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IMMUNE RESPONSES TO LIPOPOLYSACCHARIDE
IN RELATION TO ALLERGIC DISEASE,
A TLR4 GENE POLYMORPHISM AND ENDOTOXIN EXPOSURE

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During the course of the research underlying this thesis, Anna Lundberg was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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To my family, with all my love

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ORIGINAL PUBLICATIONS

This thesis is based on the following four papers, which will be referred to in the text by their roman numerals.

- I A *TLR4* polymorphism is associated with development of atopic asthma and reduced LPS induced IL-12(p70) and IL-10 responses
Fagerås Böttcher M, Hmani-Aifa M, **Lindström A***, Jenmalm MC, Mai X-M, Nilsson L, Zdolsek HA, Björkstén B, Söderkvist P, Vaarala O
Journal of Allergy and Clinical Immunology 2004, vol 114 (3), p 561-567
- II Lipopolysaccharide induced immune responses in relation to *TLR4(Asp299Gly)* gene polymorphism
Lundberg A, Wikberg LA, Ilonen J, Vaarala O, Böttcher MF
Clinical and Vaccine Immunology 2008, vol 15 (12), p 1878-1883
- III Lower LPS responsiveness in Estonian than Swedish infants associates with less allergy development and high endotoxin exposure
Lundberg A, Böttcher MF, Tomičić S, Voor T, Jenmalm MC
In manuscript
- IV Expression of toll-like receptors and immune-regulatory markers during early infancy and allergy development in Estonian and Swedish infants
Lundberg A, Jenmalm MC, Jimenez E, Böttcher MF
In manuscript

* The author's maiden name is Lindström

ABSTRACT

Background: Allergic diseases have increased during the last decades, particularly in affluent countries, possibly due to a reduced and/or altered microbial exposure during infancy. Activation of the immune system by microbes early in life is probably required for accurate maturation of the immune system and tolerance development. It is not fully understood how microbial exposure is associated with the development of allergic diseases, however. Genetic factors may influence microbial induced immune responses. A certain polymorphism, in the gene coding for the Toll-like receptor 4, *i.e.* (*TLR4* Asp299Gly), has been suggested to alter the immunological responsiveness to bacterial lipopolysaccharide (LPS).

Aim: The aim of this thesis was to study the interplay between LPS induced immune responses, LPS signalling related genetic polymorphisms, allergic disease and endotoxin exposure.

Subjects: The thesis is based on the results obtained from individuals in three different study groups, *i.e.* Estonian and Swedish children followed prospectively from birth up to five years of age, Swedish school-children eight and 14 years of age and young adults.

Methods: The study subjects were clinically evaluated regarding allergic diseases with skin prick tests, circulating IgE levels, validated questionnaires and clinical examinations by paediatricians or research nurses. The gene polymorphisms *TLR4* Asp299Gly and *CD14*-159 were analysed. Peripheral blood mononuclear cells were isolated from blood and cultured with LPS from two Gram negative bacterial strains, *i.e.* *Salmonella enterica* serotype Typhimurium (Serotype Typhimurium) and *Escherichia coli* (*E. coli*). Cytokine and chemokine secretions were analysed with Luminex or ELISA technique. Receptor expression of circulating peripheral blood monocytes was analysed with flow cytometry. The phosphorylation of intracellular proteins involved in LPS signalling pathways was analysed with Luminex technique. mRNA expression of proteins involved in LPS signalling pathways and of markers for T regulatory cells were analysed with realtime-PCR.

Results: In school-children and young adults, the *TLR4* Asp299Gly gene polymorphism was associated with reduced LPS induced I κ B α phosphorylation, IL-10 and IL-12 cytokine secretion. Interestingly, these findings were observed only when the cells were cultured with LPS from Serotype Typhimurium but not with LPS from *E. coli*. The polymorphism was positively associated with asthma, especially atopic asthma.

Several differences in immunological responses to LPS were observed between allergic and non-allergic individuals. Asthma in school-children was associated with reduced LPS induced cytokine production of IL-10 and IL-12. The phosphorylation of I κ B α was lower in adult allergic compared to non-allergic individuals. Swedish children who had developed allergic disease at five years of age had lower TLR2 mRNA expression at birth compared to children who remained healthy.

Estonian children displayed generally lower LPS induced cytokine and chemokine production as compared to Swedish children both at birth and at 3 and 6 months of age. The mRNA expression of the T regulatory associated markers Foxp3 and Ebi3 were higher in the Estonian compared to the Swedish children at birth.

Conclusion: Polymorphisms in genes coding for pattern recognition receptors can alter the immune responsiveness of the host to microbial components and may be of importance for the development of asthma. Lower LPS induced cytokine response and higher expression of T regulatory associated markers were seen in children from Estonia as compared to Sweden, suggesting an increased capacity for early immune regulation among infants from a country with a low prevalence of allergic disease.

SAMMANFATTNING

Bakgrund: Under de senaste decennierna har förekomsten av allergiska sjukdomar ökat i västvärlden. En av möjliga förklaringar kan vara en minskad eller förändrad mikrobiell exponering under uppväxttiden. Mikrobiella stimuli under de första levnadsåren tros vara viktiga för utmognaden av immunsystemet och utveckling av tolerans. Exakt hur mikroorganismers påverkan på immunförsvaret är kopplat till utvecklingen av allergiska sjukdomar är dock ännu okänt. Genetiska polymorfier kan påverka immunsvaret mot mikrobiella komponenter. En sådan polymorfi, *TLR4 Asp299Gly*, har observerats i genen som kodar för receptorn TLR4 som känner igen lipopolysackarid (LPS) från Gramnegativa bakteriers cellvägg, och har föreslagits vara associerad med en förändrad förmåga att svara immunologiskt mot LPS.

Syfte: Syftet med studierna var att studera immunsvaret mot LPS i relation till specifika genetiska polymorfier, allergisk sjukdom samt mikrobiellt tryck i form av endotoxinnivåer.

Studiepopulationer: Denna avhandling baseras på resultat från tre studiegrupper: estniska och svenska barn som är följda från födseln upp till fem års ålder, en grupp svenska skolbarn 8 och 14 år gamla samt en grupp unga vuxna.

Metoder: Två genetiska polymorfier, *TLR4 Asp299Gly* och *CD14/-159*, analyserades. Mononukleära celler isolerades från perifert blod och odlades tillsammans med LPS från två olika Gramnegativa bakteriestammar, *Salmonella enterica* serotype Typhimurium (Serotype Typhimurium) och *Escherichia coli* (*E. coli*). Cytokin- och kemokinsekretion analyserades i cellsupernatanter med Luminex eller ELISA. Ytmarkörer på monocytter i helblod studerades med flödescytometri. Intracellulära signaleringsproteiner, som är inblandade i TLR4s signalvägar analyserades med Luminexteknik. mRNA uttryck av proteiner som är relaterade till LPS signalering och markörer för regulatoriska T celler analyserades med realtids-PCR.

Resultat: *TLR4 Asp299Gly* polymorfin var associerad med lägre fosforylering av det intracellulära signaleringsproteinet I κ B α och lägre utsöndring av cytokinerna IL-12 och IL-10 efter cellstimulering med LPS hos skolbarn och unga vuxna. Skillnader i cellsvaret mellan individer med och utan polymorfin kunde påvisas när cellerna odlats med LPS från Serotype Typhimurium men inte med LPS från *E. coli*. Polymorfin var också associerad med astma och särskilt atopisk astma.

Flera skillnader i immunsvaret mot LPS observerades mellan allergiska och icke-allergiska individer. Skolbarn med astma hade lägre LPS inducerad IL-10 och IL-12 cytokinproduktion. Vuxna allergiker hade lägre LPS inducerad I κ B α fosforylering. Svenska barn som vid fem års ålder hade utvecklat allergisk sjukdom hade lägre mRNA uttryck av TLR2 vid födseln.

Estniska barn hade generellt lägre LPS inducerade cytokinsvar än svenska barn vid födseln och vid 3 och 6 månaders ålder. mRNA uttrycket av de T-regulatoriskt associerade markörerna Foxp3 och Ebi3 var vid födseln högre hos de estniska jämfört med de svenska barnen.

Slutsats: Genetiska förutsättningar kan påverka immunsvaret mot LPS och kan möjligen ha en betydelse för utveckling av astma. De generellt lägre LPS inducerade cytokinsvaren och högre uttryck av markörer för Treg celler hos estniska jämfört med svenska barn skulle kunna bero på att deras uppväxtmiljö med ett högre mikrobiellt tryck påverkar den tidiga utvecklingen av immunförsvaret och kan möjligen vara en bidragande förklaring till den lägre allergifrekvens som ses i Estland jämfört med Sverige.

ABBREVIATIONS

ARC	allergic rhino conjunctivitis
CBMC	cord blood mononuclear cells
cDNA	complementary deoxyribonucleic acid
DC	dendritic cell
Ebi	Epstein-Barr-virus-induced gene
ELISA	enzyme-linked immunosorbent assay
FSC	forward scatter
Foxp3	forkhead box P3
HSP	heat shock protein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LAL	limulus ameocyte lysate
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAMP	microbial associated molecular pattern
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
NLR	NOD-like receptor
OVA	ovalbumin
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PRR	pattern recognition receptor
RFLP	restriction fragment length polymorphism
RLR	RIG-1-like receptor
rRNA	ribosomal ribonucleic acid
RSV	respiratory syncytical virus

ABBREVIATIONS

RT	reverse transcriptase
SSC	side scatter
SOCS	suppressor of cytokine signalling
SPT	skin prick test
TCR	T cell receptor
Th	T helper
TIR	toll-IL-1 receptor
TLR	toll-like receptor
Tr1	inducible T regulatory cell type 1
Treg	regulatory T cell
TNF	tumor necrosis factor

AIMS OF THE THESIS

The general aim of this thesis was to study the interplay between lipopolysaccharide (LPS) signalling related genetic polymorphisms, LPS induced immune responses, endotoxin exposure and allergic disease.

The specific aims of each individual paper were:

- I To investigate the associations between the *TLR4* Asp299Gly and *CD14*/-159 gene polymorphisms, LPS induced cytokine production and allergic disease in children.
- II To investigate the relationship between the *TLR4* Asp299Gly polymorphism with the phenotype of circulating monocytes and LPS induced signalling *in vitro*.
- III To study LPS induced chemokine and cytokine responses in relation to domestic endotoxin exposure and allergy development during the first two years of life in Estonian and Swedish children.
- IV To study mRNA expression of genes involved in LPS signalling pathways and immune regulation in Estonian and Swedish prospectively followed birth cohorts, with special reference to domestic endotoxin exposure and allergy development.

INTRODUCTION

During the last decades, the incidence of allergic diseases has increased dramatically. This increase is most prominent in countries with a Westernised life style. The reason for this is not yet clear, but several explanations have been suggested. According to the hygiene hypothesis, a lack of microbial stimulation may affect the maturation of the immune system resulting in failure of clinical tolerance to harmless antigens, and finally in the development of allergy.

General aspects of allergic disease

Allergy is hypersensitivity reactions caused by immunological mechanisms¹. Allergic reactions can be antibody-mediated or cell-mediated. Type I hypersensitivity reactions are mediated by IgE antibodies against soluble antigens so called allergens, and induce mast cell activation in the tissue². Type II and III hypersensitivity reactions involve IgG antibodies against cell surface/matrix associated antigens or soluble antigens respectively². Type IV hypersensitivity reactions are T-cell mediated. In this thesis, the term allergy is referring to IgE mediated allergy.

Atopy is defined as tendency to become sensitised, *i.e.* produce IgE antibodies to allergens. Atopic individuals can develop symptoms of *e.g.* asthma, allergic rhinoconjunctivitis (ARC) or eczema¹. The term atopy should not be used until the presence of IgE antibodies has been documented¹. These antibodies can be demonstrated with *e.g.* skin prick testing or by analysing circulating IgE antibodies.

The different clinical manifestations of allergic disease often vary with age. Eczema and food allergy are the most common allergic diseases in the first years of life. The symptoms of food allergy mainly disappear before the age of five and are often replaced by asthma and hay fever later in the preschool and school-ages. The progress of the allergic disease is called the atopic march³. Atopic eczema in early childhood is thus believed to be a good predictor for later development of respiratory allergy such as asthma and ARC^{4,5}.

Allergy can be divided into three phases, the sensitisation phase, immediate hypersensitivity reactions and the late phase reactions. In the sensitisation phase, the antigen presenting cells,

i.e. dendritic cells (DC) take up the allergen, process it and present it to T helper cells (Th). The naïve T helper cells develop into Th1 or Th2 cells, influenced by the cytokine environment and the dose and route of the allergen and the presence or absence of inflammatory stimuli. Several other types of T cells have also been described such as Th17 cells, secreting for example IL-17, and regulatory T cells such as Tr1, Th3 and naturally occurring T regulatory cells (T reg). The Th2 cells produce IL-4, IL-5 and IL-9, and induce humoral immune responses with IgE and IgG₄ production, while Th1 cells produce IFN- γ and activate macrophages and cytotoxic T-cells (reviewed in ⁶). Interferon- γ secretion inhibits Th2 differentiation and IgE production, while IL-4 on the other hand down regulates Th1 reactivity. The T cell response to allergens in allergic individuals has a typical Th2 character indicated by a high production of IL-4, IL-5, IL-9 and IL-13. The allergen specific Th2 cells induce a B cell switch to production of IgE antibodies through the secretion of cytokines such as IL-4 ⁷. IgE produced by the B cells bind to high affinity Fc ϵ RI IgE receptors on mast cells and basophils. Monocytes, platelets and eosinophils also express IgE receptors but at lower levels ⁷. On the next encounter with the allergen a sensitised individual can develop an allergic immediate hypersensitivity reaction. Not all sensitised individuals develop allergic symptoms however, for unknown reasons. The binding of allergens to IgE on the cell surface of mast cells crosslink the IgE receptors and causes an activation of the cells with a subsequent release of chemical mediators such as histamine, chemokines, cytokines, prostaglandins and leukotrienes. These mediators cause an immediate allergic reaction producing symptoms, such as bronchoconstriction, vascular leakage from blood vessels, itch and tissue destruction. The mediators released by the mast cells also attract and activate other cells such as Th2 cells, eosinophils and basophils and leading to inflammation which may become chronic. This is the late phase reaction. Activation of the cells leads to a sustained Th2 response with further stimulation of IgE antibody production and mast cell activation.

