Maternal immune characteristics and innate immune responses in the child in relation to allergic disease

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Doctoral Thesis in Immunology at Stockholm University, Sweden 2008
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Stockholm 2008
The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" (I found it!) but "That's funny..."

Isaac Asimov (1920-1992)

To Ebba and Ines
SUMMARY

The mechanistic factors responsible for the increase in allergic diseases are still not fully understood, but a reduced microbial stimulation seems to be one of the key issues. Research is now aiming at investigating the relationship between the innate immune system, involving the toll-like receptors, and allergy development. Further, the maternal influence on the child, possibly through in utero effects, but also through the breast milk, has shown to be of great importance. This thesis aimed at understanding how the maternal immune system is influenced by early exposures and allergic disease, but also to investigate the consequences of the maternal phenotype on the innate immune system of the developing child.

The Th1/Th2 cytokine pattern in allergic diseases has been extensively studied. Here we were interested in comparing the innate cytokines in allergic and non-allergic women, and to see if the allergic status was influencing the effect of pregnancy differently. We demonstrate that IL-1β, IL-6, IL-10 and IL-12 production in cells from adult women are not influenced by allergic status, neither during pregnancy nor 2 years after. However, pregnancy had an apparent effect on cytokine levels, regardless of allergic status. Also, total IgE levels in allergic women were significantly lower 2 years after pregnancy in comparison with the levels during pregnancy, pointing to the fact that pregnancy indeed has an immunomodulatory role.

We further wanted to investigate the immune system of mothers who had migrated to Sweden in comparison with indigenous mothers. The reason for our interest here was that children born from immigrated mothers have shown to have an increased risk of developing diseases such as allergy and Crohn’s disease. The results showed that immigrants from a developing country had significantly higher levels of breast milk IL-6, IL-8 and TGF-β1. Further, regardless of maternal country of birth, a larger number of previous pregnancies was associated with down-regulation of several substances, statistically significant for soluble CD14 and IL-8. The results suggest that maternal country of birth may indeed influence adult immune characteristics, potentially relevant to disease risk in offspring.

The influence of allergic status of the mother on the expression of CD14, TLR2 and TLR4 was further investigated in monocytes from mothers and their newborn babies upon microbial stimulation. We could not find any differences in monocytic TLR levels between the groups. No significant differences regarding cytokine levels between allergic and non-allergic mothers in response to stimuli were found either. However, the cytokine and chemokine release triggered by TLR2 stimulation in CB revealed that CBMC from children with maternal allergic disease released significantly less IL-6, and a trend towards less IL-8.

As we could not find differences in TLR levels attributed to maternal allergy, but an impaired IL-6 response, we turned our focus on an intracellular event taking place after TLR ligation. The results confirmed our results of decreased IL-6 levels in CB from children to allergic mothers. At 2 years of age, the children of allergic mothers still displayed a diminished IL-6 response. Additionally, they also had a decreased activity of p38 MAPK. p38 has an important role in driving Th1 responses, suggesting that the p38 pathway could be one of the responsible mechanisms behind the impaired responses correlated to allergic heredity found in CB as well as at 2 years of age.

Infancy is a crucial time period for the developing immune system. Further, the relative composition of the two major monocytic subsets CD14 CD16 and CD14 CD16 is altered in some allergic diseases. TLR levels are different in the two subsets, proposing a possible link to the reduced responding capacity of monocytes from children with allergic heredity. We followed up our earlier studies of children at birth and at 2 years of age by looking at 5 year old children. There were no differences regarding monocytic subsets, nor in TLR levels in unstimulated cells. However, when stimulating the cells with PGN, both monocytic subsets in allergic subjects were less capable of upregulating TLR2 compared to the age-matched controls.

Taken together, the work in this thesis suggests that the maternal immune system is affected by the process of pregnancy and childhood exposures. It further suggests that maternal allergy affects the young child, in terms of impaired responses to microbial stimuli, which later in infancy correlates with allergic disease in the child. These impaired innate responses could lead to a diminished Th1 response, or alternatively to a deficiency in regulatory mechanisms, and thereby cause allergic disease.
ARTICLES

This thesis is based on the following original articles, which will be referred to by their Roman numerals:


# TABLE OF CONTENTS

## INTRODUCTION ........................................................................................................... 9

## INNATE AND ADAPTIVE IMMUNITY ....................................................................... 9

## PATTERN RECOGNITION .......................................................................................... 11

- Toll-like receptors .................................................................................................. 12
- Intracellular signaling ............................................................................................. 13
- Lipopolysaccharide recognition ............................................................................. 14
- Peptidoglycan recognition ...................................................................................... 16

## IMMUNE CELLS AND MEDIATORS ..................................................................... 17

- Monocytes/macrophages ......................................................................................... 17
- Dendritic cells .......................................................................................................... 18
- NK cells ................................................................................................................... 19
- Mast cells ................................................................................................................ 20
- Granulocytes .......................................................................................................... 20
- T cells ....................................................................................................................... 21
- B cells ...................................................................................................................... 22
- Cytokines and chemokines ..................................................................................... 23
  - Type 1/type 2 responses ....................................................................................... 25
  - TNF ...................................................................................................................... 26
  - IL-1β .................................................................................................................... 27
  - IL-6 ....................................................................................................................... 27
  - IL-10 .................................................................................................................... 28
  - IL-12 .................................................................................................................... 29
  - TGF-β ................................................................................................................ 30
  - IL-8 ....................................................................................................................... 31

## ALLERGY ................................................................................................................. 32

- The allergic reaction ............................................................................................... 32
- The hygiene hypothesis and the role of innate immunity ......................................... 34
- Monocytes/macrophages and disease ..................................................................... 36
- Pattern-recognition receptors and disease .............................................................. 37
- Gut ......................................................................................................................... 38
- Gene-environment interactions ............................................................................. 39
  - Epigenetics ......................................................................................................... 40
  - Microbial recognition for therapeutic interventions ........................................... 40

## IMMUNOLOGY OF PREGNANCY .......................................................................... 42

- Maternal influences on the child ............................................................................. 43
  - In utero ................................................................................................................ 43
  - Breastmilk ........................................................................................................... 45
  - The sibling effect ................................................................................................ 47

## THE PRESENT STUDY ............................................................................................ 48

## AIMS ......................................................................................................................... 48

## METHODOLOGY ..................................................................................................... 50

## RESULTS AND DISCUSSION ................................................................................. 51

- Impact of pregnancy and allergic status on cytokine responses (I).......................... 51
- Maternal exposures and breast milk characteristics (II) ....................................... 53
- Neonatal immune responses to microbial stimuli (III) ......................................... 56
- Maternal allergy and monocyte signaling in 2-year-old children (IV) .................... 59
- TLR2 signaling in 5 year old allergic children (V) ................................................. 61

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES .................................. 65

## ACKNOWLEDGEMENTS ......................................................................................... 67

## REFERENCES .......................................................................................................... 70
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>ASM</td>
<td>Airway smooth muscle cells</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>CB</td>
<td>Cord blood</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
</tr>
<tr>
<td>CBMC</td>
<td>Cord blood mononuclear cells</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDN</td>
<td>Eosinophil-derived neurotoxin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft-vs-host disease</td>
</tr>
<tr>
<td>HAV</td>
<td>Hepatitis A virus</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-mobility group box 1</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal epithelial cells</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>IL-12R</td>
<td>IL-12 receptor</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LBS</td>
<td>LPS-binding protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich-repeats</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88 adaptor-like</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microorganism-associated molecular pattern</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NBS</td>
<td>Nucleotide-binding site</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa –B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-oligomerization domain</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PIN</td>
<td>Peptidyl-prolyl isomerase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>sCD14</td>
<td>Soluble CD14</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
</tr>
<tr>
<td>sIL-6R</td>
<td>Soluble IL-6 receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphisms</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SPT</td>
<td>Skin prick test</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIM</td>
<td>T cell, Ig- and mucin domain</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1R</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>Toll-receptor associated molecule</td>
</tr>
<tr>
<td>T_{reg}</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>Trif</td>
<td>Toll-receptor associated activator of interferon</td>
</tr>
</tbody>
</table>
INTRODUCTION

INNATE AND ADAPTIVE IMMUNITY

The immune system of vertebrates consists of two interrelated components, the innate and adaptive responses, which are jointly required for the resolution of most infections. The innate immune response is the first line of host defense, and is responsible for an immediate recognition and control of microbial invasion. It is comprised mainly of phagocytic cells, such as macrophages and neutrophils, which can ingest and kill the invading pathogens. The effector phase of innate immunity is the process of inflammation, where the immediate response to a pathogen gives rise to a reaction characterized by the migration of cell types with defensive functions. Further consequences are alterations in vascular permeability and the secretion of soluble mediators such as cytokines and chemokines, leading ultimately to the initiation of the adaptive arm.

The specificity and memory of the adaptive immune response are mediated by T- and B cells. The specificity is generated through genetic rearrangements and selection of receptors that best recognize the specific antigens. Through the history of immunology, scientists have had difficulties integrating the innate and the adaptive systems, but the discovery of toll-like receptors (TLRs) [1] was a huge step towards bridging the two systems together. The fact that innate immune responses not only provided a first line of defense, but also were critical for setting the adaptive immune system into action, changed the former view of the innate immune system as being primitive and unspecific. Multiple studies have now revealed that it is indeed specific in the sense that different stimuli give different signals to the adaptive immune system, thereby “deciding” how the most effective response should be designed to combat a specific intruder [2]. One of the best illustrations of the now established concept that innate and adaptive immunity are not completely independent entities comes from studies showing that optimal T-cell responses require help from mast cells, cells belonging to the innate immune system [3].

A recent finding re-challenged the view of immunological memory of the adaptive immune system. It showed that the memory upon a secondary infection not only depends on memory cells of the adaptive immune system, but also on parts of the innate immune system [4].
Moreover, it was recently shown that not only does adaptive immunity have a role in combating infections, but it is also important for dampening the strong inflammatory reactions that the innate immune system gives rise to upon infection. Thus, mice unable to mount an adaptive immune response died rapidly after infection. Unexpectedly, the mice were shown not to die of unchecked microbial infection, but from damage caused by uncontrolled inflammatory cytokines released by the innate immune system [5].
PATTERN RECOGNITION

The innate immune response relies on evolutionarily ancient germline-encoded receptors, the pattern-recognition receptors (PRRs), which recognize highly conserved microbial structures, traditionally known as pathogen-associated molecular patterns (PAMPs). These microbial patterns are not uniquely found in pathogenic organisms, but also in nonpathogenic commensal microorganisms. This type of recognition enables the host to quickly identify and respond to a broad range of pathogens. The most studied PRRs are the TLRs. However, PAMP-PRR interactions are not restricted to TLRs. There are several other PPRs functioning in a similar manner in that they also recognize microbial components, although they are located in the cytosol rather than at the cell surface or in vesicles. Nucleotide-binding site and leucine-rich repeat (NBS-LRR) proteins are examples of such receptors where the nucleotide-binding oligomerization domain (NOD) is one of the most studied [6]. Examples of microbial ligands recognized by pattern-recognition molecules include lipopolysaccharide (LPS), lipoteichoic acid, flagellin, mannans, nonmethylated CpG sequences and peptidoglycan (PGN). There is further increasing evidence that TLRs also recognize host-derived ligands from the damage or death of host cells [7]. The recognized compounds are known as damage-associated molecular-pattern molecules, where the high-mobility group box 1 (HMGB1) [8] and heat shock proteins [9] belong to those that are the best studied.
Toll-like receptors

Toll-like receptors is the best characterized class of PRRs, and today 13 mammalian TLRs are known (Table 1), of which TLR2 and TLR4 are two of the most studied [10].

### What the Toll-Like Receptors See

<table>
<thead>
<tr>
<th>TLR</th>
<th>Natural Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1 (partnered with TLR2)</td>
<td>Bacterial triacyl lipopeptides and certain proteins in parasites</td>
</tr>
<tr>
<td>TLR2 (partnered with TLF6)</td>
<td>Bacterial diacyl lipopeptides, lipoteichoic acid from Gram-positive bacteria, and zymosan from the cell wall of yeast</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double-stranded RNA from viruses (e.g., West Nile virus)</td>
</tr>
<tr>
<td>TLR4</td>
<td>Endotoxin (lipopolysaccharide) from Gram-negative bacteria</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin from mobile bacteria</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded RNA from viruses (e.g., HIV)</td>
</tr>
<tr>
<td>TLR8 (inactive in mice)</td>
<td>Same as TLR7</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG DNA from bacteria or viruses</td>
</tr>
<tr>
<td>TLR10 (found in humans but not mice)</td>
<td>Unknown</td>
</tr>
<tr>
<td>TLR11 (found in mice; human form is truncated and thought to be inactive)</td>
<td>Profilin, a protein from the protozoan pathogen <em>Toxoplasmosis gondii</em> that can cause miscarriage; may also respond to components of bacteria that cause bladder and kidney infections</td>
</tr>
<tr>
<td>TLR12 and TLR13 (found in mice but not humans)</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**Table 1.** The known exogenous binding partners for each TLR (Science 2006; 312:184-187). Reprinted with permission from AAAS.

TLRs are membrane glycoproteins characterized by the extracellular domains, containing varying numbers of LRR motifs, and a cytoplasmic signaling domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R (TIR) domain. TLRs may be expressed extra- or intracellularly. While some TLRs (TLRs 1,
2, 4, 5, and 6) are expressed on the cell surface, others (TLRs 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes. TLRs are important for dendritic cell (DC) maturation and function. Additionally, it has been shown that generation of T-cell dependent antigen-specific antibody responses also requires activation of TLRs in B cells [11]. However, later studies suggest that TLR signaling in B cells amplifies, but are not required for antibody production or maintenance of memory [12]. Microorganisms, via their PAMPs, may also contribute directly to the perpetuation and activation of long term T-cell memory as T cells also express TLRs [13]. In addition to ligand specificity, the functions of individual TLRs differ in their expression patterns and the signal transduction pathways they activate.

