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**Immunological interactions between mother and child during pregnancy
in relation to the development of allergic diseases in the offspring**

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“Mommy knows best, doubt no more”

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ORIGINAL PUBLICATIONS

I. Total and allergen-specific IgE levels during and after pregnancy in relation to maternal allergy.

Sandberg* M, Frykman A, Jonsson Y, Persson M, Ernerudh J, Berg G, Matthiesen L, Ekerfelt C, Jenmalm M.C.

Journal of Reproductive Immunology 2009, 81:82-8

II. High cord blood levels of the Th2-associated chemokines CCL17 and CCL22 precede allergy development during the first 6 years of life.

Abelius M.S., Ernerudh J, Berg G, Matthiesen L, Nilsson L.J., Jenmalm M.C.

Pediatric Research 2011, 70:495-500

III. Th2-like chemokine levels are increased in allergic children and influenced by maternal immunity during pregnancy.

Abelius M.S., Lempinen E, Lindblad K, Ernerudh J, Berg G, Matthiesen L, Nilsson L.J., Jenmalm M.C.

Pediatric Allergy and Immunology 2014, in press, DOI:10.1111/pai.12235

IV. Gene expression in placenta, peripheral and cord blood mononuclear cells from allergic and non-allergic women.

Abelius M.S., Janefjord C, Ernerudh J, Berg G, Matthiesen L, Duchén K, Nilsson L.J., Jenmalm M.C.

Manuscript

* The author's maiden name is Sandberg

SUPPLEMENTARY RELEVANT PUBLICATIONS

SI. Cord blood cytokines and chemokines and development of allergic disease.

Sandberg* M, Frykman A, Ernerudh J, Berg G, Matthiesen L, Nilsson L, Ekerfelt C, Jenmalm M.C. *Pediatric Allergy and Immunology* 2009, 20:519-527

SII. A Th1/Th2-associated chemokine imbalance during infancy in children developing eczema, wheeze and sensitization.

Abrahamsson T, **Abelius M.S.**, Forsberg A, Björkstén B, Jenmalm M.C. *Clinical and Experimental Allergy* 2011, 41:1729-39

SIII. Reduced IFN- γ and IL-10 responses to paternal antigens during and after pregnancy in allergic women.

Persson M, Ekerfelt C, Ernerudh J, Matthiesen L, **Abelius M.S.**, Jonsson Y, Berg G, Jenmalm M.C. *Journal of Reproductive Immunology* 2012, 95:50-8

SIV. Increased circulating paternal antigen-specific IFN- γ and IL-4 secreting cells in allergic and non-allergic pregnant women.

Persson M, Ekerfelt C, Ernerudh J, Matthiesen L, Jenmalm M.C., Jonsson Y, **Sandberg* M** and Berg G. *Journal of Reproductive Immunology* 2008, 79:70-8

SV. Placental immune response to apple allergen in allergic mothers.

Abelius M.S. ^Ψ, Enke U^Ψ, Varosi F, Hoyer H, Schleussner E, Jenmalm M.C., Markert U.R. *Submitted to Journal of Reproductive Immunology*

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ABSTRACT

Background: Pregnancy and allergic disease have both been postulated as T-helper 2 (Th2) phenomena. Thus, the increased propensity of allergic mothers to mount Th2-responses might generate favourable effects on the maintenance of pregnancy, but might also be unfavorable, as fetal exposure to a strong Th2 environment could influence the immune development in the offspring to a Th2-like phenotype, favouring IgE production and possibly allergy development later in life. The influence of the intrauterine environment on the immunity and allergy development in the offspring needs to be further investigated.

Aim: The aim of this thesis was to explore the Th1/Th2 balance in allergic and non-allergic women during pregnancy and its influence on the shaping of the Th1/Th2 profile in the neonate and the development of allergic diseases in the offspring.