Development of allergy

The development of allergic diseases is due to an interplay between several factors including the genetic background, allergen exposure and environmental factors (figure 1).

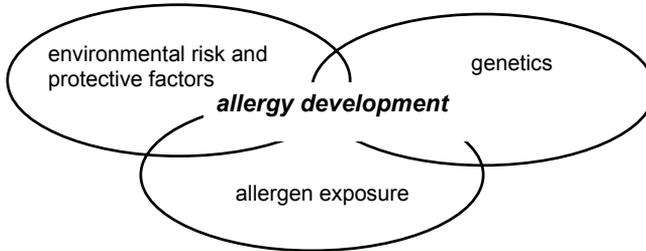


Figure 1. The development of allergic diseases is the result of an interplay between several factors.

Allergy and genetics

Studies on families with allergic diseases and on twins demonstrate a strong hereditary factor in allergy. Several genes have been identified as possible candidates that could be responsible for the inherited susceptibility for allergic disease. The high affinity receptor of IgE (FcεRI) gene on chromosome 11q13 has been linked to atopy⁸. Also linkage of atopic phenotypes to chromosome 5q31-33, a region which includes the genes coding for IL-3, IL-4, IL-5 and IL-13 and also CD14, has been reported (reviewed in⁹). In this thesis we have focused on polymorphisms in the receptors for lipopolysaccharide (LPS), namely Toll-like receptor 4 (TLR4) and CD14, and these receptors are discussed later in the introduction on page 21, 23.

Allergen exposure

In order to develop allergic sensitisation and allergic symptoms the individual needs to be repeatedly exposed to the allergen. It has been debated whether allergen exposure early in life is a risk factor or not (reviewed in¹⁰). The time of the year of birth has been shown to influence allergy development, perhaps due to different levels of allergen exposure¹¹. The allergen dose seems to be important, since animal studies have shown that low-dose allergen exposure favours Th2 associated immune responsiveness with high IgE production while high-dose exposure leads to development of clinical tolerance, *i.e.* low IgE and high IgG2a production, suppression of cytokine production and no clinical allergy¹². Whether having pets is a risk or a protective factor for allergy development is unresolved. Several recent studies indicate a protective effect of having pets^{13,14}, although, the presence of pets may also be associated with higher exposure to microbial components.

Environmental risk and protective factors

There has been a dramatic increase in the prevalence of allergic disease during the last decades¹⁵⁻¹⁷, although the incidence has been suggested to be stabilised at least in some countries¹⁸. Air pollution is one identified risk factor for allergy development but cannot explain the recent increase¹⁹. The prevalence of allergic diseases are lower in Eastern European countries in which air pollution is a greater problem than in Western European countries. The length of breastfeeding is another factor that has been suggested to have an impact on allergy development, however the association is unclear. The prevalence of allergic disease is lower among children in former socialist countries of Europe, such as Estonia, than among Scandinavian children²⁰. After the reunification of East and West Germany the prevalence of hay fever and sensitisation increased in the former East Germany²¹. In concordance with these results it has been shown that a rapid increase in atopy, *i.e.* sensitisation was found in Greenland when the native Inuit lifestyle changed towards a more modern lifestyle²². Factors associated with a Western lifestyle, *i.e.* diet, household size, improved general living conditions are proposed to be of importance for these observations, leading to the so called hygiene hypothesis.

According to the hygiene hypothesis, a lack of microbial stimulation may affect the maturation of the immune system, resulting in failure of clinical tolerance development to harmless antigens and allergy development (figure 2). This theory is supported by both epidemiological and experimental data²³. In 1989 Strachan showed that hay fever and eczema was inversely correlated to the number of siblings in an English population²⁴. He suggested that a reduced family size and improved living conditions associates with fewer infections and improved hygiene, and thereby might be responsible for an increased risk of developing allergic disease. The current concept of the hygiene hypothesis includes not only the importance of viral infections but also a discussion about microbial exposure in a broader sense, such as the influence of the normal microbial gut flora and environmental microbial components such as endotoxins.

Viral infections such as hepatitis A, measles and enterovirus infection in early life have been suggested to be protective against allergic diseases^{25,26} while other studies could not confirm this^{27,28}. Early day-care attendance might be protective against allergic disease and the proposed mechanism is that the children are exposed to more viral infections²⁹. Further, populations with anthroposophic lifestyles have been reported to have less allergic disease³⁰.

³¹. Characteristic of the anthroposophic life style includes a reduced use of antibiotics and immunisations. Respiratory syncytial virus (RSV), a common virus infection in childhood, has on the other hand been associated with an increased risk of asthma, especially in those children who develop wheezing and obstruction due to the infection ^{32, 33}.

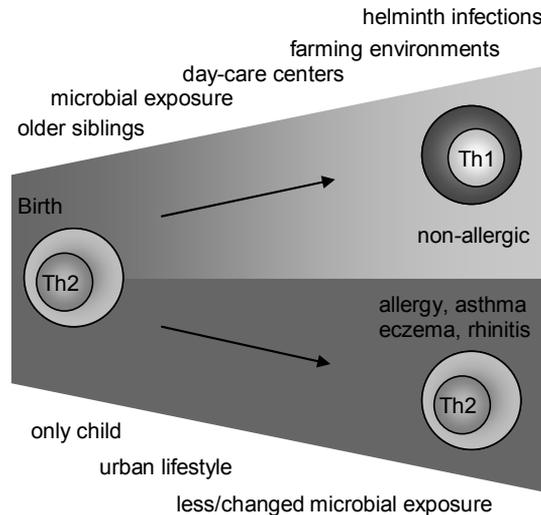


Figure 2. According to the hygiene hypothesis, the immune system at birth is immature and skewed towards T helper (Th) 2-like cytokine production. Microbial stimulation can help immunological development towards a healthy balance of Th1 and Th2-like cytokine responses. In the absence of these stimuli, the immature Th2-like pattern of cytokine production persists, leading to an increased risk of allergic diseases.

Along with the increase in allergic disease prevalence, dietary habits have also changed. Epidemiological data show an increase of ω -6 fatty acids intake and a decrease in the intake of ω -3 fatty acids over the past century ³⁴ and this alteration has been suggested to be responsible for the increase in allergic disease ³⁵. These changes seen in the Western diet is probably due to an increase in consumption of ω -6 fatty acid containing food like margarine and vegetable oils and a decrease in fish consumption, rich in ω -3 fatty acids. Fish consumption early in life has been associated with reduced risk of allergic disease ³⁶. Allergy prevention studies using ω -3 fatty acid supplementation are ongoing. Results from a randomised placebo-controlled study from our research group have shown that treatment with ω -3 fatty acids during pregnancy and lactation reduced the risk of food allergy during the first two years of life in the children (C Furuholm et al., unpublished data). The intake of ω -3 fatty acids affects the composition of the cell membrane phospholipids by competing with ω -6 fatty acids and could therefore decrease the production of arachidonic acid and its metabolites such

as proinflammatory leukotrienes and prostaglandins. The function of monocytes and lymphocytes could thus be modified, possibly affecting the risk of allergy development.

A decreased prevalence of allergic diseases has been reported in farming environments through-out the world³⁷⁻⁴⁰. There are several theories about the reasons for the farming effect, such as differences in diet, *e.g.* intake of unpasteurized milk and higher concentrations of bacterial and fungal components in house-dust. The most investigated marker for microbial exposure is endotoxin, and the allergy protective effect has been related to endotoxin exposure^{41,42}. Endotoxin or lipopolysaccharide (LPS) is a component of the cell wall of Gram negative bacteria. Endotoxin is found everywhere in the environment. Data from our group shows that endotoxin levels were inversely related to the development of atopic disease and sensitisation in Swedish children⁴³. The effect of endotoxin might be depending on time and dose of exposure and of the genetic background of the individual. High endotoxin levels have been associated with an increased *in vitro* capacity of T cells to produce IFN- γ in response to PHA or PMA Con-A, indicating that Th1 responses are influenced by environmental endotoxin exposure^{44,45}. Importantly, endotoxin level has been associated with increased risk of non-atopic wheeze⁴⁶, also in farming environments⁴¹.

Immunological mechanisms of the hygiene hypothesis

The explanatory immunological mechanism of the hygiene hypothesis has been that infections would favour Th1 associated immune responses with the production of IFN- γ inhibiting the Th2 profile, thereby preventing IgE production and development of asthma and allergies⁴⁷ (figure 2). This model has been questioned, however. In parallel with the increase in allergic disease, the prevalence of type 1 diabetes, a disease suggested to be associated with Th1 immunity have also increased⁴⁸. Furthermore, populations with high incidences of helminth infections, resulting in Th2 immune deviation and high circulating IgE levels, also to allergens, have less allergic disease⁴⁹. IgE protects against parasitic worms and helminth infections. It may be possible that immune regulating mechanisms are involved such as the Treg cells. These cells are involved in suppressing immune responses and maintaining self-tolerance. Microbial stimulation may also play a role in the function of these cells since the gut flora in animal studies have been shown to be important for the development of Treg cells⁵⁰. T regulatory cells in mice have recently been shown to respond directly to LPS via TLR⁵¹. Also, in mice, TLR2 ligands increased the proliferation and decreased the suppressive capacity of Treg cells⁵².

Several regulatory T cell subsets exist, such as naturally occurring Treg cells and inducible T regulatory cells (Tr1) and Th3 cells. The area of knowledge about these cells is expanding. Tr1 cells and Th3 cells are believed to exert their suppressive effect via secretion of the cytokines IL-10 and TGF- β , respectively. The naturally occurring thymus derived Treg cells constitute 5-15% of the CD4+Tcell population. They are characterised by high expression of for example CD25 (IL-2R α) and CTLA-4 on their cell surface and intracellularly by the transcription factor Forkhead box 3 (Foxp3). Foxp3 is important for the function of Treg cells and so is also IL-2. The mechanism of suppression by these cells is thought to be cell-contact dependent and possibly by the secretion of IL-35⁵³. IL-35 consists of two subunits, the Epstein Barr virus induced gene 3 (Ebi3) and IL-12 α . Ebi3 also forms IL-27 together with IL-27 α (p28). IL-27 has been reported to initiate Th1 responses but also to have inhibitory effects on Th1, Th2 and Th17 T cell subsets as well as on the expansion of inducible regulatory T cells (reviewed in⁵⁴). T regulatory cells may be involved in allergic disease but studies have so far shown conflicting results⁵⁵. It is challenging to investigate when these cells may play an important role, *e.g.* at the time of sensitisation or during the later allergic immune responses.

Other immune-regulatory mechanisms include the intracellular protein family suppressor of cytokine signalling (SOCS). SOCS-1 has been shown to be involved in the negative regulation of the cytokines that are induced by LPS stimulation, for example IL-6 and TNF⁵⁶. SOCS3 expression is induced by a variety of cytokines including IFN- γ , IL-3, IL-6 and IL-10 (reviewed in⁵⁷). SOCS3 is primarily expressed in Th2 cells inhibiting IL-12 signalling⁵⁸. Th2 cell mediated allergic diseases such as atopic dermatitis, asthma and increased serum IgE levels have been associated with high SOCS3 expression⁵⁹.

Monocytes are bone marrow derived leukocytes which circulate in the blood. They mature and differentiate into macrophages as they enter the tissue. The cells are characterised by their high expression of CD14. Monocytes express pattern recognition receptors such as TLRs. They belong to the innate immune system and have both antigen presenting and phagocytic capacity. It is generally considered that they can also differentiate into myeloid dendritic cells, at least *in vitro*. Monocytes are differentially activated by Gram positive and Gram negative bacteria, via TLR2 and TLR4 respectively⁶⁰⁻⁶². Activation through TLR2 may induce a more Th2-like profile while TLR4 stimulation leads to a Th1-like profile⁶³. This might not be the case *in vivo* possibly depending on dosage and time of exposure. In an animal model of

ovalbumin allergy, activation of TLR4 via exposure to LPS before or shortly after sensitisation to ovalbumin reduced ovalbumin-specific IgE production, while TLR4 activation after allergen sensitisation aggravated the inflammatory response and increased bronchial hyperresponsiveness⁶⁴.

Antigen presenting cells, especially DC are obligatory for activation of naïve T helper cells and their differentiation into Th1 and Th2 subtypes. Dendritic cells take up antigen, become activated and migrate to the lymphatic tissue and present the peptides to T cells. Microbial stimulation of the DC leads to cytokine secretion and upregulation of costimulatory molecules such as CD40, CD80 and CD86.

Development of immune responses in children

The risk for developing viral and bacterial diseases is high during infancy and early childhood, partly because the immune system is not fully developed at birth. Neonates have poor cell-mediated immunity, poor inflammatory responses and impaired defences against intracellular pathogens and an inability to produce certain immunoglobulin isotypes. Newborns have a higher proportion of naïve T cells and a lower proportion of memory T cells compared to adults, reaching adult proportions between 12 and 18 years of age⁶⁵. The proliferation and cytokine production from T cells is reduced in neonates, particularly Th1 cytokines. Immune responses are believed to be Th2 skewed in early life and during pregnancy possibly because Th1 responses could be harmful for the pregnancy⁶⁶. Neonatal immune responses towards common environmental allergens are Th2 skewed both in allergic and non-allergic children⁶⁷. The neonatal Th2 responses, however, are sustained and even upregulated in the children who later develop allergic disease⁶⁷⁻⁶⁹. Microbial stimulation is believed to be important to increase Th1 responses and thereby downregulating Th2 associated responses. This ability to down-regulate the early Th2 deviation to allergens and up-regulate Th1 responses seems impaired in children who later develop allergic disease⁷⁰.