Taken together, an increasing body of evidence emphasize the important cross-talk between different TLRs for enabling an optimal and secure response to stimuli [6;14;15]. TLR activation is essential for the ability of the immune system to combat invading pathogens, however a strict regulation is of major importance, as lack of inhibition can lead to detrimental and inappropriate inflammatory responses. TLR signaling has been shown to affect several disorders, including immunodeficiencies, autoimmune- and allergic diseases [16;17].

**Intracellular signaling**

As different responses are needed for the elimination of different microbes, TLRs operate in concert with several adaptor molecules to acquire maximum sensitivity and specificity. The most studied adaptor proteins, shown to transduce signals via the intracellular TIR domains of the different TLRs, are Myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL), Toll receptor-associated activator of interferon (Trif) and Toll receptor-associated molecule (TRAM). Most TLRs activate the MyD88-dependent pathway, leading to activation of mitogen-activated protein kinases (MAPKs) and nuclear factor -kappa B (NF-κB), a transcription factor induced within minutes after microbial challenge. NF-κB plays a critical role in the coordination of both innate and adaptive immune responses by regulating the gene expression of many cellular mediators [18].

MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK) and p38 regulate the activities of several transcription factors.
By altering the levels and activities of transcription factors, MAPKs influence transcription of genes that are important for cytokine production and involved in the cell cycle. MAPKs play different roles in IL-10 and IL-12 production [19]. A regulatory DC recently described, diffDC, have an impaired IL-12p70 production and enhanced IL-10 production compared to immature DCs. The low levels of IL-12p70 were dependent upon a suppressed p38 pathway, while the high levels of IL-10 were caused by an increased ERK activation [20]. The results from that study highlight the important role of MAPKs in directing different immune responses towards a more Th1, Th2 or regulatory response. MAPKs, especially p38, can enhance the expression of pro-inflammatory cytokines at the transcriptional level, but are also able to act on the post-transcriptional level. p38 acts on cytokine levels after transcription through mechanisms that enhance the stability and the translation of the cytokine mRNA [19].

Airway smooth muscle cells (ASM) play an important role in both hyperreactivity and remodeling in asthmatic patients. A study by Shan et al [21] showed that the different MAPKs can affect each other. If the ASM cells were pretreated with inhibitors of ERK1/2 signaling, the induced NF-κB activity and changes in ASM responsiveness in response to LPS were dampened, whereas inhibition of p38-MAPK augmented the proasthmatic responses to LPS. Their data demonstrate that p38 can have an important regulatory function, by acting on the ERK1/2 pathway after TLR4 stimulation [21]. The role of MAPK in inflammation makes them attractive targets for new therapies, when efforts are being made to identify newer, more selective, inhibitors for inflammatory diseases [22].

**Lipopolysaccharide recognition**

Host mechanisms that recognize gram-negative bacterial LPS are among the most sensitive and best studied. All gram-negative bacteria express the glycolipid component called LPS or endotoxin at their surface. There is evidence to suggest that there are structural and functional differences between LPS molecules originating from different bacterial species [23]. Myeloid cell activation by LPS involves the signaling receptor complex MD-2/TLR4 receptor, which receives LPS from CD14; a membrane bound receptor anchored by a glycerophosphatidylinositol tail. The plasma LPS-binding protein (LBP) catalytically transfers single LPS molecules from LPS
aggregates onto CD14 (Figure 1). Upon binding of the ligand, the TLR4 signaling consists of two different pathways; the MyD88-dependent and the MyD88-independent [24].

MD-2 is a small secreted glycoprotein that confers LPS responsiveness to TLR4. MD-2 associates with TLR4 in the endoplasmic reticulum, and is necessary for translocation of TLR4 from Golgi to the surface [25]. After LPS has been recognized by the receptor-mediated mechanism, it is endocytosed and transported into Golgi-like structures together with TLR4 [26]. After signaling has taken place the TLR4 is trafficked to lysosomes where it is degraded. This endosomal trafficking of the LPS receptor complex is essential for antigen presentation to Th cells as well as for termination of the signaling, thus controlling both the innate and the adaptive immune system [27].

Figure 1. Cell signaling in response to LPS (Nature 2002; 420:885-891). Reprinted by permission from Nature copyright Macmillan Publishers Ltd.

Another form of CD14, without the lipid tail, circulates as a soluble plasma protein; soluble CD14 (sCD14). sCD14 participates in cell activation by transferring LPS to CD14 on the cell membrane, or by transferring the LPS directly to the MD-2/TLR4 receptor complex on cells that do not express CD14 on the cell membrane, such as endothelial and epithelial cells [28]. LBP and CD14, which play key roles in enabling sensitive responses to LPS can also have inhibitory activities that help to
control LPS responses by limiting LPS interactions with MD-2/TLR4 [29]. sCD14 is highly expressed in breast milk and has an important role for the neonatal intestine, as it enables gut epithelial cells to respond to microbial stimuli in terms of LPS [30], a stimulation believed to be important for postnatal gut tolerance and homeostasis [31].

Excessive stimulation of monocytes and macrophages by LPS leads to endotoxin shock, a systemic disorder with a high mortality rate in humans. Studies have shown that pre-exposure to LPS reduces sensitivity to a second exposure to LPS, a phenomenon known as LPS tolerance or LPS hyporesponsiveness. This tolerance is not fully understood, but it has been suggested that the decreased TLR4 expression after LPS stimulation could be one of the underlying mechanisms [32].

**Peptidoglycan recognition**

As a major constituent of the cell wall of virtually all bacteria, and absent from eukaryotes, PGN represents an excellent target for innate immune recognition. PGN is highly abundant in gram-positive bacteria. In gram-negative bacteria the thin PGN layer is found underneath the LPS containing outer membrane [33]. Extracellular recognition of PGN is mediated by membrane-bound CD14 and TLR2, while the intracellular recognition is mediated by NOD1 and 2. There are also soluble PGN recognition molecules involved, such as sCD14 and C-type lectins. The CD14 receptor is not required for PGN signaling, even though the responses are usually enhanced by CD14 binding [33].

Peptidoglycan was traditionally believed to signal through surface TLR2, but this signaling was questioned after Travassos et al found that PGN sensing through TLR2 was lost after removal of lipoteichoic acids (LTAs) from commercial *S. aureus* PGN [34]. However, a more recent reevaluation has confirmed that it is indeed acting upon TLR2 [35]. The current belief is that there seems to be important interactions between TLR2- and NOD2 signaling pathways [6;36]. Of the 11 characterized TLRs in humans, TLR2 is unique by virtue of its ability to heterodimerize with either TLR1 or TLR6, resulting in a relatively broad ligand specificity. The signaling pathway after TLR2 ligation is similar to the TLR4 MyD88-dependent pathway, which leads to the production of pro-inflammatory cytokines.
IMMUNE CELLS AND MEDIATORS

Monocytes/macrophages

Peripheral blood monocytes are circulating myeloid precursors of antigen-presenting cells originating from the bone marrow. In humans, they constitute ~ 5-10% of the blood leukocyte pool. They vary in size and have different degrees of granularity. After being released into the periphery, where they circulate for several days, they enter tissues and replenish the tissue macrophage population. Immune stimuli, such as pro-inflammatory mediators, elicit increased recruitment of monocytes to the tissues, where differentiation into macrophages and DCs takes place.

Monocytes were initially identified by their expression of large amounts of CD14, which is highly expressed by monocytes and macrophages. Monocytes are nowadays regarded as a heterogeneous cell population that after differentiation not only contributes to host defense, but also is important for tissue remodeling and repair [37]. The monocytic subdivisions have sometimes been indistinct, but it has now been convincingly shown that the most prominent populations are the classical CD14++CD16− subpopulation and the pro-inflammatory CD14+CD16+ subpopulation, the latter representing about 10% of all monocytes in healthy individuals [38]. CD16 is the low affinity receptor for immunoglobulin (Ig) G, therefore also named FcγRIII. Monocytes are the cells within the innate immune system that exhibit the highest density of TLRs on their surface [39]. The two monocytic subsets have been shown to have different basal expressions of TLR2/TLR4 and to behave differently in response to microbial stimuli [40].

Macrophages are being divided into classically activated (M1), and alternatively activated (M2) macrophages, where M2 is a generic name for various forms of activated macrophages (M2a, M2b and M2c). In general, M1 cells are the consequence of stimulation by substances such as LPS and interferon (IFN) -γ, while M2 have been exposed to IL-4, IL-10, IL-13 or transforming growth factor (TGF) -β. M1 promote strong IL-12 mediated Th1 responses, while M2 support the Th2 – associated effector functions. M2 also have a role in resolution of inflammation [41]. Interestingly, the division of macrophages into M1 and M2 is not a permanent
phenomenon as it has been shown that both forms can be re-polarized by Th2 or Th1 cytokines, respectively [42].

The role of monocytes in disease, and more particularly in connection to allergic disease, is central in this thesis and will be described in detail in a separate section.

**Dendritic cells**

Dendritic cells are crucial mediators of immune defense, but have also been implicated in tolerance induction [43]. They are the most potent antigen-presenting cells (APC) due to their constitutive expression of high levels of Major Histocompatibility Complex (MHC) class II molecules and other co-stimulatory receptors. There are different subsets of DCs with distinct pattern-recognition receptors and functions. Most mature DCs however, have the same major function; presentation of antigen to Th cells. The myeloid dendritic cells (mDC) are derived from monocytes arising from the myeloid pathway, while plasmacytoid dendritic cells (pDC) arise from the lymphoid pathway. pDCs produce high amounts of IFN-α and mDCs mainly produce IL-12.

DCs are present in small amounts in tissues that are in contact with the external environment, mainly the skin and the inner lining of the nose, lungs, stomach and intestines. They can also be found in an immature state in the blood. Once DCs are activated, they migrate to the lymphoid tissues where they interact with T- and B cells to initiate and shape the adaptive immune responses. Located alongside epithelial cells in the airways, DCs have an important role in determining how allergic immune responses (described in detail later) are initiated and perpetuated [44]. Being an early director of the immune response, it is of no surprise that DCs initiate unwanted responses that can cause disease. However, different DC subsets also have the potential of being used as new therapeutic tools, in order to ameliorate many of the disease conditions [45]. Targeting DCs for effective vaccination is an important area taking advantage of the increasing knowledge of the important role of DCs in directing immune responses [46].
NK cells

Natural killer (NK) cells are nonspecific cytotoxic lymphocytes playing a crucial role in the innate immune system, making up approximately 10% of the circulating lymphocytes in humans. Their main function is to kill tumors and cells infected by viruses. NK cells are defined as large granular lymphocytes that do not express T-cell receptors (TCR) or the T-cell marker CD3 or surface-Ig B cell receptors, but that usually express the surface markers CD16 (FcγRIII) and CD56 in humans. They recognize the target cells by missing "self" markers of MHC class I, and induce apoptosis by releasing small cytoplasmic granules of proteins called perforin and granzyme. Human NK cells are generally subdivided into two subsets based on the expression of CD16 and CD56, where the majority of human NK cells express low levels of CD56 and high levels of CD16.

Another important localization of NK cells is the placenta. Human NK cells are massively recruited at the site of embryonic implantation. These NK cells differ in many ways from their peripheral blood NK cell counterparts in terms of gene expression, phenotype and functionality. The function of the decidual NK cells is not completely understood. However, they have shown to play an important role in the control of extravillous invasion, control of uterine vascular remodeling, and local anti-viral activity [47].

Moreover, NK cells have been shown to have a role in allergy. A study of atopic asthmatic individuals revealed a higher ratio of IL-4-producing NK cells in the patients compared to controls [48]. Aberrant NK cell frequencies and functions have also been observed in patients with atopic dermatitis [49]. Further, NK cells may contribute to allergic responses by producing several cytokines and chemokines related to an allergic reaction [50].

Natural killer T (NKT) cells are a heterogeneous group of cells, constituting 0.2% of all peripheral blood T cells. They share properties of both T cells and NK cells, as they express the TCR, CD16 and CD56. They are considered as being regulators of immune responses, as they rapidly produce large amounts of both Th1 and Th2 cytokines [51]. It is therefore not surprising that their dysfunction or deficiency have been implicated in the development of autoimmune diseases [51], cancers [52] and asthma [53].
Mast cells

Mast cells originate from bone marrow precursors that circulate in an immature form. Their characteristics as mature cells are determined by the specific tissue they are recruited to [54]. They are capable of phagocytosis, and are activated through pattern-recognition receptors to produce inflammatory mediators, a fact that makes them an interesting cell type in several diseases. Indeed, mast cells are implicated in autoimmune disorders where they are involved in the recruitment of inflammatory cells to the joints and skin [55]. Another, perhaps unexpected role for mast cells, has been elucidated in two recent murine studies showing that mast cells are important for their ability to mediate regional immune suppression [56;57]. However, the most studied role of mast cells is their contribution to the allergic reaction. They express the high-affinity receptor FcεRI. This receptor is specific for the Fc region of IgE, a class of antibody characteristic for allergic reactions (explained in a later section). This receptor-ligand interaction is of such high affinity that binding of IgE molecules is essentially irreversible. As a result, mast cells are coated with IgE. When activated by direct injury, cross-linking of IgE receptors or by activated complement proteins, they rapidly release their characteristic granules and various hormonal mediators. Mast cells are common at sites in the body that are exposed to the external environment, such as the skin. Their role in the allergic reaction is further explained in the paragraph on the allergic mechanism.