Material and methods: The study group included 20 women with and 36 women without allergic symptoms followed during pregnancy (gestational week 10-12, 15-16, 25, 35, 39) and 2 and 12 months postpartum, and their children followed from birth to 6 years of age. The circulating Th1-like chemokines CXCL9, CXCL10, CXCL11, Th2-like chemokines CCL17, CCL18 and CCL22, and the allergen-induced secretion of interleukin-4 (IL-4), IL-5, IL-10, IL-13, Interferon- γ (IFN- γ), CXCL10 and CCL17 were measured by Luminex and ELISA. The allergen-specific and total IgE levels were quantified using ImmunoCAP Technology. mRNA expression of Th1-, Th2-, Treg- and Th17-associated genes were measured by PCR arrays and real-time PCR.

Results: We found that sensitised women with allergic symptoms had increased total IgE levels and birch- and cat-induced IL-5, IL-13 and CCL17 responses during pregnancy as compared with postpartum. The non-sensitised women without allergic symptoms had elevated cat-induced IL-5 and IL-13 responses and lower birch- and cat-induced IFN- γ during pregnancy, but similar IgE levels as compared with postpartum.

Maternal total IgE levels during and after pregnancy correlated with cord blood (CB) IgE and CCL22 levels (regardless of maternal allergy status). Circulating CXCL11, CCL18 and CCL22 levels during pregnancy and postpartum correlated with the corresponding chemokine levels in the offspring at various time points during childhood. Maternal IL-5 expression in peripheral blood mononuclear cells (PBMC) was associated with neonatal Galectin-1, and placental p35 expression was negatively associated with neonatal Tbx21 expression. Increased mRNA expression of CCL22 in cord blood mononuclear cells (CBMC), and increased CCL17 and CCL22 levels in CB were observed in children later developing allergic symptoms and sensitisation as compared with children who did not. Development of allergic symptoms and sensitisation were associated with increased total IgE, CCL17, CCL18 and CCL22 levels during childhood.

Conclusions: Maternal allergy was associated with a pronounced Th2 deviation during pregnancy, shown as increased total IgE levels and birch- and cat-induced IL-5, IL-13 and CCL17 responses during pregnancy, possibly exposing their fetuses to a particular strong Th2 environment during gestation.

Correlations were shown between the maternal immunity during pregnancy and the offspring's immunity at birth and later during childhood, indicating an interplay between the maternal and fetal immunity.

Allergy development during the first 6 years of life was associated with a marked Th2 deviation at birth and a delayed down-regulation of this Th2-skewed immunity during childhood.

SAMMANFATTNING

Bakgrund: Det har länge varit känt att en atopisk hereditet är en riskfaktor för utveckling av allergiska sjukdomar hos barnet. Denna risk har visats vara betydligt större om modern är allergisk jämfört med om pappan är det. Det är därför troligt att modern bidrar med något mer än bara den genetiska faktorn. Det nyfödda barnet skulle kunna påverkas av moderns immunitet både under graviditeten och det första levnadsåret via placentan och bröstmjölken. T-hjälpar (Th) celler kan mycket förenklat anta olika profiler; Th1, Th2, Th17 och T regulatorisk, beroende av vilka signalmolekyler, så kallade cytokiner som de utsöndrar. Både graviditet och allergisk sjukdom karaktäriseras av en Th2-dominant immunitet. Den ökade benägenhet som allergiker har att rikta Th2-svar mot allergener skulle således kunna utgöra en evolutionär fördel, med gynnsamma effekter med avseende på att bli gravid och att upprätthålla graviditeten. Å andra sidan skulle en mycket stark maternell Th2-immunitet under graviditeten skulle kunna påverka barnets immunitet och leda till utveckling av allergiska sjukdomar.

Mål: Att studera det immunologiska samspelet mellan moder och barn under graviditeten och dess betydelse för utveckling av immunitet och allergisk sjukdom hos barnet.

Material och metod: Tjugo allergiska och 36 friska gravida kvinnor och deras barn har följts prospektivt, under och efter graviditeten (graviditetsvecka 10-12, 15-16, 25, 35, 39 samt 2 och 12 månader efter förlossningen) samt barnen upp till 6 års ålder. De Th1-associerade kemokinerna CXCL9, CXCL10, CXCL11 och de Th2-associerande kemokinerna CCL17, CCL18 och CCL22 i perifert blod, samt den allergen-inducerade produktionen av Th1-associerade IFN- γ och CXCL10 samt Th2-associerade IL-4, IL-5, IL-10, IL-13 och CCL17 från mononukleära celler i blodet har mätts med Luminex och ELISA. De allergen-specifika och totala nivåerna av IgE-antikroppar har kvantifierats med ImmunoCAP-teknologi. mRNA nivåerna har analyserats med PCR-array och realtids-PCR.