The monocyte function is rather mature at birth in the respect of phagocytose function but antigen presenting cells from newborns have been shown to have impaired production of IFN- α and IFN- β ^{71,72}. Although cord blood monocytes have been shown to express similar basal levels of TLR on their surface as adult monocytes^{73,74}, lower LPS induced TNF^{73,75} has been observed from CBMC compared to adult cells. Also lower LPS induced IL-12 and IFN- γ secretion from neonatal DC as compared to adult cells has been reported⁷⁶. In contrast,

TLR induced monocyte production of IL-6, IL-10 and IL-23 may instead be enhanced compared to adults ⁷⁷⁻⁷⁹.

Lipopolysaccharide recognition

Lipopolysaccharide is a component of the Gram-negative bacterial cell wall.

Lipopolysaccharide consists of a polysaccharide part and a lipid part (figure 3). The lipid A structure is largely responsible for the immune activation of LPS. The LPS molecule differ between different bacteria, which possibly explain why some bacteria may be more immunogenic than others ⁸⁰. Free LPS or endotoxin is bound by LPS binding protein (LBP) and CD14 and transported to the cell membrane of immune cells, such as antigen presenting cells. The complex is recognised by TLR4/MD2 and initiates cellular signalling.

Lipopolysaccharide is a very potent inducer of pro-inflammatory responses. Activation of cells *in vitro* with LPS leads to secretion of several cytokines and chemokines such as IL-1, TNF, IL-6, IL-10 and IL12 and to up-regulation of co-stimulatory molecules such as CD80 and CD86 ^{81, 82}. Re-exposure to LPS can lead to a phenomenon called endotoxin tolerance. Reduced cytokine secretion to LPS after LPS re-exposure has been demonstrated both *in vivo* ⁸³ and *in vitro* ^{84, 85}. Whether this applies also to environmental exposure is not known. The mechanisms behind endotoxin tolerance are not known, but it has been suggested that either TLR4 receptor downregulation, at least in mice ⁸⁶, or production of anti-inflammatory IL-10 and TGF- β could explain the phenomenon ⁸⁴.

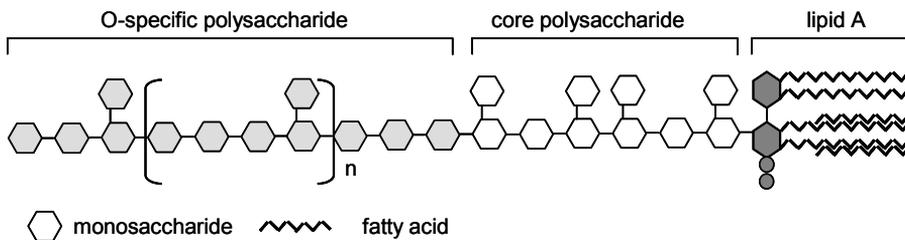


Figure 3. General structure of lipopolysaccharide (LPS) of Gram-negative bacteria. It consists of two parts, a polysaccharide and a lipid part. The O-specific polysaccharide varies among species, whereas the core polysaccharide generally considered more conserved. The lipid A component is largely responsible for the endotoxic effect of LPS.

CD14

The CD14 receptor exists both in a soluble and membrane bound form. It is found on monocytes, macrophages and neutrophils. The soluble form is important to enable TLR signalling on cells that lack the membrane bound form, such as endothelial and epithelial cells⁸⁷. CD14 is bound to the LPS-binding protein and is a receptor for LPS but functions also as a receptor for structures from Gram positive bacteria and mycobacteria (reviewed in⁸⁸). A polymorphism in the promotor region of the CD14 gene (*CD14/-159* gene polymorphism), first described by Baldini *et al.* has been suggested to increase sCD14 and reduce IgE levels⁸⁹. The polymorphism has also suggested to be associated with allergic disease but the results are contradictory⁹⁰⁻⁹³.

Pattern recognition receptors

Pattern recognition receptors (PRR) are evolutionary conserved receptors and part of the innate immune system. These receptors recognise so called pathogen associated molecular patterns (PAMPs) which are evolutionary conserved structures from bacteria, viruses, parasites and fungi structures. As these structures are both from pathogenic and non-pathogenic microbes they are sometimes instead called microbial associated molecular patterns (MAMPs). PRR are expressed on various cells of the immune system such as monocytes, macrophages, DC, NK cells, mucosal epithelial and endothelial cells. NOD-like receptor (NLR), RIG-1-like receptor (RLR) and Toll-like receptor (TLR), mannose receptors, β -glucan receptors and other C-type lectins are examples of PRR⁹⁴. The intracellular receptors NALP, NOD and IPAF, which recognises bacterial structures, belong to the NLR family. The activation of NALP and IPAF receptors leads in general to activation of caspase-1 while activation of NOD receptors leads to activation of the NF κ B pathway⁹⁵. RLR receptors detect viral structures.

Toll-like receptors

Toll-like receptors are a family of evolutionary conserved PRR⁹⁶. The Toll protein was initially discovered in the fruit-fly *Drosophila* and was found to be involved in embryogenesis, but in the adult fly the protein was important for protection against fungi infection. Binding of the ligand Spätisle to the Toll protein initiated intracellular signalling and the expression of antifungal peptide gene drosomycin⁹⁷. The first human homologue to be discovered was Toll-like receptor 4⁹⁸. At least 11 TLRs have been discovered in humans. The

receptors recognise evolutionary conserved structures from bacteria, viruses, parasites and fungi (table 1).

Table 1. Toll-like receptors and their ligands

Receptor	Localisation	Ligands
TLR1 (forms heterodimer with TLR2)	Membrane bound	triacylated lipopeptides
TLR2 (forms heterodimer with TLR1/6)	Membrane bound	bacterial diacylated lipopeptides, lipoteichoic acid from Gram positive bacteria, peptidoglycan, zymosan from yeast cell wall
TLR3	Intracellular	double-stranded RNA
TLR4	Membrane bound	lipopolysaccharide from Gram-negative bacteria, protein F from RSV
TLR5	Membrane bound	bacterial flagellin
TLR6 (forms heterodimer with TLR2)	Membrane bound	diacylated lipopeptides
TLR7	Intracellular	single-stranded viral RNA
TLR8	Intracellular	single-stranded viral RNA
TLR9	Intracellular	CpG DNA from bacteria and viruses
TLR10	Membrane bound	not determined
TLR11 (probably non-functional in humans)	Membrane bound	Profilin, a protein from a protozoan pathogen

Reviewed by Akira *et al.* 2004⁹⁹. RSV respiratory syncytial virus

Poltorak *et al.* first discovered that TLR4 is involved in LPS signalling by showing that TLR4 mutated mice were resistant to endotoxin¹⁰⁰. Endogenous ligands to TLRs such as HSP-60 for TLR4 have been suggested but are still controversial due to possible contamination of other PAMPs¹⁰¹. The exposure of endogenous ligands to TLRs might be involved in disrupting tolerance states and thus lead to autoimmunity¹⁰². The TLR genes show high variability, but how this influences gene-environment interactions and the potential changed risk or protection in diseases is not known¹⁰³. Arbour *et al.* reported a TLR4 polymorphism that was associated with a reduced response to LPS in humans¹⁰⁴. The polymorphism is a nucleotide exchange from an adenine (A) to a guanine (G) resulting in an amino acid change from aspartic acid to glycine in the fourth exon of the TLR4 gene. The Toll-like receptor 2 recognises lipoteichoic acid from Gram-positive bacteria and induces IL-12 production in monocytes¹⁰⁵.

The toll-like receptors are type I membrane proteins and have an ectodomain with leucine rich repeats important for recognition of the microbial structures, and a TIR (Toll-IL-1 receptor) domain which is required for downstream signalling⁹⁹. The TIR domain is homologous to the

cytoplasmic region of the IL-1 receptor. The TLR intracellular signalling pathways are dependent on adaptor proteins (figure 4). The MyD88 adaptor protein is involved in signalling through all TLRs except TLR3. The MyD88 independent pathway involves TRIF and is used by TLR3 and also by TLR4. TRAM is needed for recruitment of TRIF in TLR4 signalling but not in TLR3 signalling. TIRAP (MAL) is used by TLR2 and TLR4 in MyD88 dependent signalling. TLR activation with MyD88 activates the IRAK family of proteins. IRAKs interact with TRAF6, which in turn activates JAK1, a MAPKKK, and can activate either the MAPK pathway including JnK, ERK p38 MAPK and lead to activation of transcription factors such as AP-1. Activation of JAK1 can also activate IKK complex which will lead to NF κ B activation. The MyD88 independent pathway activates NF κ B, MAPK and transcription factor IRF3. IRF3 gives interferon production. Each TLR activates their own mix of adaptors which in turn activate their specific transcription factors resulting in their specific response. A newly discovered TIR adaptor is SARM which is a negative regulator of TRIF dependent TLR3 and TLR4 signalling pathways¹⁰⁶.

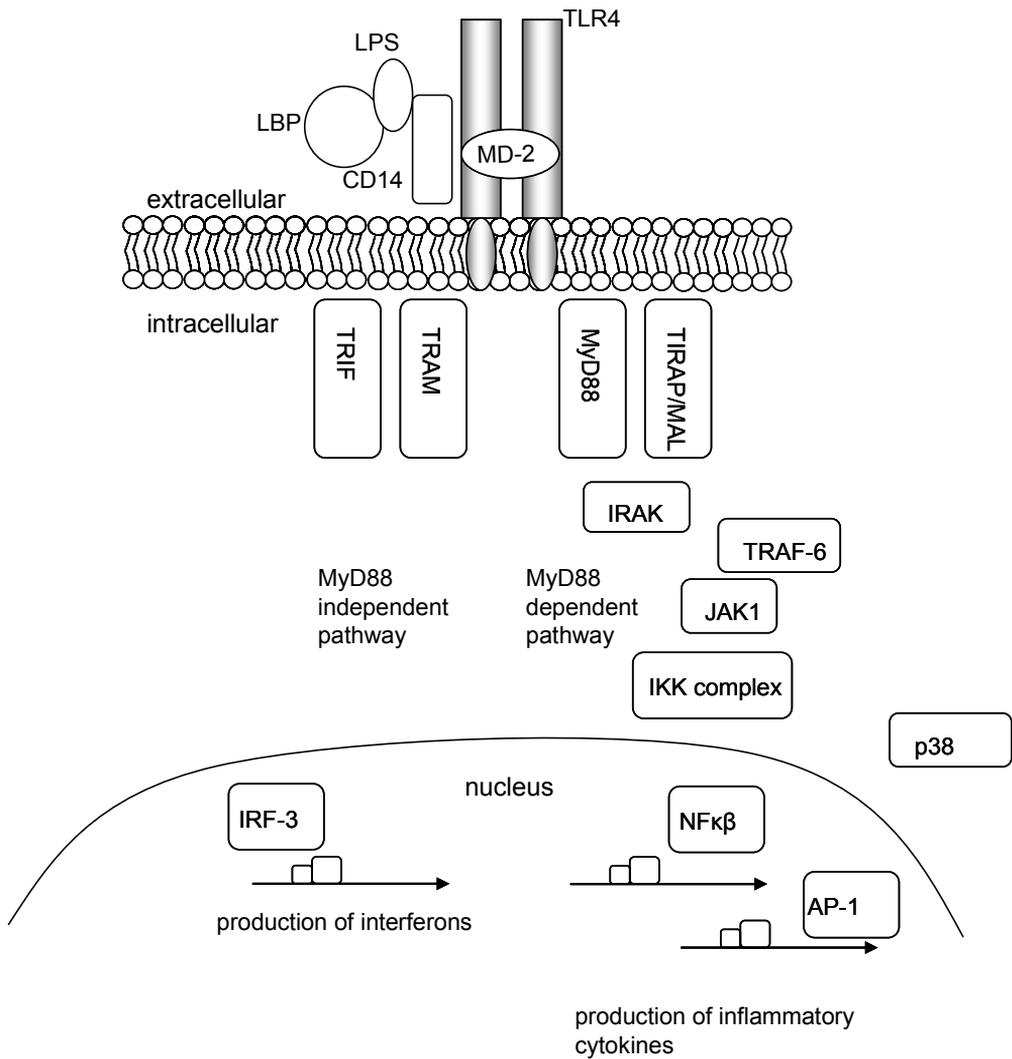


Figure 4. Schematic and simplified overview of the LPS-TLR4 signalling pathway. LPS is bound to LPS-binding protein (LBP) and sCD14 in the circulation. The complex together with MD-2 is presented to TLR4. The receptor is dimerised and a downstream signal is created. The MyD88 dependent pathway involves the adaptor proteins TIRAP/MAL. In TLR4 activated MyD88 independent pathway TRIF and TRAM are involved.

NFκβ pathway

NFκβ is a transcription factor that control expression of genes involved in immune responses but also differentiation, survival and proliferation of cells. The three main proteins involved in the signalling is the IKK complex, IκB proteins and the NFκβ dimers. The IKK proteins can be activated through several pathways, one is the classical pathway through immune receptors such as TLRs, IL-1R, and TNFR with ligands such as LPS, TNF and IL-1¹⁰⁷. The IKK complex in the LPS/TLR4 pathway includes IKKα, IKKβ and NEMO. Activated IKK proteins phosphorylate IκB proteins which lead to ubiquitination and degradation of the IκB proteins (figure 5). The NFκβ dimerase are in their activated state associated with IκB proteins in the cytoplasm (figure 5). The inhibitory IκB protein is then released from the NFκβ complex which then can be phosphorylated and translocate into the nucleus and activate gene transcription (figure 5). The most studied IκB protein is IκBα which is involved in the LPS activated pathway of NFκβ. The IKKβ is necessary for phosphorylation of IκBα on serine 32 and 36.