Granulocytes

Granulocytes are a category of white blood cells characterized by the presence of granules in their cytoplasm. They are also called polymorphonuclear leukocytes, referring to the varying shapes of the nucleus, which is usually lobed into three segments. The granulocytic cell compartment consists of neutrophils, basophils and eosinophils. Neutrophils, normally found in the blood stream, are the most abundant granulocytes. However, during the acute phase of inflammation, neutrophils leave the blood and migrate toward the site of inflammation in response to different chemotactic factors.

Eosinophils have important functions in combating infections, where their role in immune responses to parasitic infections is the most prominent one. They are also
important cells for mechanisms associated with allergy and asthma, where they have been shown to amplify type 2 responses by acting as APCs [58]. Basophils are the least common granulocytes, representing about 0.01% to 0.3% of circulating leukocytes. Functionally, they are similar to mast cells as they also store histamine, and are implicated in allergic reactions. Indeed, a recent study confirms that basophils contribute to the initiation of the allergic reaction, as they are directly activated by allergens and produce cytokines that are crucial in an allergic reaction [59].

**T cells**

T cells are CD3 carrying cells belonging to the cellular part of the adaptive immune system. They can be divided into CD4$^+$ T helper cells (Th cells), CD8$^+$ cytotoxic T cells (CTL) and regulatory T cells (T$_{reg}$). T cells originate in the bone marrow, but the development into functioning T cells takes place in the thymus. The ability of T cells to recognize foreign antigens is mediated by the T-cell receptor. The receptor is associated with CD3 on the membrane of the cell. CD3 does not influence the interaction between the antigen and the TCR, but participates in the signal transduction. The T-cell receptor undergoes genetic rearrangement during thymocyte maturation in the thymus, resulting in each T cell bearing a unique T-cell receptor, specific to a limited set of peptide-MHC combinations. The random nature of the genetic rearrangement results in a requirement of central tolerance mechanisms to remove or inactivate those T cells which bear a T-cell receptor with the ability to recognize self-peptides.

The Th cells recognize extra cellular antigens that are taken up and presented by APCs in association with MHC class II molecules. The recognition of this complex activates the Th cell, which among other functions produces cytokines and supports different events of the immune system, such as antibody production by B cells and killing of cells by the CTLs. CTLs recognize and destroy cells that are infected with intracellular pathogens, such as viruses. This recognition is dependent upon the degradation and presentation of the antigen in association with MHC I molecules on the infected cells.

T$_{reg}$ cells are important for peripheral tolerance, and today the T$_{reg}$ family comprises many types of cells. Two major classes of T$_{reg}$ cells have been described;
the naturally occurring $T_{reg}$- and the adaptive $T_{reg}$ cells. Naturally occurring $T_{reg}$ cells (also known as CD4$^{+}$CD25$^{+}$FoxP3$^{+}$ Treg cells) arise in the thymus, whereas the adaptive $T_{reg}$ cells (also known as Tr1 cells or Th3 cells) may originate during a normal immune response [60]. Genetically determined or environmentally induced abnormality in $T_{reg}$ development, maintenance and function has consequences for several diseases, where allergy is one of them [61;62].

For most antigens, the primary encounter during an initial infection or vaccination leads to an immunological memory, comprised of a memory T cell pool with cells of different specificity. It was recently shown that during a secondary infection, where antigen-specific memory cells are activated, the existence of memory T cells is not enough to eliminate the pathogen itself. The pathogen removal was dependent on innate mononuclear phagocytic cells activated by the memory T cells. Interestingly, this re-exposure to a pathogen and activation of memory T cells, leading to activation of the innate arm of the immune system, also had a non-specific bystander effect on killing another simultaneous unrelated infection [4].

Another important function of T cells that was very recently proposed is the suppression of inflammatory cytokine production that usually takes place during a normal situation where the innate immune system has triggered an inflammatory response. It was shown that without T cells, mice die of uncontrolled inflammatory events. Unexpectedly, it was not only the $T_{reg}$ cells that could carry out this suppression, but also the conventional T cells. The suppression was dependent on direct contact between T cells and the MHC II complex from cells of the innate immune system [5].

**B cells**

B cells are lymphocytes belonging to the humoral immune response, an essential part of the adaptive immune system. The principal function of B cells is to produce immunoglobulins (Ig) or antibodies against soluble antigens. Immature B cells are produced in the bone marrow of most mammals. B-cell development occurs through several stages, each stage representing a change in the genes coding for immunoglobulins. Immunoglobulins exist in a membrane-bound form as the B-cell receptor (BCR) and in a secreted form, also referred to as antibodies. After reaching
the IgM\(^+\) immature stage in the bone marrow, the B cells migrate to the spleen where they mature into B lymphocytes. An antibody comprises two light (L) and two heavy (H) chains. In the H chain loci there are three regions, V, D and J, which recombine randomly in a process called VDJ recombination, to produce a unique variable domain in the immunoglobulin of each individual B cell. Similar rearrangements occur for the L chain locus, except there are only two regions, namely V and J. When a B cell encounters its cognate antigen, and receives an additional signal from a Th cell, it differentiates into either a plasma cell secreting large amount of antibodies, or a memory B cell, which will respond quickly to a second exposure of the same antigen. Recent studies have shown that TLR signaling in B cells is an important event for optimal IgM production and memory [11;63].

During an allergic reaction IgE is synthesized and secreted by B cells that have undergone heavy-chain class switching from IgM to IgE. Synthesis of IgE by B cells occurs at a low rate compared with other immunoglobulins, even in allergic individuals. However, in the nasal mucosa of patients with allergic rhinitis approximately 4% of the B cells and 12-19% of the plasma cells express IgE, in comparison with the situation in non-allergic individuals where less than 1% of the plasma cells and 1% of the B cells express IgE [64].

Recent advances have led to the clinical use of monoclonal antibodies that deplete or inhibit development of B cells. This relatively new strategy has shown to ameliorate disease severity in several hematological malignancies as well as autoimmune disorders [65].

**Cytokines and chemokines**

The pro-inflammatory cytokines and chemokines induced in an inflammatory response direct the deviation of T cells towards an adaptive effector response. Signaling by different TLRs gives rise to differential expression of cytokines. Whereas *e. g.* signaling through TLR9, recognizing bacterial DNA, induces high type I IFN [66], signaling through TLR2 can induce high levels of IL-13 [67;68].

Chemokines, named for their ability to induce directed chemotaxis in nearby responsive cells, are responsible for the migration of leukocytes. They are released by many different cell types and serve to guide cells of both the innate and the adaptive
immune system. Different cell types express different chemokine receptors [69], which is a way for the immune system to direct the right cell to the right place. Members of the chemokine family are categorized into four groups; CC-, CXC-, C- and CX3C chemokines. The work in this thesis has investigated the production of one inflammatory chemokine; CXCL8 or IL-8. Inflammatory chemokines are produced in response to infection to trigger the recruitment of monocytes, neutrophils and other effector cells from the blood to sites of infection or tissue damage. Their release is often stimulated by pro-inflammatory cytokines such as IL-1β that are highly expressed upon TLR ligation.
Type 1/type 2 responses

The Th responses have traditionally been divided into a Th1 or a Th2 type. Recently a third independent effector population, Th-17 cells, was discovered [70]. Other non-CD4+ cells also produce cytokines influencing the balance between the Th1 and Th2 cells, and the concept of type 1 and type 2 responses has now been extended to also include cells other than Th cells. These include CD8+ cells and cells of the innate system, where many refer to polarized macrophages as M1 and M2 cells [41] and NK cells to NK1 and NK2 [71]. Thus, the division of cells into type 1 or type 2 cells is in many cases an oversimplification, and the distinction between the different subsets is not always clear.

The type 1 response is mainly effective against intracellular pathogens, and IL-2, IL-12 and IFN-γ are critical for this type of response. IL-4 is the main cytokine critical for the type 2 response, which is effective against extracellular pathogens [72]. Th-17 cells express IL-17, a cytokine that has been implicated in many functions correlated to disease, amongst others bronchial responsiveness [73;74] and promotion of autoimmune inflammation [75]. Our understanding of how pathogens, such as bacteria and viruses, induce Th1-cell responses greatly exceeds our knowledge of how Th2-cell responses are induced by allergens and parasites. However, two very recent studies on Th2-cell development show that signals derived from basophils and eosinophils are directly involved in the induction of Th2-cell responses. One of the studies, dealing with the initiation of sensitization, proposes that allergen- or helminth proteases cleave a 'sensor' that stimulates basophils to migrate to the lymph nodes, and to produce Th2-type cytokines, leading to Th2-cell differentiation [59]. The other study shows that a secretory protein, produced by eosinophils [eosinophil-derived neurotoxin (EDN)], acts as an alarm signal that is a ligand for TLR2, skewing DCs into a Th2 type upon stimulation. Splenocytes from mice immunized with ovalbumin (OVA) and EDN show a strong Th2 skewing upon restimulation, with strong IL-5, IL-10 and IL-13, and no interferon-γ production, showing that the in vivo function of EDN is of importance [76].

The products of type 1 and type 2 cells act as autocrine growth factors for further expansion of these cells, as well as reciprocal inhibitory agents for the opposite cell type [77]. Polarized T cells also express different patterns of chemokine receptors, where CXCR3 and CCR5 are mostly associated with Th1 cells, whereas
CCR3, CCR4 and CCR8 are mainly expressed on Th2 cells [69]. An imbalance of the type 1/type 2 cytokines has been linked to disorders such as autoimmune [78;79] and allergic diseases [80;81]. Even though developmental and environmental factors are important for skewing of the adaptive responses [82-84], susceptibility to different diseases show a clear linkage to genetic factors [85-88].

Transcription factors are of importance for the cytokine-induced development of naive CD4⁺ T cells into Th1 or Th2 type. Signal transducer and activator of transcription (STAT) 6 and GATA3 are important for the induction of Th2 cells and IgE responses [89], while STAT4 and T-bet are important for Th1-cell development [90]. GATA-3 and T-bet can act directly on each other to regulate the balance of Th1/Th2 cells [90;91].

As the work in this thesis deals with expression of particular cytokines and chemokines there is a more thorough background on each of them in the following section.

**TNF**

Tumor necrosis factor (TNF) is a pleiotropic pro-inflammatory cytokine synthesized primarily by macrophages and monocytes, but also by activated T-, mast- and NK cells. It exists as either a transmembrane or a soluble protein, where the soluble form is the most potent one [92]. TNF is produced early in an immune reaction, and induces the production of other pro-inflammatory mediators. It is an important factor for the induction of labor, but is also produced by intrauterine tissues in response to microbial products. TNF is of major importance for the onset of premature labor in the context of infection [93].

In connection to allergic disease, polymorphisms in the promoter region of TNF, leading to higher TNF levels, have been shown to be overrepresented in allergic patients who are sensitized to multiple allergens, compared to those being monosensitized and to controls [94]. Further, TNF is involved in chronic immune-mediated inflammatory diseases, such as asthma, rheumatoid arthritis, Crohn's disease and psoriasis. As a result of these observations, neutralizing monoclonal antibodies specific to human TNF or the TNF receptor have been developed. They are showing promising results [95], even though the treatment of asthma and rheumatoid
Immune cells and mediators

arthritis seems to benefit patients with more chronic stages of disease, and be less efficient in early disease. This is a very recent area of therapeutic interventions where the modes of action in vivo still should be carefully evaluated.

**IL-1β**

Originally described as the endogenous fever molecule, IL-1β is a pro-inflammatory cytokine, with the ability to induce several genes usually not expressed in healthy individuals. IL-1β increases the expression of many cytokines, in particular TNF and IL-6, as well as chemokines and adhesion molecules [96]. It is produced by many cells and exerts its biological effects on almost all cell types [97]. IL-1β is a key mediator of many pathophysiological events characterized by inflammation and host-environment interactions, such as graft-vs-host disease [98], gut cancer [97] and rheumatoid arthritis [99].

**IL-6**

IL-6 is a cytokine with a broad range of functions on immune and nonimmune cells [100]. It is produced by several cell types including APCs such as macrophages, DCs and B cells, at sites of tissue inflammation where it can have a pro-inflammatory as well as an anti-inflammatory effect.

Classic signaling of IL-6 involves the binding of the cytokine to its membrane bound receptor IL-6R on target cells. The receptor complex for IL-6 also consists of at least one subunit of the signal transducing glycoprotein (gp)130, that also exists in a soluble form (sgp130) in human serum [101]. However, many of the biological activities assigned to IL-6 are performed in a process known as *trans-signaling*, where IL-6 binds to a soluble form of the receptor; sIL-6R, and thereby activate cells via membrane-bound gp130 [102]. Thus, the complex is an agonist for cell types that, although expressing gp130, are normally non-responsive to IL-6 itself [103]. The naturally occurring combination of sIL-6R and sgp130 can act as a regulatory system that modulates systemic responses to circulating IL-6 [104]. IL-6 activation leads to the activation of the Janus kinase (JAK)/STAT and MAPK cascades [101].
IL-6 has a regulatory role in the process of bridging innate and adaptive immunity through its influence on leukocyte recruitment, activation and apoptosis [105]. Impaired regulation of the transition from innate to adaptive immunity where IL-6 plays a pivotal role may affect disease outcome [2]. Indeed, dysregulation of IL-6 signaling has been shown to play an important role in the onset and maintenance of several autoimmune diseases, as well as cancer and osteoporosis [102;106;107]. IL-6 has further been implicated in determining the balance between the suppression and activation of allergic responses [108;109]. A role for IL-6 trans-signaling in supporting inflammation has been demonstrated in mice where mucosal T cells become less prone to go into apoptosis upon IL-6 signaling [110]. A possible mechanistic explanation for this IL-6 regulation was demonstrated in 2003 by Pasare & Medzhitov. They showed that, in the murine system, microbial signaling via TLRs blocked the suppressive effect of CD4⁺CD25⁺ Treg cells by inducing IL-6 release from DCs and macrophages [111]. Thus, they concluded that IL-6 seems to play a critical role in activation of T cells by overcoming the suppressive effect of Treg cells. In line with this, the de novo induction of adaptive Treg cells was recently found to be abrogated in mice due to IL-6 trans-signaling in T cells [112]. Th2 differentiation is promoted by IL-6 production, probably due to the ability of IL-6 to induce IL-4 and IL-5 production [113]. Simultaneously, IL-6 inhibits Th1 polarization by upregulating suppressor of cytokine signaling (SOCS)-1 expression to interfere with IFN-γ signaling [114]. Anti-IL-6R antibody therapy has shown promising results for treatment of inflammatory disorders [115].