Resultat: De allergiska mödrarna visade högre nivåer av IgE-antikroppar samt högre björk- och katt-inducerat IL-5, IL-13 och CCL17 produktion under graviditeten jämfört med postpartum. De friska mödrarna visade en ökad katt-inducerad utsöndring av IL-5 och IL-13 och en minskad utsöndring av björk- och katt-inducerat IFN- γ under graviditeten medan nivåerna av IgE-antikroppar var oförändrade under och efter graviditeten.

Vi har observerat positiva korrelationer mellan moderns IgE (oavsett om modern är allergisk eller inte) och nivåerna av IgE-antikroppar och det Th2-associerade kemokinet CCL22 i navelsträngsblod. Moderns CXCL11-, CCL18- och CCL22-nivåer korrelerade dessutom med motsvarande kemokin hos barnet vid flera tidpunkter under de första 6 åren i livet.

De barn som utvecklat allergiska sjukdomar vid 6 års ålder hade signifikant högre mRNA-uttryck av CCL22 och högre nivåer av CCL17 och CCL22 i navelsträng samt högre nivåer av IgE-antikroppar, CCL17, CCL18 and CCL22 i blodet vid flera tidpunkter under barndomen, i jämförelse med de barn som inte utvecklat allergi.

Slutsatser: De allergiska mödrarna visade en ökad Th2-immunitet under graviditeten, vilket skulle kunna leda till att fostret exponeras för en mycket stark Th2-miljö under graviditeten. Flera samband mellan moderns immunitet under graviditeten och barnets immunitet vid födseln och under barndomen hittades, vilket tyder på ett immunologiskt samspel mellan mor och barn. En ökad Th2-immunitet vid födseln i kombination med en misslyckad nedreglering av denna Th2-immunitet under barndomen skulle kunna bidra till utveckling av allergiska sjukdomar.

ABBREVIATIONS

α -IL-4R	anti-IL-4 receptor
AD	atopic dermatitis
APC	antigen-presenting cell
ARC	allergic rhino-conjunctivitis
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
CB	cord blood
CBMC	cord blood mononuclear cells
CCL	CC ligand
CD	cluster of differentiation
Ct	threshold cycle
CTLA-4	cytotoxic T lymphocyte antigen 4
CV	coefficient of variance
CXCL	CXC ligand
CX3CL	CX3C ligand
DC	dendritic cell
DMSO	dimethyl sulphoxide
EBI3	epstein-barr virus induced 3
FCS	fetal calf serum
Fc ϵ RI	Fc ϵ receptor type I
Foxp3	forkhead box p3
GATA-3	GATA binding protein 3
gw	gestational week
hCG	human chorionic gonadotropin
HLA	human leukocyte antigen
ICOS	inducible co-stimulator
IDO1	indoleamine 2,3-dioxygenase 1
IFN- γ	interferon- γ
Ig	immunoglobulin
IL	interleukin
ILC2	type 2 innate lymphoid cells
LAG3	lymphocyte-activation gene 3
MHC	major histocompatibility complex
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSB	PBS with 1% BSA
PBS-TBN	PBS with 0.05% Tween, 0.1% BSA and 0.05% NaN ₃
PBT	PBS with 0.05% Tween
PCR	polymerase chain reaction
PD-1	programmed death 1
PHA	phytohemagglutinin
PMT	photomultiplier tube
RNA	ribonucleic acid
RORC	retinoic acid-related orphan receptor C
RT-PCR	reverse transcription polymerase chain reaction
SA-PE	streptavidin R-phycoerythrin conjugate
SPT	skin prick test

