plasma from children with allergic mothers were significantly higher than sCD14 levels in CB from children with non-allergic mothers (p<0.001). The same could be seen in the mothers, where allergic mothers had higher values than the non-allergic mothers (p<0.001). However, this could not be observed two years after delivery when the women were non-pregnant. In the non-pregnant allergic women sCD14 levels were significantly lower (p<0.001) than levels during pregnancy, while the levels in non-allergic mothers were approximately the same during and after pregnancy. This indicates that the increased sCD14 levels seen in allergic mothers and in their neonates might be a pregnancy-related phenomenon. Previous studies have shown that the maternal immune system is activated during the third trimester of pregnancy in a way resembling the activation seen during sepsis.\textsuperscript{144} This could explain
why the sCD14 levels were significantly elevated in the allergic mothers during pregnancy, but not why this was not observed in non-allergic women. We could not connect these results to a difference in infection frequency between allergic and non-allergic women, making infections an unlikely cause of this finding. However, we could speculate that the elevated sCD14 levels seen during pregnancy in allergic women could be due to activation of the immune system due to their allergic disease. A positive correlation ($r = 0.7$, p<0.001) between sCD14 levels in plasma from mother and child could be seen at birth in the group with allergic mothers, suggesting that the mothers are influencing the CB sCD14 levels, either through passage of sCD14 itself, or of factors involved in the regulation of sCD14, although this needs to be experimentally demonstrated.

We also measured sCD14 levels in plasma from 43 of these children at two years of age (22 children with allergic mothers and 21 children with non-allergic mothers). The sCD14 levels had increased in the two-year-olds, but in contrast to results from CB, no significant difference between children with allergic mothers and children with non-allergic mothers could be observed, further indicating that the differences seen in CB might be pregnancy-related.

As would be expected children with allergic mothers were more prone to have allergic disease at two years of age than children with non-allergic mothers (p<0.05). Eight out of 22 children with allergic mothers had positive SPT together with allergic symptoms. The corresponding rate in the group with non-allergic mothers was only two out of 21. To investigate whether sCD14 plasma levels could be associated with allergic disease at two years of age we grouped the children according to SPT result and allergic symptoms independent of whether the mother was allergic or non-allergic. Children with positive SPT together with allergic symptoms (probable asthma and/or atopic eczema) were compared with SPT-negative children with no symptoms of allergic disease. We could find no association between sCD14 levels either at birth or at two years and development of allergic disease. However our data from CB are in agreement with a study by Jones et al\textsuperscript{50} where no association between CB sCD14 levels and subsequent development of allergic disease at five years of age could be seen. In a recent publication, Zdolsk\'e et al\textsuperscript{23} report that they could not find a correlation between CB sCD14 levels and allergic disease at seven years of age. However, they observed lower sCD14 levels in seven-year-old allergic children compared to non-allergic children of the same age. The discrepancy between our results from two-year-olds and those reported by Zdolsk\'e et al could be explained by the differences in age of the children included in the studies. Since our children were only two years old we do not know if a further maturation of their immune system might change their allergic disease as well as their sCD14 levels. Indeed, it has been shown that both allergic and non-allergic children can mount an allergen-specific IgE response at two years of age, although this response is weak and transient in the non-allergic children and strong and persistent in the allergic children,\textsuperscript{237} suggesting that a clear difference between allergic and non-allergic children might not be evident until later in childhood. Children less than 10 years old are believed to have a weaker response to common allergens measured by SPT compared to older children,\textsuperscript{238} this might affect the diagnosis of allergy in younger children and some children who will later develop allergic disease.
might be missed. Further, it has recently been shown that postnatal exposure to environmental allergens (e.g. Der p 1) is critical for the maturation of a T cell dependent humoral immune response and that the developmental divergence between allergic and non-allergic responses requires several years of exposure to allergens.  

Further, it was recently shown that reduced amounts of sCD14 in CB were associated with increased risk of developing recurrent wheezing in the first year of life. However, the finding was not specifically correlated with other allergic symptoms in the children. Lower sCD14 levels during early life might be associated with an increased susceptibility to respiratory infections and consequently also with wheeze.

A positive correlation was seen between sCD14 and total IgE in maternal plasma taken at delivery \((r_5 = 0.5, p<0.001)\), if the allergic and non-allergic mothers were pooled together \((n = 73)\). However, this could not be observed in the mothers when they were non-pregnant, suggesting a pregnancy-related phenomenon. In contrast to our results several groups have seen a negative correlation between sCD14 and total IgE. Baldini et al were first to report the finding of a SNP in the CD14 promoter with a C to T transition at base pair -159. The TT genotype was associated with an increase in sCD14 and a decrease in IgE levels compared to individuals carrying the CC and CT genotypes. However, the differences were only significant among non-Hispanic white children who were skin test positive to local aeroallergens. Since then a lot of studies have been made but the results are not always in agreement. The studies by Baldini et al on children from Arizona was later confirmed by a study on Chinese children and one on adults from the Netherlands. However the same association could not be found in children from Germany, in that CD14 genotypes were not associated with allergic disease. Recently, Kabesch et al performed a large study on children \((n=2048)\) and adults \((n=888)\) from both western and eastern Germany. They could not find an association between this promoter polymorphism, IgE and allergic disease and suggest that CD14 genotypes may not be directly involved in allergy development in childhood.