IL-10

IL-10 is an anti-inflammatory cytokine, due to its ability of suppressing the release of pro-inflammatory cytokines by macrophages [116], but also through its ability to induce the synthesis of IL-1β receptor antagonist and soluble TNF receptors [117]. The suppression of IL-12 production by IL-10 is well established [118]. IL-10 is a product of multiple cell types, including Th1 and Th2 lymphocytes, cytotoxic-T cells, B cells, mast cells, monocytes and DCs. However in humans, monocytes and B cells are the major sources of IL-10 [119]. Treg cells exert their suppressive activity primarily via the release of IL-10 [120]. Isotype switching to IgG4 is enhanced by IL-
10 while IL-4–induced IgE is inhibited. IL-10 has been considered for therapeutic use in relation to various diseases, mainly for its ability to suppress inflammatory conditions and type 1 as well as type 2 related pathways [121].

 IL-10 can have different effects depending on environment and timing. Several studies have shown that IL-10 could play a role in the perpetuation of allergic inflammation, as high levels of IL-10 mRNA have been detected in the airways of asthmatic patients, as well as in the skin of patients with atopic dermatitis [122;123]. An IL-10 producing monocyte population, differentiating into alternatively activated macrophages (M2), has also been seen to be over represented in atopic individuals [124].

**IL-12**

 IL-12 is a pro-inflammatory cytokine produced by DCs and phagocytes early in response to infection. It forms a link between innate and adaptive immunity through its powerful effects on NK-cell function and T-cell development, where it promotes the production of IFN-γ and favors the differentiation of Th1 cells. IL-12 is a heterodimeric cytokine of 70kDa comprising of two subunits, p40 and p35. The genes encoding the two subunits are unrelated and located on different chromosomes. The p40 chain is often secreted in large excess over the p70 heterodimer. IL-12p35 is retained inside the cells and only secreted when associated with the p40 chain [72]. Many of the functions earlier attributed to IL-12 have now been shown to be carried out by a recently discovered cytokine, IL-23. IL-23 consists of two subunits, p40 that is shared with IL-12 and p19 that is specific for IL-23 [125].

 The IL-12 receptor (IL-12R) is expressed mainly by activated T- and NK cells. However, DCs [126] and B-cell lines [127] have also been shown to express the receptor, demonstrating the importance of IL-12 in the autocrine and paracrine effects on APCs. T-cell stimulation also drives IL-12 production in APCs through CD40-CD40L interactions [128;129]. IL-12 expression is controlled by upregulation of the IL-12R in response to a variety of cytokines including IFN-γ, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, as well as IL-12 itself. A combination of microbial, CD40, and cytokine stimuli [130] gives an optimal induction of IL-12 production.
A reduced ability to produce IL-12 has been shown to correlate with an allergic phenotype [131-134]. However, studies have also shown that the connection between IL-12 and allergy is not that clear, and can even be of opposite results, very much dependent upon the age of the subjects [134-136].

**TGF-β**

Transforming growth factor beta (TGF) -β1 and TGF-β2 are members of the TGF-β superfamily characterized by the presence of common sequence and structural features. TGF-β was first described as having an inhibitory action on cellular processes, but it was soon discovered that it can have both stimulatory and inhibitory effects depending on cell type and other signals present [137]. TGF-β is involved in the recruitment of blood monocytes and neutrophils to the lamina propria where they become intestinal macrophages, playing an important role in gut inflammation [138]. Eosinophil-derived TGF-β1 is critical in pulmonary immunity and fibrosis, and therefore plays an important role in asthma development, where it among other functions acts as a potent chemoattractant for monocytes [139]. A recent study, examining the role of TGF-β1 produced by eosinophils in connection to asthma, found that the production was regulated by a protein named peptidyl-prolyl isomerase (PIN) 1. Interestingly, from a therapeutic view, the authors found that inhibition of PIN1 in mouse and rat models of chronic pulmonary inflammation reduced allergic lung fibrosis [140].

TGF-β signaling is further a potent stimulus for driving the development of naïve CD25- T cells in the periphery into adaptive CD4⁺CD25⁺ T_{reg} cells, which have shown to, among other functions, be able to suppress Th1-mediated experimental colitis [141]. Recent data further show that TGF-β produced by T_{reg} cells interferes with polarization of the secretory machinery in Th cells toward APC, thus suppressing a crucial step of Th-mediated amplification of the immune response [142].

TGF-β1 is relevant to immunological development in offspring, as it is known to stimulate IgA synthesis [143;144]. In the context of an inflammatory cytokine milieu, TGF-β1 supports de novo differentiation of IL-17 producing T cells [145], cells that have been shown to be relevant in autoimmunity [75] and allergy [74]. Due
to its ability of acting stimulatory as well as inhibitory, the role of TGF-β in relation to disease is somewhat complex. TGF-β1 levels in breast milk have been positively/negatively associated with wheezing [146] and allergic disease [147] in offspring. TGF-β2 levels in breast milk have also been shown to be associated with both more and less sensitization in breast-fed infants [147;148]. A very prominent role for breast-milk TGF-β in the induction of tolerance to allergens was recently described in a mouse model of allergic asthma [149].

**IL-8**

IL-8 is a chemokine produced by various types of cells upon stimulation with inflammatory stimuli. It exerts a variety of functions on leukocytes, particularly in acute inflammation where it recruits and activates neutrophils [150].

IL-8 is present in high concentrations in breast milk, and is believed to have a function in the human neonatal gut as it remains measurable throughout simulated neonatal gastric and proximal intestinal digestion. When human fetal intestinal cells are stimulated with rhIL-8 *in vitro*, there is indeed an increase in cell migration, proliferation, and differentiation [151]. Despite concerns about potential adverse effects of IL-8 on the intestinal mucosa, there is evidence that normal levels of IL-8 have a physiological role in the gut of the newborn [152].
ALLERGY

The allergic reaction

Allergy is defined as a hypersensitivity reaction initiated by specific immunologic mechanisms [153]. Allergy can be antibody-mediated and/or cell-mediated. In most patients with allergic symptoms from mucosal membranes in the airways and gastrointestinal tract, the antibody belongs to the IgE isotype, and these patients may be said to have an IgE-mediated allergy or to be IgE-sensitized. The term *atopy* should be reserved to describe the genetic predisposition to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens commonly occurring in the environment [153].

Antigen presentation under the influence of IL-4 leads to expansion of Th2 cells and subsequent production of IL-4 and IL-13 (Figure 2). These cytokines induce Ig class switching in B cells from IgM to IgE, the latter subclass being responsible for allergic reactions [154].

*Figure 2.* Mechanisms responsible for IgE mediated allergy (Modified from Nat Rev Immunol 2008; 8:205-17). Reprinted by permission from Nature copyright Macmillan Publishers Ltd.
The head of the IgE (Fab portion) recognizes specific allergens. The activity of IgE is associated with a network of proteins; important among these are its two principal receptors, FcεRI (the high-affinity Fc receptor for IgE) and CD23 (the low-affinity Fc receptor for IgE), as well as several co-receptors for CD23, such as e.g. CD21 (Figure 2). The IgE binds to FcεRI and sensitizes these cells to allergens. FcεRI is expressed as a tetramer on mast cells and basophils, and as a trimer on human APCs, monocytes, eosinophils, platelets and smooth muscle cells. CD23, which is also found in a soluble form, is expressed on a variety of inflammatory cells, B-cells, but also epithelial cells. CD23 is implicated in negative as well as positive IgE regulation, where IgE-mediated feedback enhancement is one of the suggested mechanisms [155]. During this event, IgE antibodies administered together with their specific antigen can enhance the production of IgE recognizing this antigen by >100-fold [156].

Upon a second encounter with the same antigen, IgE antibodies bound to FcεRI are crosslinked and cause granules to rapidly empty their contents into the surrounding tissue. The preformed granules contain a variety of inflammatory substances such as leukotrienes, histamine, cytokines and chemokines. After the chemical mediators of the acute response subside, late phase responses can often occur. This is due to an inflammatory reaction causing migration of other leukocytes such as neutrophils, lymphocytes, eosinophils and macrophages to the initial site. The local inflammatory response can be seen as a red, itchy weal. This late reaction is usually seen 2-24 hours after the first reaction. Cytokines from mast cells may also play a role in the persistence of long-term effects.
The hygiene hypothesis and the role of innate immunity

The prevalence of allergic diseases has increased drastically during the past few decades. 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 play a crucial role. A notable fact is that it is not only “type 2 diseases”, such as allergy, that are increasing in the modern world, but there is also a strong global correlation between childhood wheezing and diabetes, where diabetes belongs to a more type 1 kind of disease [157]. A growing body of evidence suggests that something may lack in our modern way of living that has the capacity to provide protection against the development of such diseases. It is increasingly recognized that microbial colonization of the gastrointestinal tract, linked with lifestyle and/or geographic factors, may be important for the difference in disease prevalence throughout the world [157]. A nowadays widely accepted theory, based on the observations presented above and below, is the hygiene hypothesis, even though immunological mechanisms explaining this hypothesis are still not completely understood. Although this theory is not undisputable [158], several studies have shown that having older siblings [159-161], attending day care centers [159;162] and growing up on a farm [163;164] have protective effects against allergy development. Another fact that supports the hygiene hypothesis is that the allergic prevalence seems to increase in parallel with the affluence of a country, a recent example of this being East Germany [165].

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 [166]. Today it is believed that not only infections but also an early exposure to non-pathogenic microbes in our environment is involved in the development of the immune system [167]. The importance of stimulation of TLR4 by intestinal commensal flora in mice, in order to inhibit allergic responses to food allergens, highlights the role of the innate immune system in allergy development [168]. Indeed, it has been shown that different microbial exposures activating the innate immune system give rise to reduced IgE-specific adaptive immune responses [169]. A probable mechanistic explanation for this finding is that T-cell responses are usually
Allergy

characterized by a Th2 phenotype, unless there is a microbial stimulation of the DCs via TLRs, polarizing the CD4+ cells to a Th1 phenotype [170].

Multiple studies have shown that exposure to LPS in early life seems to have a protective effect against the development of allergy [171;172]. Something that has to be stressed in this context is that LPS might only be a marker of microbial exposure and not the causative agent. There could be other substances that are highly correlated to LPS exposure, actually conferring the immunological protective effect of microbial exposures. However, these substances would have to show a high correlation with LPS, as the protective effect has been observed in many different studies.

Lately, interest has focused on the Treg- and NKT 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. An impaired activation of the Treg cells, caused by decreased exposure to microbial agents, offers an alternative explanation for the increase in allergy frequency [173].

Our group has found that acquisition of Epstein-Barr virus (EBV) infection during the first 2 years of life is associated with a reduced risk of IgE sensitization, and that this effect is further enhanced by Cytomegalovirus (CMV) coinfection [174]. Primary infection with common virus infections such as EBV and CMV occurs within a few months to years after birth in developing countries, but only during the second and third decade of life in industrialised countries [175-178]. Crowding, poverty, and the widespread practice of breast feeding all encourage the early spread of CMV [177]. As CMV and EBV in their latent forms resides inside monocytes [179] and B cells [175] respectively, infection at a young age when the immune system is maturing, could most probably affect important events connected to allergic susceptibility.

Increasing epidemiological evidence show that obesity increases the risk of allergic and autoimmune diseases [180]. The reason for this link between obesity and allergy could also be caused by some other underlying factors, such as dietary factors or physical inactivity. However, it is known that the increasing body weight has an influence on the immune system. Obesity increases the levels of circulating IL-6, leptin, and TNF, secreted by white adipose tissue. Adiponectin, which decreases with increasing obesity, down-regulates the secretion of IL-10 from macrophages and
adipocytes. These changes in IL-6, leptin, and IL-10 are known to decrease the regulatory effect of T_{reg} cells resulting in decreased immunological tolerance to antigens, which would make individuals more prone to be sensitized to allergens. It is further speculated that in pregnant women, these obesity-induced immunological changes might be transmitted to the fetus by epigenetic inheritance (explained below), thereby increasing the risk of allergic disease [180].

Immigrants who move from a developing to an industrialized country maintain their lower disease risk of allergy [165], but also some other immune-mediated diseases such as inflammatory bowel disease (IBD) [181]. Intriguingly however, their offspring born in an industrialized country have an even higher risk of disease than the indigenous population of that country [182;183]. The reason for this phenomenon is unknown, but immunological events as a result of environmental exposures in childhood could be a clue. The difference between mother and child in childhood exposures could explain the higher incidence of disease in children to immigrants. One could speculate that since the immune system has been primed for multiple generations to fit the expected burden of exposures, this “unexpected” environment that the child grows up in could instead cause disease, as the immune system is not adapted to the new environment.