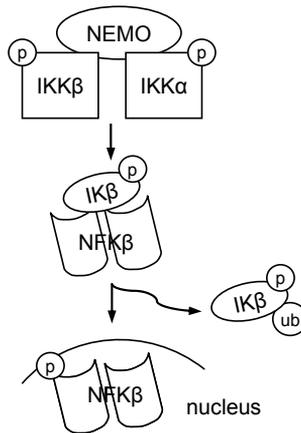


Figure 5. Activated IKK complex, consisting of IKKβ, IKKα and NEMO, phosphorylates IκBα (P) and promotes its degradation (ub) (ubiquitination). IκBα is then released from the NFκβ-dimer, which can translocate into the nucleus and act as a transcription factor.

MATERIAL AND METHODS

Study subjects

The results of the papers included in this thesis are based on three groups of subjects.

Paper I

In paper I, 115 children, of whom 69 were 8 years old and 46 were 14 years old, were included. All the children participated in larger prospective or cross-sectional studies regarding allergic diseases at the Division of Pediatrics, Allergy Centre at Linköping University Hospital. The status of the allergic sensitisation was based on skin prick tests (SPT). Whole blood for genetic studies and/or frozen peripheral blood mononuclear cells (PBMC) for functional studies was available from the children.

Paper II

To further investigate the *TLR4* Asp299Gly gene polymorphism, we recruited 16 children who had the polymorphism and/or airway allergic disease and 11 age-matched non-allergic control children from the cohort in paper I. Furthermore, to enlarge the number of individuals with the polymorphism we recruited medical students at Linköping University. Eighty-four students were screened for the *TLR4* Asp299Gly polymorphism and all four with the polymorphism and 11 age-matched controls without polymorphism, a total of 15 students, were included. The participants answered a questionnaire regarding their allergic history, present allergic symptoms and allergy medication. Venous blood samples were taken outside of pollen season. Sensitisation was studied with Phadiatop®, a screening test for circulating IgE antibodies to 11 common airborne allergens.

Paper III and IV

In paper III and IV, subgroups of Estonian and Swedish birth cohorts were studied: 14 Estonian and 36 Swedish children in paper III and 23 Estonian and 52 Swedish children in paper IV. The families of these children were enrolled during pregnancy to a large prospective study regarding environmental factors in relation to allergic disease development¹⁰⁸.

Originally, two groups comprising of 110 Estonian (Tartu) and 123 Swedish (Linköping) infants were followed from birth up to five years of age. Data about symptoms of allergy, infections and use of antibiotics were obtained by questionnaires. Clinical examinations, SPTs to food and inhalant allergens were performed at 3, 6, 12 and 24 months and 5 years of age, except that the Swedish children were examined either at three or six months. The Swedish

families were recruited between March 1996 and October 1999 from maternity wards in Linköping. The Estonian families were recruited between February 1997 and June 1998 in Tartu. Those children from whom frozen PBMC samples were available from at least three follow-ups were studied in paper III. In paper IV, the children from whom frozen PBMC were available from any follow-up point were included.

Clinical methodology

Paper I

In paper I, asthma was defined as bronchial obstruction appearing 4 times or more during the last year and verified at least once by a physician. Atopic and non-atopic asthma was defined according to the presence of at least 1 positive SPT result. Allergic rhinoconjunctivitis was defined as rhinitis and conjunctivitis appearing at least twice after exposure to a particular allergen and not related to infection. Skin prick tests were performed in duplicate on the volar aspects of the forearms with standardized birch, timothy, and cat extracts. In the group of 14-year-old children, SPTs with dog and house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) extracts (Soluprick, ALK, Hørsholm, Denmark) were also included. As a positive control histamine hydrochloride (10 mg/mL) was used, and albumin diluent was included as a negative control. The test result was regarded as positive if the mean diameter of the wheal was greater than 3 mm.

Paper II

Ongoing allergic symptoms were defined as doctor-diagnosed allergic rhinoconjunctivitis or asthma, with typical symptoms and use of medication for these symptoms during the previous 12 months. Allergic sensitisation was defined with Phadiatop test in which allergen-specific IgE antibodies against 11 common airborne allergens were analyzed in plasma with UniCap, Pharmacia CAP System, Phadiatop (Pharmacia Diagnostics, Uppsala, Sweden).

Paper III and IV

Three or more episodes of bronchial obstruction during the last 12 months period, at least once verified by a physician was the definition of asthma. Eczema was defined as pruritic, chronic or chronically relapsing dermatitis with typical features and distribution. Allergic rhinitis/conjunctivitis was defined as at least two different occasions of rhinitis and/or conjunctivitis appearing within 1h after exposure to a particular allergen and not related to infection. Urticaria was defined as allergic if it appeared within one hour after exposure to a particular allergen on at least two different occasions. Sensitisation was determined with SPT.

The tested allergens were fresh skimmed cow's milk (lipid concentration 0.5%) and thawed egg white (3, 6, 12 and 24 months of age), cat and dog (6, 12 and 24 months), birch (12 and 24 months) and timothy (24 months). Histamine hydrochloride (10 mg/mL) was used as a positive control and glycerol as a negative control. The test was considered positive when the mean wheal diameter was at least 3 mm.

Laboratory methodology

Laboratory methods in the papers

The laboratory methods used in the papers included in this thesis are listed below. The methods are described in more detail in the “Material and Methods” sections in the respective papers.

- ELISA
 - Analyses of cytokines, chemokines and sCD14 (paper I, II and III)
- Chromogenic Limulus Amebocyte Lysate (LAL) assay
 - Endotoxin analyses (paper III and IV)
- Flow cytometry (paper II)
- Genetic analyses
 - PCR amplifications (paper I and II)
 - RLFP (paper II)
 - Sequencing (paper II)
 - WAVE-sequencing (paper I)
- Cell stimulation assays of CBMC and PBMC (paper I-IV)
 - Cell isolation, culture and cell stimulation (paper I-IV)
- Luminex
 - Analyses of cytokines, chemokines (paper III and IV)
 - Phosphoprotein detection (paper II)
- UniCap
 - Analyses of total IgE (paper I) and Phadiatop® detection (paper II)
- Real time RT-PCR (paper IV)
- Skin prick testing (paper I-IV)
- Total IgE analyses (paper I)

Supplementary methods

Some supplementary data, not presented in any of the papers, is discussed in this thesis and therefore the methods are described below.

LPS and LTA whole blood stimulation

In addition to the methods and results described in paper II, the expression of monocyte markers was also studied in whole blood (1.25 mL) after incubation with 100 ng/mL LPS (*Escherichia coli* 026:B6, Sigma-Aldrich) or 1 µg /mL lipoteichoic acid (LTA)

(*Staphylococcus aureus*, Sigma-Aldrich) or without stimuli in 37°C in 96 well plates (3799 Costar® Corning Incorporated, Corning, NY, USA). The optimal cell activation time was evaluated and the tested time points were 4, 8, 16 and 24h. The highest expression of monocyte markers was observed after 16h of LPS and LTA stimulation. The cells were stained according to the same protocol as described in paper II, using antibodies directed against CD80, CD58, CCR2, TLR4, HLA-DR, CCR5, CXCR4, TLR2, CD86 and CD14. Monocytes were gated according to forward (FSC) and side scatter (SSC) and CD14 expression. Five thousand CD14+ cells were counted. The limit for positivity was set with isotype controls and only CD14+ cells were included in the analyses.

Stimulation of PBMC in paper III

In paper III, LPS/IFN- γ induced cytokine and chemokine secretion from PBMC was measured in Estonian and Swedish infants. In addition to the LPS/IFN- γ stimulation the cells were also cultured with LPS only. The same protocol was used as described in paper III. The cells were cultured with 10 ng/mL LPS *Salmonella enterica* serotype *typhimurium* (Sigma-Aldrich) for 24h and thereafter cell supernatants were collected.

Analyses of T regulatory cells in whole blood

Since the expression of TLRs has been investigated in this thesis it was also of interest to explore the expression of these receptors on human Treg cells. Venous heparinised whole blood was collected from 10 healthy volunteers in pilot experiments and stained according to manufacturer's instructions. Briefly, antibodies were added to tubes with 200 μ L blood. After 15 minutes of incubation in room temperature, 200 μ L optilyse B (Beckman Coulter, Bromma, Sweden) was added and the samples were vortexed vigorously. After 10 min, one mL H₂O was added. The following monoclonal flouochrome conjugated antibodies were used: CD4-PerCP, CD25-APC, TLR2-FITC, and TLR4-PE. Matching isotype control antibodies were used to set the limit of positivity. The samples were analysed on a four-coloured FACSCalibur (Becton-Dickinson, San José, CA, USA), and the acquired data was analysed with CellQuest Pro software (Becton-Dickinson, San José, CA, USA). The lymphocyte population was gated according to FSC and SSC. The cells expressing higher intensity of CD25 than the CD4 negative population were defined as CD25^{high} cells^{109, 110}. CD4+CD25⁺⁺ were the cells with the absolutely highest CD25 intensity and a slightly reduced CD4 intensity¹¹¹.

Statistical methods

Samples with cytokine and chemokine levels below the sensitivity limit were given half the value of the cut off to enable statistical analyses. As the data of cytokine, chemokine, IgE, sCD14 and mRNA gene levels and most of the receptor expression was not normally distributed, non-parametric statistical tests, corrected for ties, were employed. Unpaired data was analysed with Mann Whitney-*U* test and paired data with Wilcoxon signed rank test. Correlations were calculated with Spearman rank correlation test. The χ^2 test was used for nominal variables, and Fisher's exact test was used when the expected frequency for any cell was less than 5. The odds ratios for associations between different genotypes and atopic symptoms were calculated in a multivariate logistic regression model adjusting for potential confounders (paper I). A difference together with a p-value below 0.05 was considered as statistically significant, and a p-value below 0.1 was considered as a trend. In paper II and III many variables were measured and analysed. Mass significance can be a problem when performing many statistical tests if conclusions are drawn from single significances which presumably appear in random patterns. However as no conclusions were drawn from occasional significant results, we did not adjust any individual tests for mass significance. The statistical package Statview 5.0 for PC (SAS institute inc. Cary. NC) was used for the calculations.

Ethical considerations

The separate studies were approved by the Regional Committee for Human Research at the University Hospital of Linköping (I-IV) and by the Ethics Review Committee on Human Research of the University of Tartu (III and IV). All participants (paper II) or their parents (I, III, IV) gave their written informed consent.

In paper I, III and IV samples were taken from children and infants. The participants included children with or without allergic disease. It is questionable to take samples from small children for research purpose. They might not benefit from the results themselves, however the family may benefit from an earlier diagnosis of allergic disease and advice is given by an experienced research nurse and by a paediatrician. It is important for society and for the development of strategies for prevention and treatments that research is performed not only on children with allergic diseases but also includes non-allergic children in the studies. Participation in the studies was voluntary and the families were informed that they could at

any time point discontinue without any explanations. To minimize discomfort of taking blood samples a topical anaesthetic cream (EMLA) was applied prior to sampling. In paper I, III and IV skin prick tests are performed. The pain of skin prick testing where a small amount of allergen is placed on the skin which then is perforated by a lancet is minimal. Children could get allergic reactions from skin prick testing but it is very unusual (7 out of 5908 children, 0.12%, showed a generalised allergic reaction ¹¹²).

In paper II, the participants were genotyped for a *TLR4* gene polymorphism. All participants were informed that they could have access to this information if requested but were also informed that it is not possible, on an individual level, to say what consequences the different genotypes may have.

In all, we judge that the ethical advantages outweigh the disadvantages.

RESULTS AND DISCUSSION

Methodological aspects

Polymorphism detection

Genetic analyses of the *CD14*/-159 gene polymorphism was performed with restriction fragment length polymorphism. Amplified PCR products of 497 base pairs were digested with a restriction enzyme (*Ava*II), which cuts the product only if the T allele exists. The products are run on an agarose gel (figure 6). If the individual has the TT genotype the whole product is cleaved, yielding two bands of 144 and 353 base pairs. Three bands of 144, 353, and 497 base pairs will be seen for the CT genotype and one band of 497 base pairs for the CC genotype (figure 6). The results of this restriction fragment length polymorphism assay were confirmed by direct sequencing of the -159 promoter region of the *CD14* gene.

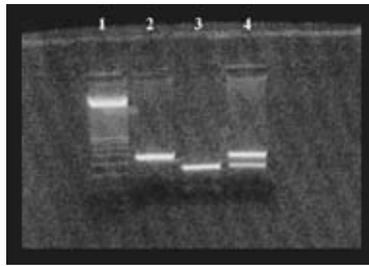


Figure 6. Genotyping of the *CD14*/-159 gene polymorphism.

Lane 1. marker, 2. CC homozygote individual, 3. TT homozygote individual, 4. CT homozygote individual

The detection of the *TLR4* Asp299Gly polymorphism was performed by denaturing HPLC analysis on a WAVE DNA Fragment Analysis System (Transgenomic Inc, San Jose', Calif) (**paper I**) and by direct sequencing in **paper II**. Figure 7 shows patterns obtained with the WAVE method for two individuals with the *TLR4* Asp299 genotype and two individuals with the *TLR4* Asp299Gly genotype.