In contrast to our findings for sCD14, we observed significantly higher percentages of CD14+ cells in CBMC than in the PBMC from two-year-olds \((p<0.001)\) and mothers \((p<0.05)\). The high percentage of CD14+ cells suggests that factors involved in the regulation of sCD14 are suppressed or not yet produced in sufficient amounts in the infant, and would be in agreement with the low sCD14 levels observed in the CB plasma. Both IL-4 and IL-13\(^{241}\) have been shown to down-regulate sCD14 at the transcriptional level. If the general dogma of a T\(_h\)2-skewed fetus holds true, these findings would correspond well with our low sCD14 levels in CB. Percentages of circulating CD14+ cells were significantly correlated between mothers and two-year-olds \((r_5 = 0.5, p<0.01)\), suggesting a strong maternal influence, possibly genetic, on the cellular distribution in the peripheral blood of the child. No correlations were observed between CBMC and PBMC from 2-year-olds or between CBMC and PBMC from the mothers. Further, there were no differences in percentage of CD14+ cells between the groups irrespective of the child’s and the mother’s allergic status, suggesting that the significantly higher proportion of CD14+ cells in the CBMC might be a developmental phenomenon or be caused by labor-associated activation of the fetal immune system.  

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Results and Discussion

Although the levels of sCD14 are very low in CB, the child will encounter very high levels of sCD14 through breast milk. The function of this is not known, but might involve regulation of the early gastro-intestinal colonization, suggested to play a role in the generation of the allergic phenotype. Further, this sCD14 could be of importance in the early development of the immune system, serving to regulate T cell and/or B cell function. Soluble CD14 has been shown to act as a negative regulator of T cell activation and function by inhibiting IL-2 production. Further, interaction of sCD14 with B cells was shown to increase the levels of IgG and inhibit IgE production by activated tonsillar B cells and Ag-stimulated PBMC. In a recent study by Jones et al it was shown that reduced sCD14 in amniotic fluid and breast milk are associated with development of allergy and eczema in children.

These findings highlight the complexity of the interaction between innate and adaptive immune responses and lead us to conclude that sCD14 in CB is not a reliable marker for subsequent allergic disease. CD14 might be involved in the regulation of IgE production, but we suggest that CD14 could also be important for the maturation and development of the neonatal immune system.
5 CONCLUSIONS

[I]
In this study we presented the novel finding that IgE’ cells are abundant in the human placenta independent of both maternal allergy status and maternal and fetal total serum IgE levels. No evidence was found regarding local fetal IgE production or the expression of FcεRIα. However, the IgE correlated well with FcγRI on placental macrophages, suggesting that IgE might be bound to macrophages in complex with IgG, maybe to protect the fetus from the potential harmful effects of the IgE. The finding of numerous IgE’ cells in the placenta irrespective of maternal allergy could indicate a physiological role of IgE in utero during pregnancy.

[II]
In this study we demonstrated for the first time that TLR2 and TLR4 are highly and continuously expressed on the trophoblast cells covering the peripheral chorionic villi, as well as in intermediate trophoblast cells in free cell islands, the cell columns and the decidua in full term placentas. We also conclude that the receptors are functional since stimulation with LPS and zymosan induced a release of IL-6 and IL-8 into culture supernatants. These data suggests a mechanism by which the fetoplacental unit could interact with microorganisms and protect the fetus against placential infection.

[III]
Cord blood (CB) monocytes from children with allergic mothers had significantly lower expression of TLR2 and TLR4 compared to maternal monocytes both before and after microbial stimulation, in contrast to CB monocytes from children with non-allergic mothers. CB mononuclear cells from children with allergic mothers had a lower IL-6 response after stimulation with PGN than CBMCs from children with non-allergic mothers. However, no significant differences in receptor expression could be seen between the children. Our results imply that CB monocytes and CBMC immune responses are influenced by maternal allergy. Based on these findings we speculate that monocytes from children with allergic mothers could have a reduced capacity to respond to microbial stimuli.