**Monocytes/macrophages and disease**

Monocytes/macrophages play a pivotal role in many diseases such as cancer [184], parasite infections [185], and rheumatoid arthritis [186]. The role of the two different monocyte subpopulations; the classical CD14^{++}CD16^{-} subpopulation and the pro-inflammatory CD14^{+}CD16^{+} subpopulation, in allergic disorders is not fully elucidated. One study showed that blood monocytes from untreated adult asthmatics have a higher percentage of the pro-inflammatory CD14^{+}CD16^{+} subset than non-allergic subjects [187]. Atopic eczema has also been linked to an increased population of CD14^{+}CD16^{+}, which was diminished in connection to clinical improvement [188]. However, another study on atopic dermatitis did not find any differences in the two monocytic subsets or in TLR levels, but an impaired IL-1β and TNF production in response to a TLR2 ligand [189]. A recent study [124] showed that an IL-10-producing monocyte subset is over-represented in allergic compared to non-allergic
individuals, and that these monocytes differentiate into alternatively activated monocytes (M2). Another study found that M2 inhibit the generation of M1, and that this inhibition is dependent upon CCL17 and IL-10 production in M2 [185;190]. As IL-10 has been shown to be over-expressed in other studies of allergic individuals [191;192], and as M2 support Th2-effector functions [41], this would provide a possible explanation for how IL-10 could act in promoting allergic disease, rather than acting anti-inflammatory. A study in mice infected with a gastrointestinal parasite showed that activated Th2 cells induce macrophages, required for parasite clearance. Their study emphasizes the role of macrophages as essential effector cells in protective Th2 responses, and provides an evolutionary role for the alternatively activated M2 cells, believed to play a role in allergic disease [185].

Pattern-recognition receptors and disease

Multiple studies have investigated the role of TLR4, CD14 and sCD14 in relation to allergic disease. The reason for this interest is the involvement of these receptors in immunological responses to infections, since exposure to microbial compounds is suggested as being a protective factor for allergy development. Moreover, increasing evidence shows that TLRs also recognize host-derived (endogenous) ligands, a fact that also connects TLRs to diseases that may not have an etiology that is associated directly with infection [193].

The gene coding for TLR4 is highly polymorphic, and to date 44 TLR4 single-nucleotide polymorphisms (SNPs) have been identified [194]. Some of the investigated polymorphisms of TLR2 and 4 have been linked to altered systemic inflammatory reactions [195], cancer [194], viral responses [196], inflammatory bowel disease [197;198] and allergy [199;200].

Polymorphisms within the CD14 receptor have been found to be associated with Crohn’s disease [201] and allergy [202-204]. The results from studies of associations of sCD14 with allergic disease are inconsistent [205-208]. This variation in direction of association may depend upon the dual role of sCD14, depending on concentration and environment. Thus, while it may have systemic anti-inflammatory effects, it can also exert a pro-inflammatory influence in specific tissues to increase resistance to bacteria [29]. Another fact, that may complicate interpretations of the
observed associations with disease, is that there are many other influencing factors and confounders, including gender-gene-environment interactions [207]. Indeed, multiple studies show that polymorphisms can be differently important depending on the environmental exposure [200;209].

Gut

Immune modulation of the gut is influenced by factors derived prenatally from the placenta, and after birth from the breast milk and oral contact with the neonatal environment [210]. The fetal gut is structurally mature from week 19 of gestation, and all cellular components of the gastro-intestinal immune system are already present at birth. However, at birth, the gastrointestinal tract is functionally immature and immunoincompetent. After birth, it undergoes rapid growth and maturational changes, including the colonization with an immense benign biotic mass [211]. This protects the gut from colonization of pathogens, and is also believed to be an important stimulus for the developing immune system. During homeostatic conditions, these commensal bacteria do not evoke inflammatory immune responses. However, in some cases such as IBD, this flora can also cause disease [212]. The important role of the gut for the developing immune system is underlined by the B- and T cell development taking place in the fetal gut [210].

The importance of very early innate events in the context of stimulation of the gut has been convincingly shown in several studies. One study demonstrated that TLR4 dependent signals, provided by the intestinal commensal flora, can inhibit the development of allergic responses to food antigens [168]. Another study [31] showed that a rapid postnatal acquisition of epithelial tolerance to microbial ligands is dependent upon LPS activation of intestinal epithelial cells (IEC). This homeostatic state in the gut was dependent on LPS exposure in both studies. Factors such as vaginal delivery, oral exposure to endotoxin, and the presence of TLR4 were coupled to protection, while antibiotic treatment and lack of TLR4 showed opposite results. More supporting evidence for the importance of early stimulation of the infantile gut comes from studies using pro- and pre-biotic treatment. These studies show that probiotic treatment given to the mother during late pregnancy, and to the young infant after birth significantly protected against atopic eczema at the age of 2 [213;214]. Indeed, differences in the gut flora between allergic and non-allergic children have
Allergy

been detected in several studies [215;216]. Our group recently found that children developing allergy up to five years of age were significantly less often colonised with lactobacilli during their first two months compared to non-allergic children [Sjögren et al, unpublished data].

Another immunological influence of the commensal flora on the immune system is the induction of sCD14 release from monocytes and DCs. A recent study showed that sCD14 induced by commensal bacteria inhibits birch allergen-induced Th2 differentiation by suppressing IL-13 production [217]. IL-13 is known to diminish LPS-mediated cell activation by downregulating TLR4 signaling [218]. Keeping in mind that TLR4 signaling is important for gut homeostasis [31;168], this induction of sCD14 by the commensal bacteria, leading to lower IL-13 levels, could be one of the molecular mechanisms behind the beneficial effects of a balanced gut flora.

**Gene-environment interactions**

Growing up on a farm, with a constant exposure to microbial products present in an environment with live-stock, is associated with protection against allergy development later in life [219]. One of the main causes for this protective effect has shown to be the consumption of farm milk [163;220]. However, Eder et al [200] demonstrated that genetic variations in the TLR2 gene are important for the susceptibility of farmer’s children to allergic disease. The protective effect of endotoxin exposure in early life in relation to allergy development is further dependent upon genetic differences regarding the LPS recognition receptor CD14 [203].

In relation to allergy development, another noteworthy finding exemplifying the importance of gene-environment interactions was reported in 2003 [221]. McIntire et al found that individuals with specific alleles of HAVCR1, a gene encoding the T cell, Ig- and mucin domain (TIM)-1 glycoprotein expressed preferentially on Th2 cells, were protected against allergic disease. There was an increased protection in individuals previously exposed to the hepatitis A virus (HAV), probably due to the fact that TIM-1 also functions as the receptor for HAV [222].
Epigenetics

Although much effort has focused on the gene-environment interactions, there is a growing body of evidence suggesting that environmental influences may extend beyond the DNA sequences of our genes. Something that underscores the important role played by the environment in shaping the genetic constitution is that monozygotic twins are genetically indistinguishable early in life, but with age exhibit substantial differences shown to be linked to events such as replication, transcription, recombination and repair of DNA [223]. The emerging field dedicated to the study of this form of biologic regulation is termed “epigenetics”. Epigenetics constitutes the study of changes in gene expression not accompanied by alterations in DNA sequence [224]. It has been shown that environmental factors that alter epigenetic events such as methylation processes play a role in disease susceptibility [224]. As gene-environment interactions play a pivotal role for allergy development, this field of research opens up and broadens our way of looking at how gene expression is influenced by the environment. Epigenetic events in form of cellular acetylation have further been shown to be important for maintaining the pre-established Th1/Th2 like responses. This fact offers an epigenetic regulatory mechanism explaining how the environment could influence the maintenance of an excessive Th2 skewing, characteristic for allergic diseases [225]. Another recent study in mice, confirming the important role of epigenetics in the gene-environment context, shows that in utero or neonatal exposure to a chemical can change the phenotype of the offspring by stably altering the epigenome. Interestingly, this effect could be counteracted by maternal dietary supplements [226].

Microbial recognition for therapeutic interventions

New insights of the role of TLRs in relation to disease are of interest for various therapeutic strategies. The possibility of using TLR agonists as vaccine adjuvants and as immunomodulators is now a very active area of research, having shown promising results [227]. The TLR ligand/allergen vaccination approach depends on the capacity of treated patients to respond to TLR ligands, and at the same time recognize the used antigen. The advantage of this action is that while the allergen is being processed, the TLR stimulation will give rise to a type 1 response counteracting a type 2 response to the antigen used [136]. CpG, recognized by TLR9, has demonstrated preventive and
therapeutic activity in murine models of atopic airway inflammation [228-231]. A synthetic TLR4 ligand acting as adjuvant in vaccination programs has proven safe and active in human trials [232], and TLR4 and TLR2 agonists have proven to ameliorate allergic airway symptoms in mice [233-235]. For a successful use of TLRs as targets in the context of allergic disease, there is still however a lot of important knowledge lacking.

Another potential therapeutic strategy regarding allergy is to take advantage of the interplay between some species of helminths and the immune system. During helminth infection there is a selective immune suppression that gives rise to a regulatory environment which also protects from other diseases such as allergies. Helminth infections seem to modulate dendritic cells and macrophages to induce a T-cell hyporesponsiveness [236]. Studies in mice have shown that the mechanism whereby worm infection modulates immunity is dependent on different kinds of macrophages that can prevent inflammation [185], and that it is these macrophages that can render infected mice refractory to induced colitis [237].

Factors that modulate the induction and function of T_{reg} cells are other potentially interesting areas for therapeutic interventions regarding inflammatory diseases. IL-6 trans-signaling, that has recently been found to abrogate the induction of T_{reg} cells and inhibit apoptosis in T cells in Crohn’s disease [110;112], could be one of the targeted pathways in order to restore a balance between protective and damaging immunity. Indeed, by using neutralizing antibodies towards the IL-6R or sIL-6R, inflammatory conditions have shown to be ameliorated [115].
IMMUNOLOGY OF PREGNANCY

Implantation is an early critical process where the fetus adheres to the wall of the uterus. Human implantation is unique, and there is no ideal animal model for human placentation [238]. This uniqueness is characterized on the maternal side by a spontaneous and massive decidualization, the process by which the inner membrane of the uterus, endometrium, transforms itself into a dense cellular matrix. On the embryonic side there is an almost unlimited invasive process taking place [239]. The implantation requires a synchronized cross-talk between maternal and embryonic tissues in terms of molecular and cellular events resulting in healthy uterine growth and differentiation, invasion and placental formation. Besides the main role of sex steroids, the complexity of embryo implantation and placentation is exemplified by the number of cytokines and growth factors with established roles in these processes. Disturbances of expression and action of these factors can result in absolute or partial failure of implantation and abnormal placental formation [240].

During pregnancy, immune responses belonging to the adaptive part are suppressed, while the innate branch of the maternal immune system is activated [241]. The activation of the innate system leads to an increase in numbers of granulocytes, and both monocytes and granulocytes enhance their phagocytic capacity [242]. The cytokine balance during pregnancy is very complex and has been debated over the last decade. Until recently, it was postulated that successful pregnancy induces an immune type 2 bias [82]. However, when summarizing old and recent data, it turns out that for reproductive success, both type 1 and type 2 immunity are important in a dynamic interplay [243]. A recent study investigated how the maternal IFN-γ and IL-6 levels, used as markers of the type 1/type 2 immune status, fluctuate during pregnancy. They found that the type 1 bias with higher levels of IFN-γ was dominant at the beginning of pregnancy, balanced in the middle of pregnancy and replaced by a type 2 bias with higher IL-6 levels at the end of pregnancy [244]. The higher level of IL-6 at the end of pregnancy not only promotes a type 2 immunity, but are also important for the pro-inflammatory events taking place during parturition [245].

Inflammation has long been recognized as a key feature of both preterm, but also term labour. An influx of inflammatory cells into the uterus and elevated levels
of pro-inflammatory mediators such as IL-6 and IL-8 are key events during parturition [246]. These mediators are especially important in cervical ripening, a prerequisite for parturition [245]. The inflammatory characteristics of labor are further demonstrated by the role for NF-κB in the physiology and pathophysiology of labor [247].

An interesting observation, demonstrating the effect of pregnancy on the immune status on the mother, is that the more children women give birth to, the less likely the women are to be IgE sensitized [248-250].

The immune system of the newborn child is also believed to be Th2 skewed [251]. However, IL-5 and IL-13, two important cytokines for the Th2 development are showing different patterns at birth where IL-5 is being suppressed, while IL-13 is not, illustrating the difficulty in attributing a strict Th2 type to the immune system of the neonate [252].

**Maternal influences on the child**

*In utero*

The potential effects of environmental stimuli on the immune system are greatest in early life including fetal life, when the immune system is going through important developmental processes. Maternal influences during fetal life could be particularly important for the development of immune regulation and tolerance induction. Studies indicate that maternal allergy influences the *in utero* environment [253], which could further shape neonatal immunity. The fact that maternal but not paternal total IgE levels correlate with cord blood (CB) total IgE [254;255] also indicates that maternal factors, placental factors, or both have an impact on perinatal allergic sensitization. Allergens have been shown to cross the placental barrier [256]. A fact that further illustrates this event is that T cells from unborn as well as newborn babies can exhibit a memory phenotype, specific for common allergens [257]. However, studies have failed to show a consistent dose-response relationship between allergen exposure in fetal life and allergen specific reactivity in cord blood. A probable cause for this lack of correlation is that most of the allergens that cross the placenta are retained in the placenta [258]. Studies comparing exposure levels to
pollens indicate that even though there was a trend towards increased risk of sensitization in children whose mothers were exposed to high allergen levels during pregnancy [259], the effect was not as evident as if the children were exposed to high levels during early infancy [260], or if the mother was suffering from allergic disease [261]. A very recent study questioning the sensitization of the child during the in utero period interestingly showed that the allergen-specific IgE in cord blood is NOT produced by the fetus, but seems to be the result of transfer of maternal IgE to the fetus [262]. Following the observations of that study, our earlier results of presence of IgE in the placenta [263], strongly suggests that the IgE found in placental tissues is of maternal, and not fetal origin.