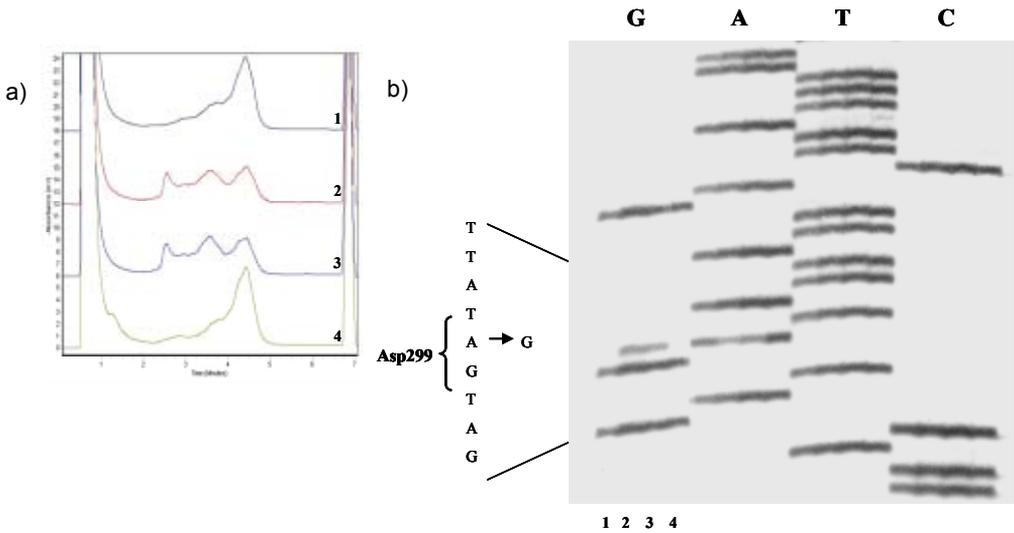


Figure 7. Results from analyses performed with the WAVE method are shown in a) where nr 1 and 4 are individuals with the *TLR4* Asp299 (AA) genotype and 2 and 3 have the *TLR4* Asp299Gly (AG) genotype. The corresponding sequencing result is shown in b).

Evaluation of Real-time PCR reactions

In paper IV, mRNA expression of several genes was analysed. Before running the samples, the primers and probes were tested to control that the reaction did not amplify genomic DNA. This was done by running reactions with RNA samples instead of cDNA samples. No reactions amplified genomic DNA. Each gene was run separately and according to the standard curve method as described in User Bulletin no 2 (Applied Biosystems). At optimal amplification reactions the slope of the standard curve is -3.3. All genes studied had an interassay variation of less than 15% and the slopes were between -3.3 and -4.2. rRNA was used as internal controls, *i.e.* the amount of the expressed gene was calculated relative to the amount of rRNA in each sample.

TLR4 Asp299Gly gene polymorphism and LPS induced immune responses

Cytokine secretion

In **paper I**, including 8 and 14 year old Swedish children, the *TLR4 Asp299Gly* gene polymorphism was associated with lower *in vitro* LPS induced IL-10 and IL-12p70 cytokine secretion from PBMC. Significantly more *TLR4 Asp299* (AA) individuals (23/57, 40%) than *TLR4 Asp299Gly* (AG) individuals (1/11, 9%) secreted detectable levels of IL-12p70 ($p=0.042$). Similar results were seen for LPS induced IL-10 secretion. Detectable levels were found in 46/58 (79%) AA individuals compared to 5/11 (45%) of the AG individuals ($p=0.029$). The LPS induced IL-12p70 cytokine secretion was lower among AG individuals ($p=0.04$) and a similar trend was seen for IL-10 secretion ($p=0.09$). Asthma as such was also associated with lower LPS induced IL-10 and IL-12p70 secretion and with the *TLR4 Asp299Gly* gene polymorphism (discussed later). The polymorphism was independently associated with lower cytokine secretion as demonstrated by analysing only children without asthma (figure 8).

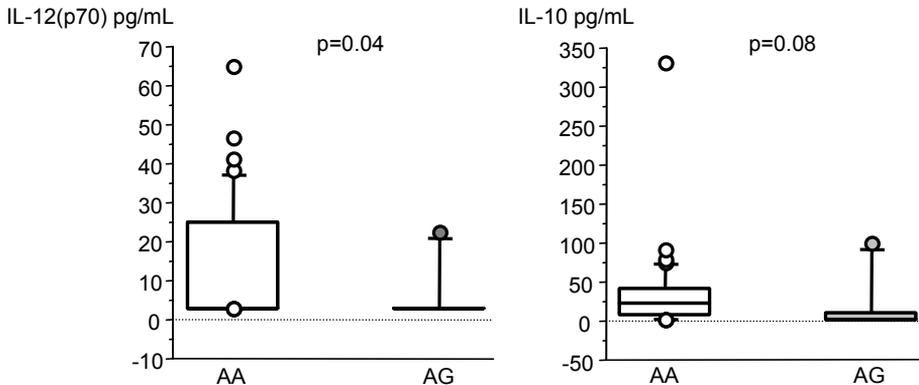


Figure 8. LPS induced IL-12p70 and IL-10 secretion in individuals without asthma divided into *TLR4 Asp299Gly* (AG) and *TLR4 Asp299* (AA) individuals.

The finding of lower IL-12p70 secretion in AG compared to AA individuals was confirmed in **paper II** where the study population consisted of young adults. In **paper II** PBMC were cultured with LPS from two strains of bacteria, *Salmonella enterica* serotype Typhimurium (Serotype Typhimurium), also used in paper I, and *Escherichia coli* (*E. coli*). The reduced cytokine secretion in AG individuals as compared to AA individuals was only seen when the cells were stimulated with Serotype Typhimurium derived LPS, giving a stronger response than *E. coli* derived LPS (**paper II**) (figure 9).

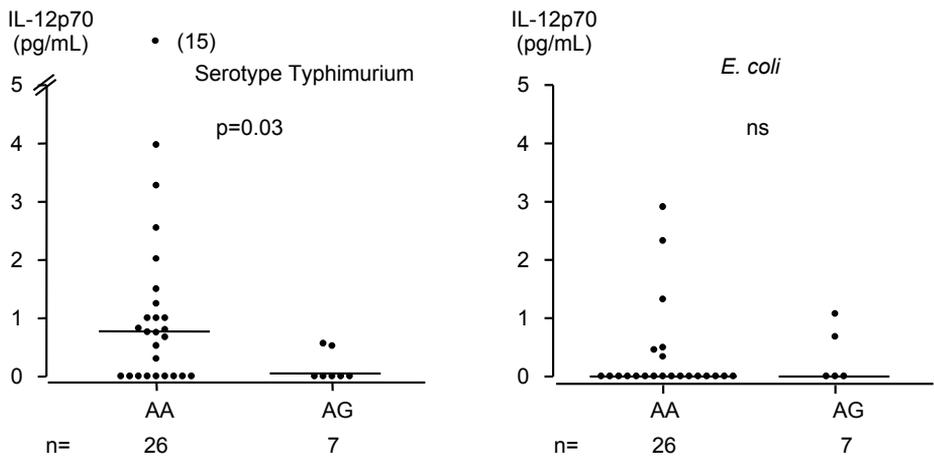


Figure 9. *TLR4* Asp299Gly (AG) individuals had lower IL-12p70 secretion compared to AA individuals when cultivating PBMC with LPS from Serotype Typhimurium, but these results were not observed when using *E. coli* derived LPS.

Other studies have not found any associations between the *TLR4* Asp299Gly gene variant and *E. coli* LPS induced cytokine secretion in stimulation experiments using isolated monocytes, PBMC or whole blood¹¹³⁻¹¹⁵. In the study by van der Graaf *et al.*¹¹⁵ PBMC from individuals homozygote for the wild type, heterozygotic and one homozygotic for the *TLR4* Asp299Gly polymorphism were cultured with HSP-60, which has been suggested to be the endogenous ligand of TLR4. No differences between the genotypes in TNF or IL-10 cytokine secretion were observed¹¹⁵. Erridge and co-authors¹¹³ tested several LPS strains, however not *Salmonella* derived LPS, with negative results. They suggested that the *TLR4* Asp299Gly polymorphism may be of importance in some cell types as it has been demonstrated to result in lower TLR4 expression in airway epithelia¹⁰⁴ but it does not necessarily result in changes of TLR4 signalling in circulating immune cells. On the other hand, von Aulock *et al.* have reported reduced LPS induced IL-10 secretion in a whole blood model using a *Salmonella*

strain derived LPS, in concordance with our results ¹¹⁶. The LPS induced IFN- γ cytokine secretion and PHA induced cytokine secretion was similar in the two genotype groups in **paper I**, implicating that the finding of reduced TLR4 signalling in individuals with the TLR4 Asp299Gly genotype in our study is specific for antigen presenting cells and their cytokine production.

The *TLR4* Asp299Gly polymorphism studied in this thesis is often co-segregated with another polymorphism, Thr399Ile in European populations. In our initial experiments the individuals with Asp299Gly were also shown to have Thr399Ile. Therefore, when screening the whole material the analysis was developed for the Asp299Gly and not Thr399Ile detection which thus was not determined in all samples. In transfection studies the *TLR4* Asp299Gly polymorphism and not the Thr399Ile polymorphism has been shown to have a functional effect ^{104, 117}. *TLR4* Asp299Gly gene polymorphism also exists without the cosegregation of Thr399Ile, especially in African populations ¹¹⁸. Ferwerda *et al.* has suggested that it is only the *TLR4* Asp299Gly haplotype that has any functional effects due to their findings of no differences in LPS induced TNF and IL-10 production between *TLR4* Asp299Gly/ Thr399Ile individuals and individuals with wild type alleles. In contrast, *TLR4* Asp299Gly individuals had higher TNF production compared to wild type in a whole blood model system ¹¹⁹.

In **paper I**, 104 of the 115 (90%) children had the *TLR4* Asp299 wild type genotype and 11 (10%) had the *TLR4* Asp299Gly genotype. The frequency of the polymorphism was in line with other reports ^{104, 120} and the observed and expected frequency did not differ, indicating that the study population was in Hardy-Weinberg equilibrium. No homozygotes GG for the genotype was found in this material. Homozygotic individuals exist but are rare ¹²¹.

Intracellular signalling pathways of LPS

Supporting our findings of the importance of the *TLR4* Asp299Gly polymorphism in LPS signalling, we demonstrated that individuals with the *Asp299Gly* polymorphism had a diminished phosphorylation of I κ B α protein after LPS stimulation of PBMC (**paper II**) (figure 10).

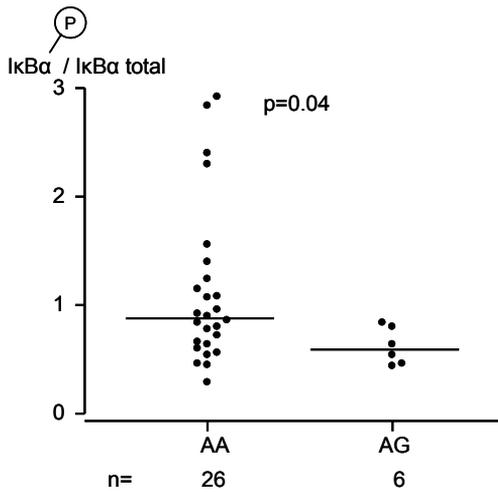


Figure 10. The Serotype Typhimurium derived LPS induced phosphorylation of IκBα was lower in individuals with the *TLR4* Asp299Gly (AG) genotype than individuals with the *TLR4* Asp299 (AA) genotype.

Diminished phosphorylation of IκBα protein results in impaired translocation of NFκB into the nucleus, leading to a reduced activation of cytokine transcription, in concordance with the reduced LPS induced IL-12 cytokine secretion in the same individuals. However, these results were only seen when using LPS from Serotype Typhimurium and not with LPS from *E. coli* (**paper II**). The bacterial origin of the LPS seems to be of importance for the magnitude of induced immune responses in circulating human cells. Interestingly, the *TLR4* Asp299Gly polymorphism may differentially modulate the signal transduction induced by LPS from different bacterial origin. Our results are in line with an earlier report of reduced IκBα protein phosphorylation in LPS activated human PBMC from heterozygous individuals for *TLR4* Asp299Gly polymorphism¹²². We also analysed phosphorylation of p38MAPK and Erk-2 protein, but in our assay the phosphorylation degree of these proteins did not increase to a measurable extent after LPS activation. Therefore we cannot conclude anything regarding these proteins and the *TLR4* Asp299Gly gene variant. Imahara *et al.* however showed that p38-MAPK activation was similar in *TLR4* Asp299Gly and Asp299 individuals¹²³. The *TLR4* Asp299Gly gene polymorphism was associated with reduced LPS induced cytokine secretion when monocyte derived cytokines were studied (**paper I and II**) and these findings were strengthened by the association with impaired intracellular TLR4 signalling (**paper II**).

Serotype Typhimurium and *E. coli* derived LPS differently induces immune responses in vitro

We observed reduced cytokine secretion and reduced I κ B α protein phosphorylation in individuals with the *TLR4* Asp299Gly genotype with Serotype Typhimurium derived LPS only (**paper I and II**). We speculate that the changes in the receptor conformation, due to the polymorphism, may affect the ability of the receptor to recognise different LPS molecules somehow. The amino acid change in the *TLR4* 299Gly allelic variant, is believed to change the extracellular domain of the TLR4 receptor and the expression of TLR4 in airway epithelia was reported to be lower in AG individuals¹⁰⁴. The transfer of the 299Gly allele to a monocytic cell line interrupted LPS induced NF κ B activation and IL-1 α release. Lipid A is the most conserved part of the LPS molecule but the number and position of acyl chains can vary between bacterial strains and it has also been suggested to affect the binding and signalling properties of LPS⁸⁰. Lipid A from *Salmonella* consists of seven acyl groups whereas *E. coli* has six¹²⁴. Another explanation for the diverging results could be that *E. coli* is found practically everywhere in our environment while *Salmonella* is not encountered as often. The continuous exposure of *E. coli* might result in lower immune responses. This explanation might be supported by the fact that both the magnitude of LPS induced cytokine responses and level of phosphorylated I κ B α protein was higher when activating the cells with Serotype Typhimurium than with *E. coli*. Stimulation time points and concentrations for both Serotype Typhimurium and *E. coli* LPS were evaluated and the conditions giving the maximum responses were chosen. In contrast to our findings, Tulic *et al.* were able to demonstrate *TLR4* Asp299Gly in association with reduced IL-10 cytokine secretion and reduced IL12p35 mRNA in PBMC stimulated with *E. coli* derived LPS¹²².