[IV]
In this study we found higher sCD14 levels in CB from children with allergic mothers compared to children with non-allergic mothers. However, this could not be observed when the children were two years old, and could not be associated to allergy development at this time point. In contrast to previous studies we could find a positive correlation between total IgE levels and sCD14 in CB as well as in the mothers, but no correlation was found when the children were two years old or when the mothers were non-pregnant, indicating that the elevated levels of sCD14 might be pregnancy-related. The immune system of the mother is altered during pregnancy and a positive correlation could be observed between sCD14 in plasma from allergic mothers and in
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CB from their children, supporting this hypothesis. Membrane CD14 expression on monocytes did not significantly differ between children with allergic and children with non-allergic mothers, neither in CB nor at two years of age, and no correlation could be made to development of allergic disease, although age-related differences could be seen. These findings highlight the complexity of the interaction between innate and adaptive immune responses and leads us to conclude that sCD14 in CB is not a reliable predictor for subsequent allergic disease. CD14 might be involved in the regulation of IgE production, but we suggest that CD14 could also be important for the maturation and development of the neonatal immune system.
6 FUTURE PERSPECTIVES

My studies have provided answers to some questions but also generated new questions. I will briefly outline what I think would be interesting to explore further.

The finding of numerous IgE⁺ cells in the placenta raised the question whether the IgE is of maternal or fetal origin and if the IgE has a physiological function in the placenta. The IgE seen in the placenta could originate from the fetal circulation or from the mother in the form of IgG/IgE complexes. It could be difficult to reveal the origin of the placental IgE since there are no IgE allotypes. However, one approach could be to explore the possible transfer of IgG/IgE complexes across the placenta by performing transfer experiments in vitro. Further, it would be of interest to delineate the ontogeny of IgE in the placenta by studying placental tissue from different gestational ages, possibly revealing clues to the function of the placental IgE. Recently, we observed that IgE expression in the placenta is higher during second trimester compared to third trimester (Rindsjø et al to be published), this might reflect an unknown physiological role for IgE at this time point. An animal model might be a good way to address the question of the in vivo role of IgE during pregnancy as well as the origin of the IgE. The human pregnancy is unique which makes it difficult to draw any real conclusions from an animal model. However, a mouse pregnancy is short and one can study the different stages in detail as well as generating KO mice. Other animal models like the pig or sheep might also be suitable for studying the importance of IgE in pregnancy and a lot of research related to pregnancy has already been done in sheep. Studies to explore this further could be of great importance for understanding the physiological role of IgE in the in utero environment during pregnancy.

The report of the association between a lack of TLR signaling in mice and increased IgE responses makes it interesting to study the expression of TLRs in placenta from allergic and non-allergic mothers. Studies on farmers’ children have shown that their blood cells express significantly higher levels of CD14 and TLR2 compared to non-farmers’ children. This indicates that the immune system of farmers’ children respond to the microbes in their environment and may protect from allergic disease. We have observed individual differences in the expression of TLR2 and TLR4 with immunohistochemistry but no significant differences was observed between placenta from allergic and non-allergic mothers (Rindsjø et al to be published). An expression of TLR2 and TLR4 in all placentas indicates that these receptors are important during pregnancy, probably as a defense against placental infection. It would therefore be of interest to elucidate the impact of placental infection in TLR KO mice as well as to further study TLR expression from complicated pregnancies (e.g. placental infection). We have preliminary data that TLR4 expression on trophoblast cells is lower during second trimester compared to first and third trimester of pregnancy (our unpublished data). It would be interesting to further reveal the ontogeny of TLRs in placenta and also in the fetus. This could be done by studying human material from different gestational ages in parallel with in vivo studies in an animal model.

A difference in cytokine response after PGN stimulation between children with allergic mothers and children with non-allergic mothers was observed in my third study: the children with allergic mothers had significantly lower IL-6 release after PGN
stimulation compared to children with non-allergic mothers, indicating a reduced capacity to respond to microbial stimuli. It would therefore be interesting to follow children prospectively from birth and during the first years of life to determine whether this difference will persist or even increase with age. However, it is important to take into account the environment influencing each specific child, to try to reveal how a combination of inherited differences in the ability to respond to microbial stimuli and the exposure to different environmental factors influence the child’s future allergy development. Reports on differences in cell-surface expression of CD14 and TLR2 in children exposed to different amounts of environmental endotoxins (e.g. living on a farm)\textsuperscript{44} as well as an association between maternal exposure to farm animals during pregnancy and increased CD14, TLR2 and TLR4 mRNA expression\textsuperscript{47} makes it interesting to elucidate if any differences exist in TLR signaling events between cells from children living in different environments. It would also be interesting to compare cell signaling events between children with allergic mothers and children with non-allergic mothers as well as between cells from children who have developed allergic disease and those who remain healthy. A way to reveal these potential differences might be to use flow cytometry to study intracellular signaling such as phosphorylation of transcription factors.

Recently, the involvement of epigenetic inheritance in allergy development in childhood has been implicated\textsuperscript{110}. In some infants who later will develop allergies, the T\textsubscript{H}2-mediated cytokine responses seems to be preserved in infancy\textsuperscript{111,112}. This might indicate that there is a persistence of the fetal stage in the children who develop allergic disease, and that the persistence is due to a defect in T\textsubscript{H}2 dampening mechanisms. It would be of great interest to follow children prospectively and investigate how epigenetic inheritance together with different environmental factors might direct the immune response of the child towards allergy development.
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8 REFERENCES


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