However, a murine study by Hertz et al suggests that in utero effects of a maternal allergic phenotype do exist. They could observe that offspring to OVA-sensitized mothers had an increased Th2 response at birth. Further, when immunized with a novel antigen, the young mice born to allergic mothers had an enhanced allergic response as compared to offspring derived from non-allergic mice [264]. The reason for this influence of maternal allergy is not fully understood, but it has been demonstrated, among other things, that the fetus is exposed to intact amniotic fluid IgE via the gastrointestinal tract [265].

Studies of prenatal exposure in humans have shown to result in measurable phenotypic responses in the newborn [164;266;267]. Farming environment, levels of endotoxin in house dust and presence of cat or dog have been suggested as factors affecting the IFN-γ and IL-6 producing capacity during the first 3 months of life [267], a fact that could have implications for allergy development. In a similar study looking at exposure of stable environment, the strongest protective effect against IgE sensitization of the child was found for maternal stable work during pregnancy [164]. They could further observe that maternal exposure to stable environment during pregnancy, rather than the current exposure to several farm characteristics, was associated with increased gene expression of CD14, TLR2 and TLR4 in the children. Results, in line with this, were further established in a study where maternal allergies were associated with lower levels of CD14, TLR2 and TLR4 mRNA in cord blood samples [268]. The idea that LPS exposure during pregnancy can modulate the development of allergies in the offspring has also been demonstrated in mice [269;270]. Another indication of an in utero effect on child sensitization is that IgE in
cord blood is reduced with increasing birth order [255]. The importance of gene-environment interactions in the context of allergic disease also apply to the in utero environment. A study recently suggested that different IL-13 gene polymorphisms in the child modify the effect of in utero exposure to tobacco smoke on persistent wheeze and asthma in childhood [271].

Understanding the maternal influences on the child is of major importance when it comes to the important area of how to avoid sensitization in the child. The increased knowledge of tolerance induction has shifted the former belief of allergen avoidance into a current view of a more deliberate exposure. As an example, a very recent study showed that maternal intake of fish oil during pregnancy decreased several compounds, relevant to allergic susceptibility, in the CB, such as IL-4 and IL-13 [272].

A murine study from 2001 proposed that breast feeding by mothers that are immune to an antigen suppresses the development of an allergic response to the same antigen in the pups [273]. A more recent study [274] further investigated the influence of preconception immunization of the mother on sensitization in the newborn. Neonatal sensitization was induced by allergen exposure during pregnancy and breastfeeding. Maternal immunization efficiently decreased the passage of free antigens through breastfeeding, which inhibited the enhanced IgE antibody response after postnatal antigen exposure in the pups. Together, these findings suggest that early life sensitization could be avoided by preventive vaccination of the mother.

Breastmilk

Human milk is the communication medium between the maternal immune system and the child. It has several immunological roles for the child; it promotes maturation of the gut barrier function, protects from pathogenic insults, and can also actively attenuate early inappropriate inflammatory reactions [275]. Colostrum is the specific first diet of mammalian neonates, and it contains a variety of antimicrobial substances that function to protect the lactating mammary gland, as well as the newborn child at a time when its immune system is still immature. Human milk contains its own immune system with a wide range of soluble and cellular factors. The cellular compartment comprises 55-60% macrophages, 30-40% neutrophils and
5-10% lymphocytes (where >80% are T cells) [276]. In animal models, lymphocytes from the milk are able to traverse the neonatal intestine, showing that the breast milk indeed can have a systemic influence on the child [277].

Mammary epithelial cells are the source of chemokines such as IL-8 and RANTES [278], and as a part of a functional immune system, lactating mammary glands also have a local production of antibodies, mainly secretory IgA (sIgA). These antibodies are highly directed against infectious agents in the mother’s environment, mainly respiratory and intestinal pathogens that the child most likely will encounter [279]. Colostrum and mature milk contain many cytokines and chemokines, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TGF-β, IFN-γ and TNF [280-283]. These cytokines and chemokines, in combination with the ingested maternal immunoglobulins and nonspecific antibacterial components, likely play an important role in modulating immunologic development in the newborn child.

Additionally to child survival, breastfeeding reduces the risk of developing various immune-related conditions [276]. Its connection to allergic disease is somewhat complex. It is speculated that in IgE-mediated allergy, manifested as dermatitis or asthma, breastfeeding is less protective than in non-IgE-mediated gastrointestinal disease. Breast milk has been suggested to be a transmission route of allergic susceptibility from mother to child, even though these results are from animal model systems [284;285]. In one of the studies both serum and cell membrane-bound IgE were undetectable in newborn foals before colostrum uptake and peaked on days 2–5 after birth. At the peak, the IgE levels in the mothers’ milk were strongly correlated to the serum IgE levels in offspring, demonstrating that the milk is most probably the transference medium [284]. Breast milk contains dietary antigens [286], and a very recent murine study showed that airborne antigens that the female is exposed to during lactation can also be found in the breast milk [149]. In that study they demonstrated an interesting tolerogenic effect where pups receiving breast milk containing airborne allergens acquired an antigen-specific protection from allergic airway disease later on.
The sibling effect

Several studies have shown that having older siblings is protective against the development of allergic diseases [165]. The most widely accepted explanation for this phenomenon is that having older siblings results in higher levels of exposure to common infectious agents at a younger age. Despite this, no specific infectious agent or exposure has been linked convincingly and consistently with allergy risk [287]. A study investigating the protective effect of older siblings interestingly showed that the onset of IgE sensitization was delayed in younger siblings, but not prevented per se, suggesting that the apparent protective effect of older siblings on allergic diseases reported elsewhere only delays the onset of IgE sensitization [288]. Recent interest in the mechanisms behind the sibling effect has turned the focus to prenatal events. Thus, at birth, IgE levels in cord blood and cellular responses to allergens show a decrease with increasing birth order, indicating that the sibling effect may instead have its origin in utero [255].
THE PRESENT STUDY

AIMS

Increasing data suggest that immunological events taking place in a pregnant woman have a large impact on the immune system and allergy development of the neonate. Further, early life innate immune reactions are nowadays considered to be of major importance in relation to allergic conditions. The work presented in this thesis concerns several immunological aspects regarding the maternal immune system and its influences on the immune system of the newborn. More precisely, paper I and II consider maternal immune characteristics that could influence the child, paper III and IV study the maternal influence on the child, while the last paper (V) considers the children when they have a manifested allergic disease.

The precise aims of each paper are listed below

**Paper I:** Pregnancy is an immunological challenge for the maternal immune system. Disease could further modulate the maternal immune system during pregnancy, which could have implications for the fetus. In paper I, we wanted to investigate how *in vitro* PBMC cytokine responses (IL-1β, IL-6, IL-10 and IL-12) are influenced by pregnancy and allergic status in women, before and after microbial stimulation.

**Paper II:** Children of immigrants from developing countries have a higher risk of certain immune mediated diseases than the indigenous population. Here, we wanted to investigate if maternal immunological characteristics, in terms of TNF, IL-1β, IL-6, IL-8, IL-10 IL-12, TGF-β1 and 2 and sCD14, are influenced by factors such as maternal country of birth, and more recent exposures such as number of siblings.

**Paper III:** Maternal allergy could influence the immune system of the fetus. Here, we wanted to examine how a maternal allergic phenotype influences the innate immune system of the newborn. Therefore, we investigated CD14, TLR2 and TLR4 surface expression on monocytes in CB, as well as cytokine production (TNF, IL-1β, IL-6, IL-8, IL-10 and IL-12), following *in vitro* culture of CBMC with LPS or PGN.
**Aims of the study**

**Paper IV:** In paper III we found a defective IL-6 response to PGN in newborns from allergic mothers, in spite of unaltered TLR expression. This finding suggested that there could be important events downstream the TLRs accounting for the decreased IL-6 levels. Here we aimed at analyzing the important intracellular component; p38-MAPK, involved in both LPS and PGN-mediated signaling. We further aimed at investigating how the responses to innate stimuli develop with age, by examining the same group of children at birth and at 2 years of age.

**Paper V:** As we in paper II and III revealed a defective response to PGN in children of allergic mothers at birth, as well as at 2 years of age, we here wanted to investigate children at 5 years of age when they have a more manifested disease. Further, we wanted to study the monocyte population following microbial stimulation in more detail, with regard to the distribution of monocyte subsets. To do this, we analyzed monocytic CD14, CD16, TLR2 and TLR4 expression, as well as PBMC production of TNF, IL-1β, IL-6, IL-8, IL-10 and IL-12 upon *in vitro* stimulation.
METHODOLOGY

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

- Clinical evaluation (paper I, III and IV)
- Phadiatop/Cap-FEIA™ (paper I, III and IV)
- Skin prick test (SPT) (paper I, III and IV)
- Questionnaire (paper I-IV)
- ELISA (paper I-III)
- ELISpot (paper I)
- Separation of CBMC and PBMCs (paper I, III and IV)
- In vitro activation of CBMC and PBMCs (paper I, III and IV)
- Cytometric Bead Array (CBA) for cytokine and chemokine analysis (paper II-IV)
- Flow cytometric analyses (paper II-IV)
- Statistical analyses (paper I-IV)

The studies were approved by the ethics committees of the Karolinska University Hospital, Stockholm Söder Hospital and the Uppsala region of Sweden. All families gave their informed consent.
RESULTS AND DISCUSSION

Impact of pregnancy and allergic status on cytokine responses (I)

The involvement of type 1 and type 2 cytokines in allergic disorders has been extensively studied [289;290]. In paper I we also wanted to assess the role of the innate immune system with regards to allergy and pregnancy. This was achieved by looking at production of IL-1β, IL-6, IL-10 and IL-12 in response to LPS in allergic and non-allergic women. As pregnancy induces several innate and pro-inflammatory components of the immune system [241], we also compared cytokine levels at pregnancy with 2 years after.

We demonstrate here that IL-1β, IL-6, IL-10 and IL-12 production in PBMCs from adult women are not influenced by the allergic status, neither for unstimulated cells, nor for cells stimulated with a microbial substance (LPS). We also show that the lack of differences attributed to allergic status is not affected by pregnancy. Differences in receptors recognizing microbial products have been shown to influence the likelihood of developing allergic diseases [200]. A subsequent diversity in cytokine production would be a natural consequence of these differences [199;291]. However, our results indicate that either these differences are not seen in adult women, or alternatively, differences in receptor expression does not arbitrarily mean that the final response (here cytokines) would have to differ.

Further, we could not find any differences between allergic and non-allergic individuals in terms of IL-10 or IL-12 production when stimulating the adaptive immune system (PHA and allergen extracts). This lack of differences in allergen-induced responses between allergic and non-allergic individuals might be explained by the fact that single allergen-specific T cells constitute a very small fraction of the whole CD4+ T-cell repertoire [292]. Another possible explanation is that as the allergen extracts used were not tested for LPS contamination, an allergen-specific reaction might have been masked by an innate response induced by LPS. Earlier studies in our group have shown that a diminished number of IL-12 producing cells in CB, in response to allergen extracts, is associated with IgE sensitization at 2 years of age [133]. This could also be the result of a failure to respond properly to LPS, and not to the specific allergens. The finding of no differences in IL-12 production in
response to allergen extracts between allergic and non-allergic women in paper I could be explained by the same reasoning. This would agree with results showing that monocyte-derived DCs from highly atopic adult individuals are not impaired in their IL-12 responses to toll-like receptor ligands [136].

Further, we compared the cytokine pattern in women during pregnancy and 2 years after pregnancy. We observed elevated spontaneous innate cytokine levels (IL-1β, IL-6) during pregnancy, findings in line with previous publications illustrating the activated innate immune components during pregnancy [241]. Also here, we found that PHA, a T-cell stimulus, induced lower production of IL-10 and IL-12 during pregnancy than 2 years after. This is in agreement with the theory of a suppressed adaptive immunity during pregnancy. IL-10 and IL-12 are produced by cells of both the innate and the adaptive immune system. Thus, the lower IL-10 and IL-12 production in response to PHA could be attributed to a diminished production of these cytokines from T- and B cells, respectively. However, stimulation of T cells has been observed to upregulate APC activity via stimulation through CD40-CD40L interactions [293]. Then, the diminished IL-10 and IL-12 production during pregnancy, in response to T-cell stimuli, could also be originating from APCs that have received lower stimulation signals from “pregnancy-suppressed” T cells.

Whether IL-12 production is influenced by pregnancy is debated [294;295]. We did not find any statistically significant alterations in IL-12 production due to pregnancy, neither for unstimulated cells, nor for LPS- stimulated cells. No differences in IL-1β and IL-10 production/producing cells in response to LPS were detected, when comparing the pregnant state of the women versus the non-pregnant state. The greater effect of LPS on IL-6 production 2 years after pregnancy than during pregnancy is perhaps an unexpected result. It is possible that pregnancy-associated immune modulation results in an increased ability to respond to microbial stimuli after pregnancy. It should also be noted that cells of the adaptive immune system express PRRs that could respond to microbial stimuli such as LPS [11;13;296].

Studies have pointed towards an important role of pregnancy and birth regarding immunological changes in women [248;249]. In this study, we found significantly lower total serum IgE levels in allergic women 2 years after than during pregnancy. Numerous studies have shown that having older siblings is protective
against the development of allergic diseases [165], which is usually attributed to a “healthy” immunological shift due to early acquired infections. In the light of our findings however, it is tempting to speculate that the protective effect of having older siblings is at least partially explained by the modulated immune system in the mother following multiple pregnancies.