Expression of monocyte markers

Even though epithelial airway tissue of Asp299Gly polymorphism individuals express reduced levels of the TLR4 receptor¹⁰⁴ it is not known if the polymorphism affects TLR4 expression on circulating monocytes. Investigation of the TLR4 expression on circulating CD14 positive monocytes analysed *ex vivo* in whole blood directly after blood sampling, displayed no differences between AA and AG individuals (**paper II**). Furthermore, no differences were found between the two genotype groups in TLR4 expression on monocytes from whole blood cultures incubated with *E. coli* derived LPS or gram positive derived lipoteichoic acid (LTA) from *Staphylococcus aureus* (not published, shown in figure 11). This is in contrast to a recent study where lower TLR4 expression on cryopreserved PBMC was reported in *TLR4* Asp299Gly individuals¹²². Another study showed that after *in vivo* infusion of LPS no differences were found between the two genotype groups regarding expression of surface TLR4 monocytes¹²⁵. The discrepant findings might be due to handling of the cells, using isolated PBMC or whole blood or also due to the gating strategies in flow cytometry analyses. We used whole blood as an experimental system to investigate receptor expression of circulating monocytes since our initial experiments demonstrated that Ficoll gradient isolation of monocytes downregulated TLR expression (data not shown).

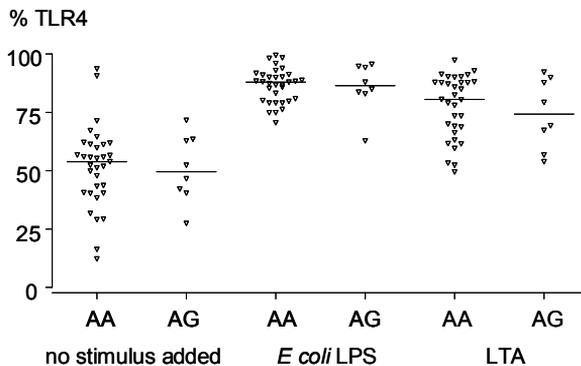


Figure 11. The percentage of TLR4 expressing circulating monocytes was similar in *TLR4* Asp299 (AA) and *TLR4* Asp299Gly (AG) individuals both before and after incubation of whole blood with LPS and LTA. The median value is indicated with a horizontal line.

While TLR4 expression on CD14 positive cells was similar in the two genotype groups (figure 12a), the percentage of TLR2 expressing CD14+ cells was higher among heterozygote AG as compared to AA individuals (**paper II**) (figure 12b). Furthermore, the percentage of CCR5 expressing CD14+ cells was higher among AG individuals *ex vivo* and after LPS stimulation (figure 12c, d).

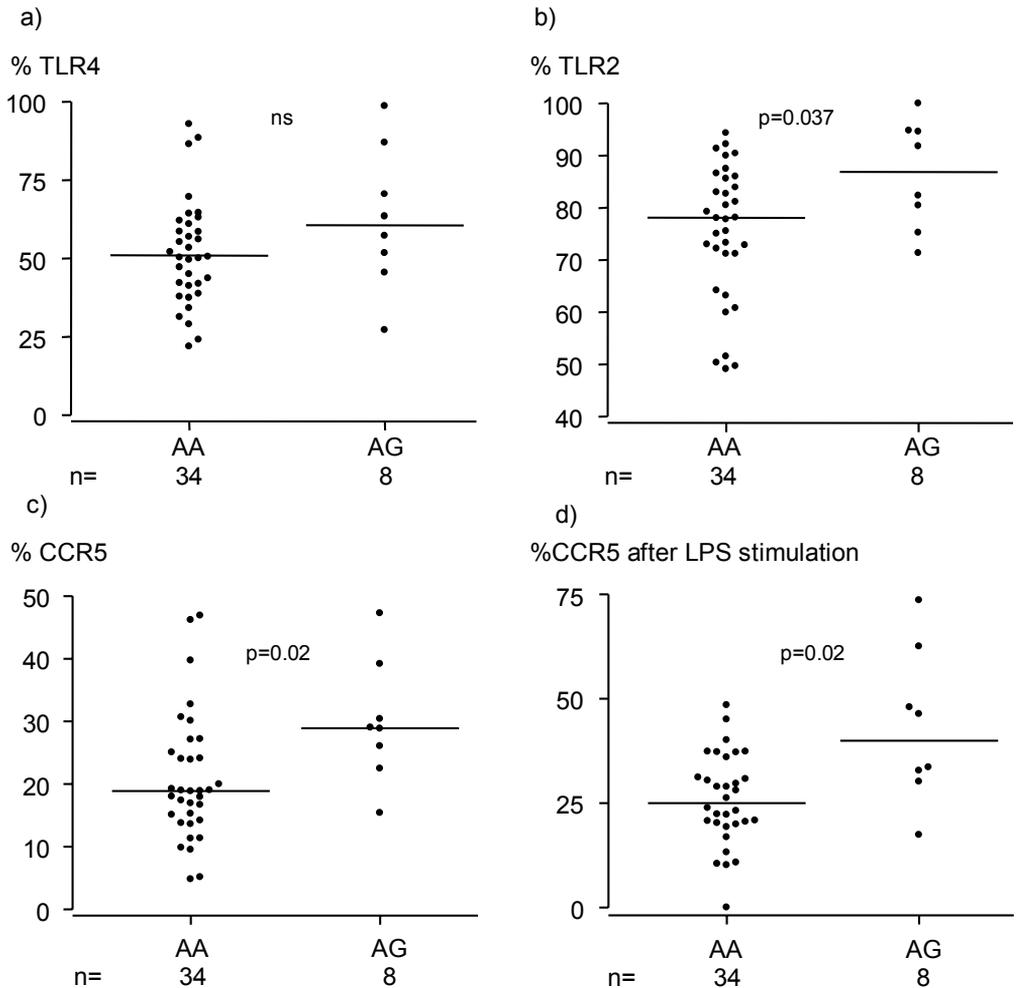


Figure 12. Percentages of TLR4 (a), TLR2 (b), and CCR5 (c) expressing CD14-positive monocytes in whole-blood samples from individuals with (AG) and without (AA) the *TLR4* (Asp299Gly) polymorphism. Expression of CCR5 after whole blood stimulation with 100ng/mL *E. coli* derived LPS is shown in d). The median value for each group is indicated by a horizontal line. ns, not significant.

TLR2 recognises a variety of microbial patterns and associates with other TLRs to confer signalling. CCR5 is a chemokine receptor which binds CCL3, CCL4 and CCL5. An explanation for the higher percentages of TLR2 and CCR5 expressing monocytes in *TLR4* Asp299Gly heterozygote individuals might be an immunological crosstalk between TLRs. Pre-treating mouse macrophages with neutralising anti-TLR4 antibodies was shown to result in an up-regulation of TLR2 and an increased cytokine secretion after stimulation with superantigen¹²⁶. An intact TLR4 signalling was important for down-regulation of TLR2 expression. Re-challenge of cells with LPS results in impaired responsiveness, endotoxin tolerance, but other pattern-recognition molecules may become activated instead. An *et al.* showed that activation of mouse macrophages through TLR4 up-regulated the expression of TLR9, thus rendering the cells more capable to detect CpG DNA¹²⁷. Thus, the increased expression of TLR2 and CCR5 in *TLR4* Asp299Gly individuals could be due to the impaired signalling via TLR4.

In conclusion (figure 13), TLR4 Asp299Gly gene polymorphism was associated with reduced LPS induced phosphorylation of IκBα and reduced LPS induced cytokine secretion. These results were observed when cells were cultured with LPS from Serotype Typhimurium but not with LPS from E. coli. The polymorphism was also associated with lower TLR2 and CCR5 expressing CD14 positive cells in blood.

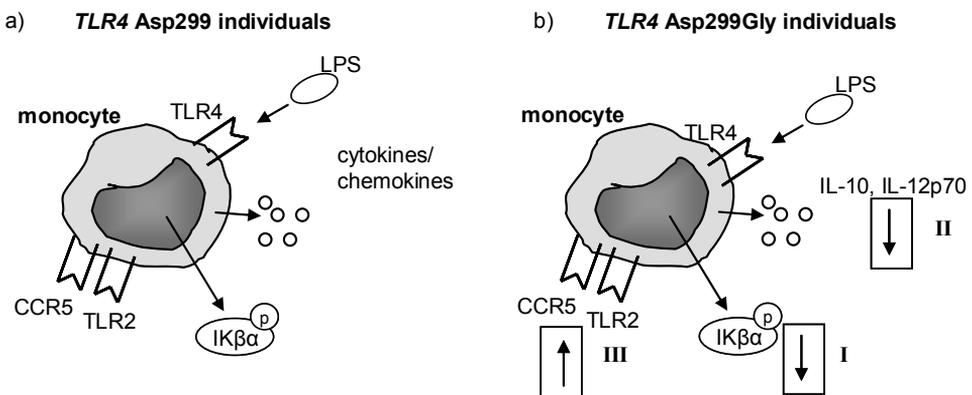


Figure 13. A schematic figure that summarizes our results regarding LPS induced immune responses and the *TLR4* Asp299Gly gene polymorphism. Individuals with the *TLR4* Asp299Gly genotype, shown in b), had lower LPS induced IκBα phosphorylation (I) and cytokine secretion (II) (↓) as compared to individuals with the *TLR4* Asp299 genotype, shown in a). The levels of TLR4 expressing circulating monocytes were similar but individuals with the *TLR4* Asp299Gly genotype had more TLR2 and CCR5 expressing monocytes (III)(↑).

TLR4 Asp299Gly polymorphism and asthma

In **paper I**, the *TLR4* Asp299Gly gene polymorphism was found to be associated with a 4-fold increased prevalence of asthma and a 7-fold higher prevalence of atopic asthma (*i.e.* asthma combined with sensitisation which was defined by a positive skin prick test), in Swedish children (figure 14).

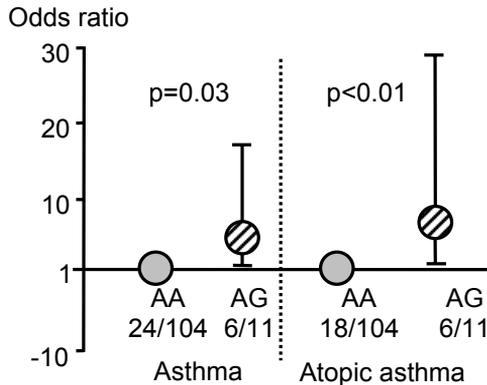


Figure 14. Adjusted odds ratio for asthma and atopic asthma in individuals with the *TLR4* Asp299 (AA) or *TLR4* Asp299Gly (AG) genotype.

This *TLR4* Asp299Gly gene polymorphism was earlier shown to be associated with a reduced capacity to respond to inhaled LPS in humans^{104, 128}. The polymorphism was not associated with allergic symptoms other than asthma and not to SPT positivity or circulating IgE levels as such. Our finding is in discrepancy with other studies where the association between the *TLR4* Asp299Gly gene polymorphism and asthma has not been demonstrated^{121, 129}. The limitation of our study is the low numbers of participants, although the clinical material is very well diagnosed and categorized. Possible explanations for the different results could be both genetic heterogeneity among the populations and gene environment interactions. Eder *et al.* also investigated allergic phenotypes and the *TLR4* Asp299Gly gene polymorphism in children of school-age. In environments with low amounts of endotoxin, the polymorphism was associated with a trend of increased risk of atopy, *i.e.* sensitisation defined by specific IgE measurements, (OR 1.93), whereas in high endotoxin environments the polymorphism was associated with a decreased risk of atopy (OR 0.17)¹³⁰. Werner *et al.* also showed that the *TLR4* Asp299Gly gene polymorphism was associated with a decreased risk of asthma only in environments with high or moderate endotoxin levels, in an adult population¹³¹. This

could be one contributing explanation to our results since the endotoxin levels in the environment where the Swedish children live are probably rather low, a suggestion supported by results from our group with lower endotoxin levels in comparison to Estonia⁴³.

The TLR4 receptor does not only detect LPS but also syncytical virus F protein from RSV virus¹³². Earlier, the *TLR4* Asp299Gly gene polymorphism has been associated with an increased risk for severe RSV infections in healthy infants^{133, 134} or in high risk (based largely on prematurity) infants¹³⁵. A mechanistic explanation is suggested by a study where the TLR4 Asp299Gly gene polymorphism was associated with lower RSV virus induced secretion of IL-8, TNF- α , IL-10, and IL-6 from PBMC¹²². RSV infection is believed to increase the risk of later developing asthma (reviewed in³²), which could be one possible explanation for the association of the receptor polymorphism and asthma.

The *TLR4* Asp299Gly gene polymorphism studied in this thesis has been associated with higher incidence of Gram-negative infections¹³⁶ and has also been implicated in other diseases such as atherosclerosis, Crohn's disease, tuberculosis and malaria (reviewed in¹³⁷).

Environmental factors both before birth and early in life might affect disease development and gene expression. Research on inherited modifications of gene expression patterns that do not depend on changes in DNA sequences is called epigenetics. The changes are believed to be enzymatic changes in DNA or alterations in histone proteins. Different methylation, acetylation or phosphorylations of histone proteins are suggested to be important for changing the gene expression. Genetic variation might alter the individuals' susceptibility for developing allergic diseases such as asthma¹³⁸ and also to be of importance for the interaction with the environment as has been observed regarding polymorphisms in for example several TLR genes and the CD14 gene.

In conclusion, the TLR4 Asp299Gly gene polymorphism was associated with asthma and atopic asthma in Swedish school-children.