Maternal exposures and breast milk characteristics (II)

Populations in high infectious exposure countries are at low risk of immune-mediated diseases such as Crohn’s disease [181] and allergy [165]. This low risk is maintained upon immigration to an industrialized country, but the offspring of such immigrants have a higher immune-mediated disease risk than the indigenous population [182;183]. In paper II we hypothesized that early life exposures in a developing country shape the maternal immune system, which could have implications for the offspring born in a developed country with less infectious exposures. To measure one means of maternal influence, we measured breast milk compounds that are abundant in colostrum, and have been shown to be of relevance to the immune system of the newborn. It has previously been shown, in a murine study, that breast milk is a transmission route for allergy susceptibility from mother to offspring [285], indicating that mediators in the breastmilk indeed reflects the immune status of the mother.

To compare women with different childhood exposures, breast milk was collected from 64 mothers who either; 1) grew up in Sweden, considered to have had low levels of microbial exposures in childhood or 2) spent their first ~10 years of life in a developing country, but now living in Sweden, considered as having been exposed to higher levels of microbial substances in childhood.

Our results show that immigrants from a developing country had statistically significant higher levels of breast milk IL-6, IL-8 and TGF-β1 compared to the native Swedish mothers (Figure 3). Further, regardless of maternal country of birth, a larger number of previous pregnancies was associated with down-regulation of several substances, statistically significant for soluble CD14 and IL-8. The results suggest that maternal country of birth may indeed have immunological consequences still in adulthood, which could be relevant to disease risk in offspring. Such a mechanism
may explain the higher immune-mediated disease risk among children of migrants from a developing to developed country.

The mechanisms behind the differences in breast milk compounds due to childhood exposures are not clear. However, one could speculate that epigenetic events play a role. It has been shown that environmental factors alter epigenetic events such as methylation processes [224]. LPS exposure that have been negatively associated with allergic disease [219] plays a role in gene silencing, acting as a potent inflammogen that stimulates another epigenetic event; acetylation of histones [297]. If the immigrant mothers were exposed to high levels of LPS and similar substances during childhood, a probable cause for the long time effects could be at the epigenetic level.

With the design of our study, the possibility that contemporaneous exposures such as dietary habits and stress could have influenced the results can not be ruled out. However, adjustment for the person-per-room ratio (crowding) did not notably influence the reported associations. It is known that lifestyle factors influence the composition of the gut flora [298], which might be transferred to the offspring. However, this fact cannot readily explain the higher incidence of inflammatory diseases in offspring to immigrants when they are born in a developed country instead of their native country.

To have a “control group”, we also included women who were raised and gave birth in Mali (Africa). Unfortunately, we did not have access to complete demographic data of these women, and the results from that group were therefore not included in the paper. The breast milk components that were investigated in these women living in Africa did not differ from the ones in breast milk of women who had migrated to Sweden (Figure 3). Interestingly, and to our surprise, a comparison of the milk from women living in Africa with the milk from native Swedish mothers, revealed no differences regarding inflammatory mediators such as IL-6 and IL-8. However, TGF-β1 and sCD14 levels were higher in the women living in Africa, compared to native Swedish mothers. The observation of higher TGF-β levels in breast milk, both from African- and immigrated mothers compared to women being raised in Sweden, is of great interest as inflammatory diseases are more prevalent in the western world compared to developing countries. Since breast milk TGF-β was recently shown to be of major importance for tolerance induction and protection from...
allergic asthma in mice, these higher breast milk TGF-β levels in women raised in developing countries might provide a partial explanation to why children born in Africa have a lower incidence of allergy than children in the western world. The finding of no differences between women living in Africa and women who had migrated to Sweden, confirm our hypothesis of the important childhood exposures in modifying the immune system. Additionally, it argues against the possibility of current exposures as being the cause of differences found between immigrant- and Swedish mothers.

![Figure 3. Summary of results including women from 3 groups; native Swedish mothers, immigrated mothers in Sweden and women living in Mali.](image)

Even though we did find differences in breast milk composition due to maternal country of birth or previous pregnancies, the major question is how these differences affect the newborn child. To investigate this issue we stimulated cord blood mononuclear cells (CBMCs) with the breast milk alone, or with the addition of microbial stimuli, and analyzed the cytokine responses. Preliminary data from this ongoing study indicate that breast milk from native Swedish mothers and African women living in Mali show no differences in their stimulatory capacity (Figure 3). However, breast milk from the immigrant mothers, together with LPS stimuli, induced higher IL-6 levels than breast milk from the two other groups. As breast milk
from immigrant mothers had higher levels of IL-6 and IL-8 than the Swedish native mothers, these augmented responses could explain why children of immigrants have a high risk of inflammatory diseases. Intriguingly though, we would have expected the breast milk from African women to have the same stimulatory capacity as the breast milk from mothers who had migrated from developing countries, as there were no differences in breast milk composition. Perhaps there are other factors, not measured in this study, which are lost or increased upon migration to a developing country, which would explain the differences in stimulatory capacity of the breast milk.

It is possible that the well known sibling effect seen for diseases such as allergy [159;161] and multiple sclerosis [299] influences disease risk in the child through the action of previous pregnancies on maternal immune characteristics. Further, a maternal influence on neonatal immunity has been shown in previous studies, although mainly in animal studies. Both in utero [164;257;264;266;267], as well as breast-milk related effects have been reported [149;284]. The difference in breast milk composition in this study is of course only one part of the maternal influences that the mother exerts on the child. However, we believe that the immune status of the mother is reflected in the breastmilk, and together with the influences of the in utero environment has a large impact on the immune system of the newborn. We further suggest that the imbalance in pattern of early life infection between mother and offspring among immigrants from a developing to an industrialized country may help to explain the increased risk of some immune-mediated disease in such offspring.

**Neonatal immune responses to microbial stimuli (III)**

TLR-mediated signaling plays an important role in diminished IgE-specific immune responses. This, and the fact that susceptibility to allergic diseases can be transferred from mother to child in utero [253;257], prompted us to look at how innate CB responses to microbial stimuli were affected by maternal allergy. More precisely, in paper III, we investigated the influence of allergic status in the mother on the monocytic CD14, TLR2 and TLR4 expression in their newborn babies upon microbial stimuli (LPS and PGN). We also measured cytokine and chemokine (TNF, IL-1β, IL-6, IL-8, IL-10 and IL-12) responses from stimulated PBMCs using the CBA method.
Results and discussion

Even though we could not detect any significant differences in CD14, TLR2 or TLR4 receptor expression between mothers with different allergic status, the allergic mothers showed a tendency towards a higher surface expression of TLR2 and TLR4 on their monocytes.

Further, examination of CB monocyte expression of TLR2, TLR4 and CD14 revealed no significant differences between children with allergic mothers and children with non-allergic mothers. However, when we compared the mothers with their children, CB monocytes from children with allergic mothers had a significantly lower cell surface expression of TLR2 and TLR4 than monocytes from their mothers. This was the case both before and after PGN and LPS stimulation. No corresponding difference was observed when we compared TLR2 and TLR4 expression on CB monocytes and maternal monocytes within the non-allergic group. In addition, there was a positive correlation between mothers and children regarding TLR2 expression on monocytes regardless of maternal allergy, suggesting a maternal influence. The children’s “true” response to microbial stimuli might therefore be distinguishable only if analyzed later in infancy and/or if investigating the effector responses.

Many studies have demonstrated a down regulation of TLR4 in response to LPS, and this has been proposed as being a possible mechanism for LPS tolerance [300]. In our study, the down regulation after LPS stimulation was similar in monocytes from mothers and in monocytes from CB of their children regardless of allergic status. The same pattern was seen for TLR2, as the upregulation upon PGN stimulation was similar in all groups. These data suggest that the monocyte population is functioning very well already at birth, and that the allergic status does not influence the responding capacity of TLR2 and 4.

However, the apparent absence of significant differences in receptor expression on monocytes from women with different allergic status, does not rule out that there are genetic differences in TLRs or in the signaling cascades following TLR triggering. Indeed, Poltorak et al [301] 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 occurs independently of a TLR4 dependent signaling pathway.
We did not detect any significant differences regarding cytokine levels between PBMCs of allergic and non-allergic mothers in response to LPS or PGN. However, a trend towards an augmented release of pro-inflammatory cytokines after PBMC stimulation was seen for the 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.

However, the cytokine- and chemokine release triggered by TLR2 stimulation in CB revealed some interesting differences. CBMC from children with maternal allergic disease released significantly less IL-6 in response to PGN stimulation, compared to CBMC from children without maternal allergic disease, and there was also a trend towards less secretion of IL-8. This observation might indicate that subtle differences at the cell surface receptor level could have implications for the signaling cascade triggered by TLRs. It is of interest that Pasare et al [111] showed that microbial signaling via TLRs in mice abrogated the suppression of CD4⁺CD25⁺ T_{reg} cells by inducing IL-6 release from DCs and macrophages.

![Diagram](image)

**Figure 4.** Innate immune cells control T_{reg} cell development and activation. The activation state of APCs determines the CD4⁺ T cell response. Resting APCs may promote the development of CD4⁺CD25⁺ T_{reg} cells by inducing Foxp3 expression in CD4⁺ T cells. During infection by pathogens, recognition of PAMPs by TLRs results in activation of APCs. The APCs then produce IL-6 and other soluble factors that together override the suppressive effects of T_{reg} cells, allowing efficient generation of effector T-cells (T_E) cells against the pathogen (Science 2003; 299:1030-1). Reprinted with permission from AAAS.

They speculate that a reduced IL-6 production following microbial exposure could result in an over-regulated immune response, not allowing proper Th1-type of immune responses to develop (Figure 4). Relating this to our findings, it would...
implicate that a lowered anti-microbial response early in life could result in a hampered/slower Th2-Th1 shift, which results in an increased risk for allergy development. A study supporting this theory very recently showed that if monocyte derived DCs are not exposed to microbial stimuli, the CD4+ population is Th2 skewed [170].

For sCD14 levels in plasma, no significant differences between allergic and non-allergic mothers or between their children could be observed, although we observed a tendency towards higher sCD14 levels in allergic mothers and their children, which is in agreement with previously published data from our research group [206]. The association of sCD14 with allergic disease is hard to interpret as studies have shown inconsistent results, possibly due to gene-environment interactions [205].

Even though the sample size in this study was small (9/10), other studies have provided results that are in line with ours [268;302]. In a study from 2005, maternal allergies were shown to be associated with significantly lower levels of CD14, TLR2 and TLR4 mRNA in cord blood samples [268]. In contrast to our study, where we did not detect any differences between the mothers in terms of cell surface expression of CD14/TLR2/TLR4, they found that maternal allergy was associated with decreased levels of mRNA from the same receptors. The discrepancy between their results and ours may be due to sample size, or more likely to the fact that they investigated mRNA expression, when we looked at receptor levels on the cell surface. Another study found that PGN-induced IL-10 production and induction of FOXP3, a transcription factor expressed in T_{reg} cells, were higher in CBMC without than with maternal allergy [302]. Interestingly, this lower T_{reg} cell activation coupled to allergic status of the mother could have a link to our results of lower IL-6 levels in CB from children of allergic mothers, as IL-6 has been shown to have a regulatory role in the process of bridging innate and adaptive immunity [105].

Maternal allergy and monocyte signaling in 2-year-old children (IV)

In paper IV we aimed at investigating if the decreased IL-6 production in newborns of allergic mothers seen in paper III persisted during infancy, and if the responsible mechanism could be found intracellularly. We considered p38-MAPK to
be a suitable intracellular target for this study since it is involved in both LPS [303] and PGN signaling [304], and a key factor in inflammatory responses [22]. By applying a novel flow cytometry-based technique [305] we analyzed p38-MAPK phosphorylation in the same infants at birth, and at 2 years of age after microbial (LPS, PGN) stimulation.

The results showed that there were no significant differences between the groups of infants concerning basal levels (unstimulated cells) of IL-6, neither at birth nor at the age of 2. However, CBMCs from newborns with allergic mothers tended to have a lower IL-6 response following a microbial challenge compared with the group without maternal allergy, while p38-MAPK activation levels did not differ significantly between the groups. Although the results did not reach statistical significance, they confirm our results in paper III. LPS stimulation did not result in statistically significant differences between the two groups of children in either paper III or paper IV, although trends were seen in the same direction as for PGN.

One should keep in mind that a microbial encounter in reality is not strictly divided into LPS or PGN, but a more heterogeneous simultaneous exposure from several compounds. By mixing several microbial substances in these types of experiments one would receive further information about an individual’s capacity to respond to this type of challenge.

Further, stimulation of PBMCs from the same children at the age of 2 revealed a significantly lower IL-6 production upon PGN stimulation in children with allergic mothers compared with age-matched infants with non-allergic mothers. Increasing the sample size of 2 year olds made the significance stronger, which reinforces the observed results. Confirming the inability of children with maternal allergic disease to respond, a tendency towards a similar difference was also seen for LPS stimulation. The extended group of 2 year olds further strengthened this tendency.

To investigate whether the decreased release of IL-6 after LPS and PGN stimulation could be a consequence of reduced activity of p38-MAPK in monocytes, we analyzed the p38-MAPK phosphorylation ability in the stimulated CD14⁺ monocytes. Two-year old infants with allergic mothers displayed markedly reduced CD14⁺ monocyte p38-MAPK phosphorylation after LPS and PGN challenge compared to children with non-allergic mothers (Figure 5).
Results and discussion

Figure 5. Representative histogram plots displaying phosphorylated p38-MAPK upon peptidoglycan challenge (unshaded lines) in 2-year-olds with allergic mothers (a) and with non-allergic mothers (b). Unstimulated controls are shaded.