CD14 and CD14/-159 gene polymorphism and allergy

In **paper I**, sCD14 levels in serum and a genetic polymorphism in the promoter region of the CD14 gene, *CD14/-159*, were investigated in 8- or 14- year old Swedish children. The frequency of the genotypes was 43 of 115 (37%) for CC, 48 of 115 (42%) were heterozygotes (CT) and 24 of 115 (21%) had the TT genotype. The observed and expected frequency of the polymorphism did not differ, indicating that the study population was in Hardy-Weinberg equilibrium. No associations of the CD14 genotypes and asthma, ARC or sensitisation were found. Further, in our material the polymorphism was not associated with either sCD14 levels or total IgE levels. Soluble CD14 levels were not associated with allergic disease or total IgE levels. One study showed that non atopic asthma in adults was associated with the T allele¹³⁹ while others have not shown any association with the polymorphism and asthma^{90,91}.

However, individuals with the TT genotype had higher LPS induced IL12-p70 secretion in PBMC than CT/CC individuals. Similar results have been shown by Keskin *et al.* in Turkish 6 to 18 year old asthmatic children, where the TT individuals had higher LPS induced IL-10 and IL-1 β secretion from PBMC than the CC individuals¹⁴⁰. In the report where the polymorphism was first described, sensitised children with the TT genotype had lower IgE levels and higher sCD14 serum levels than the CT and the CC individuals⁸⁹. Several studies have confirmed the findings of lower IgE levels^{92,141} and higher sCD14 levels¹⁴¹ among the TT individuals while others could not confirm these associations^{93,142}. Lauener *et al.* showed a higher CD14 mRNA expression in PBMC from farmer's children who are at a lower risk of developing allergic disease¹⁴³. Reduced sCD14 in human breast milk was associated with development of eczema at 6 months of age in an English study¹⁴⁴.

The presence of the T allele of this particular CD14 polymorphism resulted, in a monocytic cell line, in a higher transcription rate of the CD14 gene¹⁴⁵. The CD14 receptor detects LPS and also other Gram-positive bacteria and mycobacteria (reviewed in⁸⁸). An increased expression of a pattern recognition receptor, such as CD14, could enhance the responsiveness to microbial products in early life, skew the responses towards Th1 rather than Th2 thus decreasing the risk of developing IgE responses to allergens. Also, sCD14 has been shown to inhibit IgE production in human antigen stimulated PBMC and to favour IgG1 production¹⁴⁶. One reason for the discrepant results regarding the *CD14/-159* polymorphism and allergic disease might be due to complex interactions between gene and environment in the different

study populations. The TT genotype of the *CD14/-159* polymorphism has been associated with a decreased risk of asthma in environments with low endotoxin exposure, but an increased risk in environments with high endotoxin exposure¹⁴⁷. In another study, the protective effect of endotoxin exposure for development of atopy was stratified for the *CD14/-159* gene polymorphism. The effect of endotoxin exposure on IgE formation was mainly observed among CC homozygotes and the association among the other genotypes was weaker¹⁴⁸. In low endotoxin environments the CC individuals may carry the risk of sensitisation but in high endotoxin environment they may be protected from sensitisation.

In conclusion, the studied CD14 gene polymorphism showed no association to allergic disease, IgE levels or sCD14 levels, but the TT individuals had higher LPS induced IL12p70 secretion, which might suggest a more Th1 deviated immune response.

Allergic disease and innate immune responses

Children who later develop allergic disease show immunological differences already very early in life. In **paper IV**, lower TLR2 mRNA expression was seen at birth in Swedish children who were allergic at 5 years of age as compared to children who did not develop allergy. One study reported that children with allergic mothers had lower TLR2 mRNA expression in CBMC than children with non-allergic mothers, possibly supporting our results suggesting that impaired transcription of TLR2 is associated with allergy¹⁴⁹. It has been reported that CRP levels are low in children who later develop allergies¹⁵⁰. Several allergy prevention studies use supplementation with probiotic Gram positive bacteria, which seems to be associated with increased inflammation, seen as increased CRP levels¹⁵⁰. It seems that low-grade inflammation in infancy could be a factor that protects against allergy most likely by modulation of the maturing immune system. Our findings of reduced TLR2 mRNA expression may be a factor that reflects poor bacterial signalling, low inflammation and thus could be related to the development of allergy by this mechanism. mRNA expression of TLR4 in PBMC correlated with several of the LPS induced cytokines and chemokines (**paper IV**) indicating that TLR4 transcripts are related to the LPS mediated cytokine responses. Interestingly, additional data from cell stimulations of the study group from **paper II** showed that young adults with asthma and/or rhinoconjunctivitis had an impaired upregulation of TLR2 on CD14+ cells in whole blood after LTA stimulation (figure 15a). Also, the numbers of CCR3 expressing CD14+ cells *ex vivo* and after LTA stimulation were decreased in the group of allergic subjects. Furthermore, the expression of CD80 on CD14+ cells increased after LTA stimulation in the majority of non-allergic individuals (p=0.01) but tended to decrease in allergic individuals (figure 15b). In general, LPS and LTA stimulation of whole blood upregulated the expression of TLR2 and TLR4 on CD14+ monocytes in accordance with other studies^{151, 152}. Thus, it could be speculated that innate immune responses to Gram-positive LTA may be poor in allergic individuals.

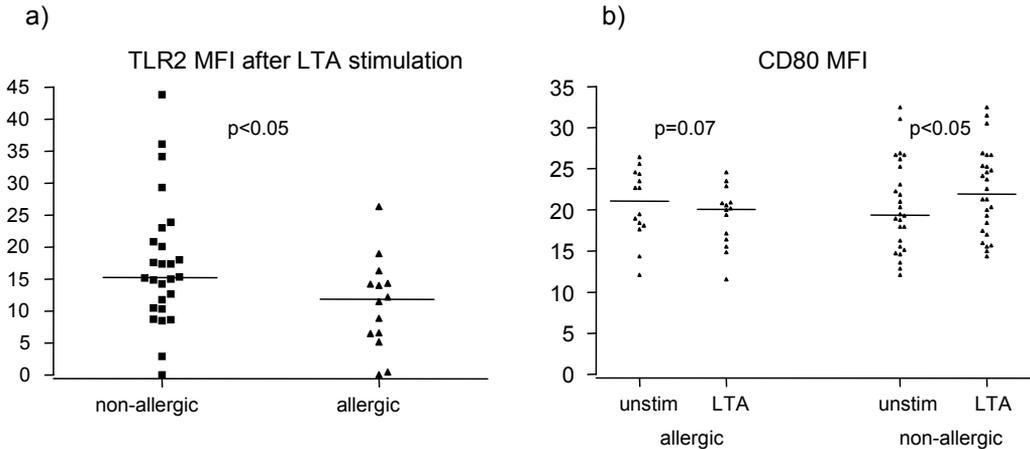


Figure 15. Expression (MFI) of TLR2, (the unstimulated is subtracted) in a) and CD80 (b) on CD14 positive monocytes after 1 μ g/mL LTA for 16h in 37°C or no added stimuli (unstim) in the allergic and non-allergic group. Medians are indicated in each figure.

In **paper I**, 8- and 14-year-old children with allergic asthma had reduced Serotype Typhimurium derived LPS-induced IL-10 and IL-12p70 levels as compared to non-allergic children, which is supported by the findings of others¹⁵³. Since our results show that the *TLR4* Asp299Gly gene polymorphism affects LPS induced cytokine secretion, we compared the AA individuals separately and the result remained. In line with these results, the I κ B α phosphorylation was lower in allergic than non-allergic individuals (**paper II**) after stimulation with LPS derived from Serotype Typhimurium but not *E. coli*. However, no differences were observed in LPS induced cytokine secretion between allergic and non-allergic individuals in **paper II**.

Sensitised Swedish children up to two years of age showed increased LPS/IFN- γ induced IL-10 secretion at 3 and 12 months of age with similar trends observed for sensitised Swedish infants with allergic symptoms compared to non-allergic, non-sensitised infants (**paper III**). The possible reasons for the diverging results are many. As the children in paper I were of school-age, asthma was a common allergic manifestation, whereas eczema represents the primary allergic manifestation in **paper III**. Also, **paper III** is a prospective study investigating immune responses before development of allergic symptoms, whereas current established allergic symptoms were related to LPS responsiveness among the school-age children and the young adults in paper II. Also, in paper III, LPS in combination with IFN- γ was used to enhance the cellular response.

In conclusion, several differences in innate immune responses were observed between allergic and non-allergic individuals. Asthma in school-children was associated with reduced LPS induced cytokine production. LPS induced phosphorylation of I κ B α was lower in adult allergic compared to non-allergic individuals. Lower TLR2 mRNA expression was found in Swedish children who had developed allergic disease at five years of age.

Immune responses in Estonian and Swedish children

In **paper III**, we followed innate immune responses in Estonian and Swedish children from birth to two years of age. Peripheral blood mononuclear cells or cord blood mononuclear cells were cultured with LPS or LPS in combination with IFN- γ . The cytokine and chemokine secretion after LPS or LPS in combination with IFN- γ stimulation was evaluated during method development. The results from both stimulations correlated relatively well but the combination with IFN- γ was used as it enhanced the responses. It has been shown in macrophages that IFN- γ priming or costimulation increases the ability of the cells to respond to bacterial substances such as LPS affecting among other things, cytokine and chemokine secretion, and production of toxic mediators¹⁵⁴⁻¹⁵⁶. Another aspect of adding IFN- γ in the experimental setting is that *in vivo*, activated Th1 cells produce IFN- γ , but another important source of IFN- γ in early innate immune responses are NK-cells which can directly respond to bacterial stimuli and thus potentiate the macrophage function¹⁵⁷. LPS induced cytokine and chemokine responses were in general higher in Swedish than Estonian infants at birth and at 3 and 6 months of age (table 2).

Table 2. Observed differences between Estonian and Swedish children regarding cytokine and chemokine secretion (pg/mL) from cord blood mononuclear cells at birth and peripheral blood mononuclear cells at 3 and 6 months of age cultured with or without LPS/IFN- γ . A statistically significant increase or decrease in the Swedish as compared to the Estonian infants, calculated with Mann Whitney-*U* test, is marked \uparrow Swe and \downarrow Swe, respectively.

Age	<i>no stimulus</i>		<i>LPS/IFN-γ</i>		
	3 m	6 m	birth	3 m	6 m
CCL4	ns	\uparrow Swe	\uparrow Swe	ns	Ns
CXCL8	ns	ns	ns	ns	Ns
IL-10	ns	ns	ns	ns	Ns
IL-1 β	ns	ns	ns	\uparrow Swe	Ns
IL-6	ns	ns	\uparrow Swe	ns	\uparrow Swe
IL-12p70	ns	ns	ns	\uparrow Swe	Ns
IL-17	ns	ns	ns	\uparrow Swe	Ns
TNF	ns	\uparrow Swe	ns	\uparrow Swe	Ns
CCL2	\uparrow Swe	\uparrow Swe	ns	\downarrow Swe	Ns

The results of higher LPS/IFN- γ induced responses in Swedish infants are also strengthened by our earlier reported results of higher allergen induced cytokine responses in Swedish than Estonian children¹⁵⁸. mRNA expression of TLR4 was similar in the two countries but was correlated with several of the LPS induced cytokines and chemokines (**paper IV**). Estonia is a country with a lower prevalence of allergy than Sweden¹⁰⁸. The house dust endotoxin levels were higher in the homes of the Estonian than the Swedish infants (**paper III** and⁴³). It has been speculated that a reduced microbial pressure during early childhood or even during pregnancy is a risk factor for developing allergic diseases. Endotoxin levels were inversely associated with the development of allergic disease and sensitisation among the Swedish children⁴³. Low environmental endotoxin levels early in life have also been associated to an increased risk for allergic symptoms and sensitisation in other studies^{42, 159}. The differences in immune responses between the two study groups was not dependent on the fact that the allergy frequency was higher in the Swedish cohort, as the results remained when comparing non-sensitised non allergic Swedish infants with the Estonian infants. Further, there were no differences between the groups in PHA induced cellular responses. We acknowledge that the number of infants unfortunately is low in **paper III** and larger studies are needed to further investigate eventual bacterial induced immune response differences between countries or populations with different microbial conditions. It is possible that the higher environmental

endotoxin load in Estonia results in so called endotoxin tolerance, and that the response of PBMC to LPS stimulation *in vitro* thus is lower in Estonian than in Swedish children who are exposed to lower amounts of environmental endotoxin. However, although the endotoxin levels were higher in Estonian compared to Swedish homes no correlations between endotoxin levels and LPS induced cytokine and chemokine responses were found. In **paper III**, we observed in Swedish infants higher LPS/IFN- γ induced levels of TNF at 3 months and IL-6 at 6 months of age than in Estonian infants. Lappalainen *et al.* reported an inverse association with mitogen induced TNF and IL-6 and the levels of 3-hydroxy fatty acids, which is a chemical marker for LPS, in mothers and their 3 months old infants¹⁶⁰. In that study, no correlations were found for endotoxin measured with LAL assay, which measures the bioactive form of endotoxin and is the method used in our studies. Braun-Fahrlander *et al.* have shown a protective effect of endotoxin on allergic sensitisation⁴¹ in a German/Swiss/Austrian population of school-children. They further showed that endotoxin levels in mattresses were inversely correlated to the capacity to respond to LPS *in vitro*, and suggested an *in vivo* effect of long term exposure of endotoxin that affects the innate immune responses. Unresponsiveness to LPS after an earlier encounter is called endotoxin tolerance and has been shown both in humans and *in vitro*¹⁶¹. Production of anti-inflammatory IL-10 and TGF- β has been suggested as possible mechanisms behind endotoxin tolerance⁸⁴. We did not detect any increased production of IL-10 in our cell cultures but an *in vivo* role can not be excluded.