This altered anti-microbial response was attributed to maternal allergy rather than to being IgE-sensitized at 2 years of age. The activity of p38-MAPK is important for driving Th1 responses [306]. Thus, the lower p38 activity found in connection to allergic heredity in this study would suggest that there is a reduced capacity to induce Th1 responses in these children. As Th1 and Th2 responses are known to counteract each other [77], a consequence of the reduced p38 activity would be an increased Th2 bias, which is a hallmark of allergic diseases [80;307]. The strong correlation between p38-MAPK phosphorylation and IL-6, following both LPS and PGN stimulation, suggests that an altered monocyte function may play an important role in the reduced anti-microbial IL-6 response in PBMCs from children with allergic mothers.

TLR2 signaling in 5 year old allergic children (V)

In paper III and IV we could show that children of allergic mothers have a reduced capacity to respond to microbial stimulation in terms of decreased IL-6 responses at birth as well as at 2 years of age, possibly caused by the observed reduction of p38-MAPK phosphorylation in CD14+ monocytes. In line with these results, other studies have also demonstrated a selective impairment of TLR2 responses in terms of IL-10 production in children with allergic heredity [302], and in adults with allergic disease [189].

Age has shown to be of importance for innate immune responses in mice [310;311]. Also, the immune system of the growing child is going through multiple
important maturation steps, making interpretations from different studies in humans difficult due to study populations with different ages. As an example, age is known to effect responses to LPS in terms of IL-12p70 production [312].

The fact that at 2 years, the IgE sensitization of the children was not correlated to defective signaling (paper IV) prompted us to look at children with a more manifested allergic disease. To do this, we looked at the innate immune responses in relation to allergy development of the child by stimulating PBMCs from 5 year old allergic/non-allergic children with LPS and PGN. We thereafter analyzed the CD14, CD16, TLR2 and TLR4 expression as well as the p38-MAPK activity in monocytes. The inflammatory cytokine responses after 24h of in vitro stimulation were also measured.

The results showed that for unstimulated cells there were no differences in frequency of the monocytic subsets or their toll-like receptor levels between allergic and non-allergic children. Another study on atopic dermatitis (AD), confirming our results, also failed to detect any differences in the two monocytic subsets or in TLR levels that could be correlated to disease [189]. Interestingly, they found an impaired IL-1β and TNF production in response to a TLR2 ligand in the subjects suffering from AD. In line with this, we could show that children in the allergic group did not upregulate the TLR2 receptor upon stimuli to the same extent as the non-allergic children (Figure 6).

![Figure 6. Stimulation index (ratio of TLR expression in stimulated/unstimulated monocytes) for TLR2 after PGN stimulation in the different monocyte populations in allergic (n=10) and non-allergic (n=10) 5 year old children. °=outlier. The line represents stimulation index=1, which means no up/downregulation. □ CD14⁺CD16⁺ ■ CD14⁺⁺CD16⁻ □ All CD14 positive cells.](image)
A very recent study found the same defective upregulation of both TLR2 and TLR4 together with a reduced upregulation of HLA-DR in monocytes from patients with AD in response to *Staphylococcus aureus* enterotoxin B [312], a compound that act upon both TLR4 and TLR2 in human monocytes. Even though the allergic children in our study had a reduced TLR2 upregulation in response to PGN we did not observe any differences in p38-MAPK activity. The reason for this remains unclear, however it should be emphasized that the signaling cascade in response to PGN involves many other pathways that could have compensatory roles [6].

We could not observe any differences in TLR4 signaling attributed to allergic disease in this study, showing that the allergic phenotype here seems to have a larger impact on the TLR2- rather than the TLR4 signaling.

Another interesting result concerning the TLR4 expression in response to LPS is that in the CD14⁺CD16⁻ subset TLR4 was upregulated, while in the CD14⁺⁺CD16⁻ subset TLR4 was downregulated, regardless of allergic status. There has been conflicting results regarding the regulation of TLR4 in response to LPS [300]. A reason for the diverging results could be that a lot of evidence for TLR regulation is based on mRNA levels, and the cell surface expression of TLR4 do not parallel data obtained at the mRNA level [313]. Our results of different regulation of TLR4 in the two monocytic subsets also show that gating and phenotyping of monocytes when performing this type of experiments are crucial events for the interpretation of the results obtained.

As we had earlier found a defective IL-6 response to PGN in cells from children with allergic heredity, at birth (paper III) and at 2 years of age (paper IV), we also analyzed the production of cytokines in response to PGN at the age of 5. For unstimulated cells there was a higher production of IL-6 for allergic children. IL-8 and IL-1β levels were also slightly elevated for the allergic children, however not statistically significant. The pattern of higher spontaneous pro-inflammatory cytokines in allergic children suggests that once the disease is declared, there is a more inflammatory state of the immune system in allergic children compared to non-allergic. Further, there were no differences between the two groups regarding p38-MAPK activity or cytokine and chemokine production upon stimulation. Our results are supported by a study in 2006 showing that TNF secretion following in vitro stimulation of PBMCs was similar in women with and without allergy [302].
However another study using a different ligand (Pam3Cys) for TLR2 activation could observe a diminished TNF response in allergic adults compared to non-allergic individuals [189]. This discrepancy could be the effect of the different microbial stimuli used in the studies.

Taken together, the results of higher spontaneous cytokine production, and a decreased TLR2 responsive capacity in monocytes from the allergic subjects in this study suggests that there is an activated monocytic state in allergic individuals, that seems to lead to a hypo-responsiveness to further stimulation. This type of hypo-responsiveness in monocytes has previously been demonstrated in patients with atopic dermatitis [312].
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Even though reduced stimulation of TLRs might effect the development of different diseases, the immunological mechanisms responsible for this protective effect are still largely unknown. The fact that non-immune cells and cells of the innate immune system [10] as well as the adaptive immune system [11;13] all express PRRs, further highlight the important interaction and regulation of the traditionally separated innate- and adaptive immune systems.

The importance of age for the outcome of adaptive immune responses is readily acknowledged. The results in this thesis further show that age is crucial also for innate immune responses, as TLR regulation and cytokine production differed between children at different ages.

A main finding from paper I and III is that the maternal immune system is affected by the process of pregnancy. This is demonstrated by the association of lower breast milk cytokine levels in mothers with previous pregnancies (paper II), plus the lower IgE levels of mothers two years after giving birth (paper I). These results indicate that the immune system of the mother is modulated as a consequence of giving birth, but that this modulation also seems to increase with increasing number of births. Thus, it is possible that the apparently protective effect of older siblings in allergy [165] and multiple sclerosis [299] may be mediated though maternal immune characteristics and their influence on the developing infant. Today, women in the Western world tend to have fewer children, and also to give birth later in life, which might be important for disease development, both in the women themselves and in their children.

Summarizing the results from study III, IV and V, one should note a decreased capacity to respond to microbial stimuli in individuals with allergic heredity, or later in infancy with an allergic phenotype. This is interesting in relation to the discussion regarding microbial exposure and allergic disease. Stimulation through PRRs seems to inhibit allergic disorders, but the findings from this thesis also show that maternal allergy confers an altered capacity to respond to microbial stimuli; implying that not only exposures are important. This might also have important implications for immunomodulatory therapy, as compounds stimulating toll-like receptors are being aimed at ameliorating inflammatory diseases in the future. The observed influence of
maternal allergy on the immune system of the neonate is fascinating, but future studies are needed to further dissect the responsible immunological mechanisms, and if the influence of the mother disappear with time.

In conclusion, our results from the children show that monocytes from newborns with allergic mothers are less responsive to bacterial challenge than monocytes from children with non-allergic mothers, and that this impairment persists during the first 2 years of infancy. However, at 5 years of age, the manifested allergic disease seems to be more important for the immune responses than the allergic heredity. It seems as if the events responsible for allergy development in infancy are dependent upon heredity, but once the disease is declared, the additional inflammatory state that the individual suffers from is of more importance.

In a future perspective one would have to take into consideration additional factors when investigating the maternal influences on the child. Results of the influence of breast milk in our study, as well as in most other studies, comes from in vitro set-ups, where only the substances included in the liqous fraction are investigated. It would be of great interest to also investigate the cellular fractions, as these most surely play a role for the maturation of the neonatal immune system, but perhaps also are implicated in susceptibility to disease. Moreover, understanding how the epigenome responds to environmental exposures will be of great importance for understanding the gene-environment interactions that seem to be of great importance for allergy development. Epigenetics also provides an important link between early developmental environment and later disease [314], and elucidation of these events will be an important contribution to the knowledge of maternal influences on the child. A plausible mechanism behind the effects of pregnancy on the immune system of the mother might indeed be found in epigenetic events, as these effects seem to be kept in time. Another question mark that needs further investigations is whether the observed impairment in response to PGN, found in connection to allergic disease in this thesis, also extends to other microbial ligands and receptors. A hint of that it might be the case comes from a very recent study showing that DCs from allergic adults have a reduced capacity to produce IFN-α after stimulation with TLR9 ligands [315].
ACKNOWLEDGEMENTS

I would like to express my warm gratitude to everybody that has contributed in any way in making this thesis a reality, and most especially I would like to thank;

Eva Sverremark-Ekström, for simply being the best supervisor you could ever imagine. Working with her is easy and inspiring. And thanks also to Mattias for letting her work so much late at night 😊. Revisions were usually done to the next day…

All colleagues at the department; especially my room mates Nora Bachmayer, Yvonne Sundström and Ylva Sjögren for making our room into a nice place to come to in the morning. I really appreciated our mutual support during the tougher days Yvonne. John Arko-Mensah, Nnaemeka Iriemenam, Salah Eldin Farouk, Amre Nasre, Jubayer Rahman, Stefania Varani, Nancy Awah, Maria Johansson, Ebba Sohlberg, Judith Anchang, Charles Arama, Hedvig Perlmann, Khosro Masjedi, Margareta “Maggan” Hagstedt, Ann Sjölund, Gelana Yadeta and Anna-Leena Jaarva. Shanie Saghafian-Hedengren for her class and enthusiasm whether it is science, wine or life. Ulrika Holmlund for our co-operation and social stuff which have always been very valuable. Lisa Israelsson, my toast madame, thanks for everything!! The Tip Tops, the joy, your true interest and help with my dissertation and thesis, but also for the sharing of our common passion; horses! You know how to be a good scientist without forgetting other things in life. Halima Balogun for being so cool and nice to work with. Manijeh Vafa Homann for our discussions about science, dissertations, love and cultural differences. You also provided us with the name of Zelzele, we are SO greatful. Pablo Giusti, you were the coolest 😊 newcomer. The “boys” at the physiology department, Olle, Damir and Thomas, for suddenly giving the Wenner-Gren “meetings” a new meaning. Former students at the department; Anna Tjärnlund, Camilla Rydström, Ariane Rodríguez, Nina-Maria Vasconcelos, Qazi Khaleda Rahman, Mounira Djerbi and Anna-Karin Sigfrinius (Larsson).
The “seniors” at the department; **Klavs Berzins, Marita Troye-Blomberg, Carmen Fernández** and **Eva Severinsson** for making me a better scientist.

My co-authors **Ulrika Holmlund, Shanie Saghafian-Hedengren, Jacob T Minang, Marita Troye-Blomberg, Gunnar Lilja, Caroline Nilsson, Christina (Tina) Trollmo, Vivianne (Vivi) Malmström, Katarina Bremme, Annika Scheynius** and **Jens Schollin. Scott M Montgomery** for his professionalism, our fruitful discussions and nice co-operation.

My outstanding conference partners (best privileges for a PhD student): **Gunnar, Jacob, Anna-Karin, Lisa, Manijeh, Halima, Shanie, Ylva, Yvonne, Per Thunqvist, Monica Nordlund, Nicolas Brodszki** and **Philippe Cabelduc**.

**Gunnar Lilja**, for always being there for me, whether it is work issues or only a concerned mother who needs to discuss childhood diseases. I’m forever grateful that you encouraged me to choose the course “Immunology” back in 1999, that’s where this journey all started.

Thanks to **Vivi** and **Tina** at CMM for offering me an exiting future in a stimulating and nice environment.

The “Uppsalagirls”; **Ewa Wredle, Anna Larsson, Mikaela Patel, Monica Roman and Linnea Nygren-Babol** for our regular get togethers that are filled with laughter, food, chocolate and friendship. We are still waiting for Ewa to get that job close to a beach so we can have even nicer meetings in the future…

I would be a less happier person if it wasn’t for things and people that light up the sometimes harder days, special thanks to **Peter, Lotta, Tuva, Täby galopp** with the whole **Bendik Bö stable**, the **GAFF board**, the **SÅEF** people and **Stall Zygot**. Special thanks to **Leif Wretman**, and let us hope for **Zelzele** to be a star.

Thanks to **Pär** for your professionalism and support. Let this thesis inspire you to finish yours, it will probably be so good that even your supervisor won’t understand….
All my other friends that are a part of why I am what I am; Pia, Katta, Caroline, Karin, Marie, Christian, Daniel, Jeanette, Krille, Kidd, Anders, Johan F, John, Yann, Johan L, Jocke and Patrik.

Thanks to my family; Pappa, for the sculpture and your growing interest in my world of horses, Irene, Jonas, Ullis, Fanny, Iris, Valter, Hugo, the Boëthius family and most of all to my mother Karin for her warm heart and for always being there for me.

Best of them all, filling my life with so much love, Ebba and Ines. The future is yours.

This work was supported financially by grants from the Swedish Research Council (grants: 57X-15160-05-2, 74X-15160-03-2, 74XD-15160-01A, K2004-74X-15042-01A), the Swedish Medical Society, The Swedish Asthma- and Allergy Research Foundation, Örebro University Hospital Research Committee; KI Fonden; Visceral; the Cancer- and Allergy-, The Golden Jubilee Memorial-, HRH Crown-princess Lovisa & Axel Tielman-, Hesselman-, Vardal-, Golje-, Jeansson-, Magnus Bergvall-, Swärd/Eklund-, Apotekare Hedberg-, Salén- and the Åhlén foundations.
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