An altered gut flora has been observed in Swedish as compared to Estonian infants. Swedish infants have less lactobacilli and more frequently clostridia than Estonian infants¹⁶². Furthermore, the gut flora differs in allergic and non-allergic children^{163,164}. Higher secretory IgA levels in saliva has been found in the Estonian as compared to the Swedish infants up to two years of age¹⁶⁵ suggesting a delayed maturation of the mucosal soluble IgA system among the Swedish infants, possibly due to low microbial exposure. Recent results demonstrate that among the Swedish children, the relative amounts of the Gram negative *Bacteroides fragilis* colonisation early in life was negatively correlated to TLR4 mRNA expression at 12 months (**paper IV**) and also to LPS-induced production of IL-6 and CCL4 at 12 months of age (Sjögren *et al.*, submitted). Also, in the Swedish children from this cohort, high endotoxin levels were associated with increased faecal bifido bacterial diversity early in life (Sjögren *et al.*, submitted). These results suggest a link between endotoxin levels and

microflora diversity and also emphasize the importance of the gut flora and its possible impact on the development of innate immune responses.

In the Estonian infants, a positive correlation between the level of spontaneous secretion of CXCL8 and CCL2 from PBMC cultured in medium alone and levels of endotoxin in mattresses was found (**paper III**). The reason and relevance for this finding is unknown but possibly the microbial load in Estonia is enough to produce this association between endotoxin levels and innate immune responses, whereas in Sweden the endotoxin levels are low and therefore no association is observed.

In **paper IV**, Estonian infants at birth were found to have higher mRNA expression of the Treg associated markers, Foxp3 and Ebi3, than Swedish infants in blood mononuclear cells. Ebi3 forms together with p35 the immunoregulatory cytokine IL-35 secreted by murine Treg cells⁵³. Ebi3 also forms, together with p28 (IL-27 α) the cytokine IL-27. Ebi3 expression was correlated at all time points with the gene expression of Treg associated Foxp3. Additional data shows that the expression of p35, but not p28, also correlated to both Ebi3 expression and Foxp3 expression at birth (table 3). Therefore it seems that the mRNA expression of Ebi3 in our study contributes to IL-35 and immune regulation functions. The increased mRNA expression of Ebi3 together with an increased expression of Foxp3 support the idea of increased activity of Treg cells in Estonian than Swedish infants at birth. The increased expression of Treg associated markers implies that there are more cells with regulatory functions or more Treg cell activity. This in turn could mean that there might be an *in vivo* production of anti-inflammatory cytokines.

Table 3. The mRNA expression of T regulatory markers Foxp3 and Ebi3 correlated at all time-points. The mRNA expression of Foxp3 and Ebi3 also correlated to p35 mRNA expression at birth. The expression of Foxp3 and Ebi3 was not correlated to the expression of p28 however.

	Foxp3 and Ebi3		Foxp3 and p35		Foxp3 and p28		Ebi3 and p35		Ebi3 and p28	
	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value
CB	0.67	<0.001	0.7	<0.0001	0.4	ns	0.58	<0.0001	0.48	0.09
3m	0.48	<0.01								
6m	0.50	<0.01								
12m	0.56	<0.0001								
24m	0.56	<0.0001								

The proposed increased involvement of Treg cells or function might be an explanation for the reduced LPS induced cytokine and chemokine production results seen in Estonian children in **paper III** (figure 16). It is possible that an increased microbial pressure in the Estonian infants, suggested by higher endotoxin levels in the house dust and an altered gut flora, is involved in the upregulation of Foxp3 and Ebi3 in cord blood (figure 16). At birth the infant has been exposed to the vaginal microbes for several hours, which may be enough for the upregulation of the immune system. Alternatively, the environmental microbes may affect the infant already during the pregnancy.

Interestingly, sensitisation up to 5 years age was associated with low Ebi3 expression at birth in the Swedish children (**paper IV**), although this finding is based on a small number of children and should be studied further. Schaub *et al.* demonstrated a correlation between *in vitro* IL-10 secretion and expression of Treg markers ¹⁶⁶. In their study, CBMC from children with atopic mothers produced less IL-10 after stimulation with peptidoglycan, a TLR2 ligand. However the role of Treg cells in allergy is not clear. In one-year old egg-allergic children the amount of cord blood derived Treg cells were not different compared to non-allergic children but they had a somewhat reduced function ¹⁶⁷.

Estonian and Swedish infants had similar TLR2 and TLR4 mRNA expression (**paper IV**). A higher mRNA expression of TLR2, TLR4 and CD14 has previously been reported in farmers' children of school-age ¹⁴³. Also, Ege *et al.* found that children of mothers who worked in stables during pregnancy showed increased spontaneous expression of TLR2, TLR4 and CD14 mRNA at the age of 10 years ¹⁶⁸. In those studies it was proposed that high microbial

exposure resulted in increased expression of the pattern recognition receptors, and reduced risk of developing allergic disease.

In **paper IV**, we also analysed the mRNA expression of the Th2 associated regulatory protein SOCS3. SOCS3 has been shown to be expressed primarily in Th2 cells and to inhibit IL-12 signalling⁵⁸. Also, allergic disease has been associated with high SOCS3 expression⁵⁹. We could not find any association with SOCS3 expression and allergic disease. Also, the expression was similar in Estonian and Swedish infants at all ages.

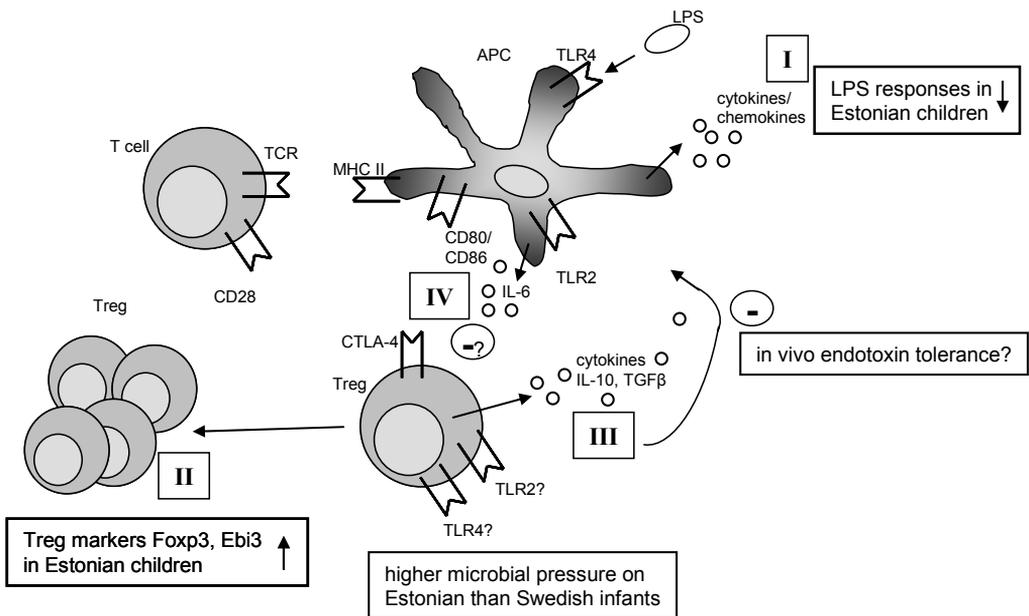


Figure 16. A summary and a possible explanation model of our results, regarding innate immune responses and expression of immune regulatory markers in Estonian and Swedish children, can be visualised with this schematic figure. Reduced LPS induced responses in Estonian children (I) could be due to an *in vivo* endotoxin tolerance effect due to high environmental endotoxin levels. Treg cells have been proposed to directly respond to TLR2 ligands, which reduce their suppressive function and induce proliferation of the Treg population. A higher microbial pressure on Estonian infants could lead to an increased number of Treg cells and thus explain our observed higher expression of Treg associated markers (II). That in turn lead to suppression of immune responses (III). Stimulation of TLRs, lead to *e.g.* IL-6 cytokine production which has been suggested to reduce the suppressive function of Treg cells, enabling immune responses (IV).

In conclusion, Estonian infants have lower LPS induced cytokine and chemokine responses during the first year of life and higher mRNA expression of Treg associated markers than Swedish infants. These results may implicate a more rapid development of immune regulation in Estonian children.

TLR expression on Treg cells

Since the monocyte expression of TLRs has been investigated in this thesis, it was also of interest to explore the expression of these receptors on human T cells. Human T cells have been reported to express many of the TLRs^{169, 170} and it has also been reported in mice that Treg cells express both TLR2 and TLR4⁵¹. In a pilot experiment of 10 individuals, TLR2 and TLR4 expression was analysed on T cell populations from whole blood in relation to CD4 and CD25 expression. The cells with the absolutely highest expression of CD25 (CD25⁺⁺) showed the highest expression of both TLR2 and TLR4 (figure 17).

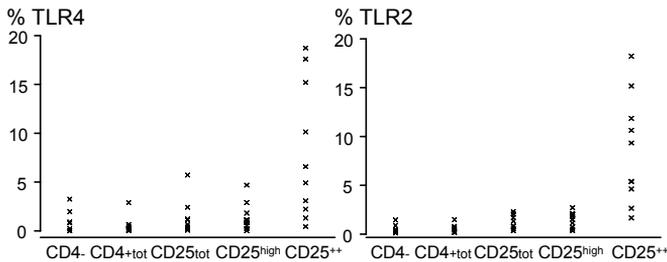


Figure 17. TLR2 and TLR4 expression on different T cell populations

The CD25^{high} cell population was between 3-10% of the total CD4+ cell population and the CD25⁺⁺ were only approximately 0.2% of the total CD4+ cell population, approximately only 200-500 cells. Despite the limitations related to low numbers of cells and subjects analysed, it is tempting to speculate that also human Treg cells may have a direct link to bacterial exposure via the pathogen recognition receptors. In continuation we would have wanted to explore if Treg cells were induced by LPS also in human cells as has been shown in mice⁵¹. Stimulation of PBMC with microbial antigen, LPS and LTA, had no effect on the TLR expression on CD4+CD25+ cells in our initial experiments. Neither did the number of Treg cells, nor the expression of their surface markers change. The mouse data regarding LPS expansion of Treg cells has not been confirmed in a human system, however^{171, 172}. Suttmüller *et al.* showed in mice that Treg cells activated via TCR and IL-2 together with a TLR2 ligand Pam3Cys showed increased proliferation, but the cells suppressive function was decreased⁵². This direct link between Treg cells and innate immune responses would suggest that during an infection the number of Treg cells are expanded but their function is reduced, allowing immune responses to clear the infection. Once the infection is cleared, the Treg cells suppress the immune response and the immunological balance is restored.

SUMMARY AND CONCLUDING REMARKS

The *TLR4* Asp299Gly gene polymorphism was associated with reduced LPS induced cytokine production in Swedish school-children. The expression of TLR4 on the monocyte cell surface was not altered either in the natural state or after LPS cultivation in individuals with this polymorphism. However, the expression of TLR2 and CCR5 was higher in individuals with the polymorphism, which might imply a crosstalk between the receptors. Individuals with the *Asp299Gly* genotype had reduced LPS induced phosphorylation of the intracellular protein $\text{I}\kappa\text{B}\alpha$, important for $\text{NF}\kappa\text{B}$ activation. Since the phosphorylation of $\text{I}\kappa\text{B}\alpha$ enables the transcription factor $\text{NF}\kappa\text{B}$ to be translocated into the nucleus and start transcription of several genes, among them genes coding for cytokines such as IL-12 and IL-10, this is a probable explanation to the reduced cytokine production observed in individuals with the *TLR4* Asp299Gly gene polymorphism.

Asthma and atopic asthma in Swedish school-children was associated with the *TLR4* Asp299Gly gene polymorphism (adjusted OR 4.5 and OR 7, respectively). This association was not seen with other allergic diseases or to sensitisation as such, however. The *CD14*-159 genotypes were not associated with asthma or allergic rhinoconjunctivitis, or sensitisation or sCD14 levels. However, individuals with the TT genotype had higher LPS induced IL12-p70 secretion in PBMC than the CT/CC individuals, which support the hypothesis that the T allele is associated with more Th1 immune responses.

Several differences in immunological responses to LPS were observed between allergic and non-allergic individuals. Asthma in school-children was associated with reduced LPS induced cytokine production of IL-10 and IL-12. The phosphorylation of $\text{I}\kappa\text{B}\alpha$ was lower in adult allergic compared to non-allergic individuals. Swedish children who had developed allergic disease at five years of age had lower TLR2 mRNA expression at birth compared to children who remained healthy. Furthermore, Swedish children who had a cumulatively positive skin prick test at five years of age had lower *Ebi3* mRNA expression at birth compared to non-sensitised children. These results together suggest that innate immune responses can be different between allergic and non-allergic individuals and perhaps even at birth. The findings of poor response to LTA in allergic individuals and reduced TLR2 mRNA expression early in life in children later becoming allergic are interesting and further studies should be done to make reliable interpretations.

In this thesis, innate immune responses to LPS were followed from birth up to two years of age in infants from Estonia and Sweden. In general the cytokine and chemokine responses were higher in the Swedish than Estonian children. However, no differences were observed in mRNA expression of innate immune receptors TLR2 or TLR4 between the populations. The mRNA expression of Treg associated markers Foxp3 and Ebi3 was higher at birth in Estonian than Swedish infants. That the expression of Ebi3 stands for a regulatory function is supported by the result that the expression of Foxp3 and Ebi3 was correlated at all time-points and that the expression of Ebi3 and p35, which together forms the cytokine IL-35, also correlated at birth. Increased LPS responses during infancy and a higher expression of Treg associated markers at birth in Estonian than in Swedish children could indicate a less rapid immune regulation in Sweden, a country with a high prevalence of allergic disease and reduced microbial exposure.

Polymorphisms in genes coding for receptors to microbial components can alter the immune responsiveness of the host to microbial agents and may be of importance for the development of asthma. Lower LPS induced cytokine response and higher expression of T regulatory associated markers were seen in children from Estonia as compared to Sweden, suggesting an increased capacity for early immune regulation among infants from a country with a low prevalence of allergic disease.

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