ABBREVIATIONS

STAT	signal transducer and activator of transcription
Tbet	T-box expressed in T cells
Tbx21	T-box transcription factor 21
TCR	T cell receptor
TGF β	transforming growth factor- β
Th	T-helper
TLR	toll-like receptor
Treg	regulatory T cells
TREM-2	triggering receptor expressed on myeloid cells 2
TSLP	thymic stromal lymphopoietin
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

REVIEW OF THE LITERATURE

General aspects of allergic disease

There are four types of hypersensitivity reactions; immediate hypersensitivity (type I), antibody mediated (type II), immune complex mediated (type III) and T cell mediated (type IV). Type I and IV hypersensitivity reactions include allergy. Type I represents IgE antibody mediated allergy with engagement of mast cells and their mediators and type IV represents T-helper (Th) 1/Th17 cell mediated inflammation (non-IgE antibody mediated allergy) such as allergic contact dermatitis. Type II and III hypersensitivity reactions involve Immunoglobulin G (IgG) and IgM antibodies to antigens on the cell surface or extracellular matrix (type II) or to soluble antigens forming immune complexes (type III) and activation of the complement system [1]. In this thesis, the term allergy refers to IgE mediated allergy.

Some common environmental antigens, *e.g.* allergens, are able to induce immediate hypersensitivity reactions. Allergens are in general small, highly stable, soluble and glycosylated proteins, with enzymatic activity. Everyone is exposed to allergens, which are acknowledged by the immune system, and allergen-specific IgG- and IgM antibodies are produced. The majority of individuals do not synthesise IgE antibodies in response to allergens [1].

Atopic individuals have a genetic predisposition, personal and/or familial, to become sensitised and produce allergen-specific IgE antibodies. Thus, the clinical definition of atopy is an IgE antibody high-responder and confirmation of sensitisation with positive skin prick test (SPT) or presence of allergen-specific IgE antibodies in the circulation must be done when using the term atopy [2].

Sensitised individuals usually develop symptoms of asthma, food allergy, atopic dermatitis (AD) and allergic rhinoconjunctivitis (ARC), but some sensitised individuals do not develop allergic symptoms despite repeated allergen exposure, for unknown reasons.

The clinical symptoms of allergic disease are common in Sweden, with a prevalence of 7% for ARC symptoms, 10% for asthma symptoms and 22 % for symptoms of eczema at 6-7 years of age, according to the International Study of Asthma and Allergies in Childhood, phase III [3]. Furthermore, the Swedish population based cohort (BAMSE) reported that 58% of the children had had any allergic disease at some time during the first 12 years of life [4].

An increased prevalence of allergic diseases has been reported during the last decades, predominantly in countries with a westernised lifestyle [3, 5, 6]. The occurrence of allergic symptoms tends to vary with age, with a typical pattern called “the atopic march” [7]. AD and food allergy are the most prominent allergic manifestations during infancy. These symptoms decline with age and asthma and ARC usually develop in pre-school age.

The early origin of AD and food allergen sensitisation has shed light on their capacity as predictors for development of sensitisation and other allergic symptoms. They are both associated with an increased risk for development of sensitisation to inhalant allergens, asthma and ARC later in life [7-15]. Lowe *et al.* demonstrated that children with atopic dermatitis were at greater risk for development of asthma and allergic rhinitis if they were sensitised than if they were not [14]. Furthermore, sensitisation in asymptomatic infants independently predicted development of wheeze, asthma and rhinitis [13], underlining the importance of early sensitisation.

Genetic and environmental factors

Allergic diseases are probably caused by interactions between genetic and environmental factors in combination with allergen exposure. The strong hereditary component in allergic disease has resulted in identification of over 100 genes associated with disease susceptibility, with a few overlapping associations for different allergic diseases, reviewed in [16]. Genetic factors are clearly important, but cannot independently explain the increased prevalence of allergic diseases during the last 40-50 years. A time period of a few decades is far too short for human genetics to undergo such substantial changes causing this increasing prevalence. Thus, a lot of research has focused on environmental factors associated with a westernised lifestyle. Several environmental factors have changed along with the increase in allergy prevalence, many of them leading to reduced microbial exposure, such as increased cleanliness, reduced family size and living in urban areas with an improved general living standard. The “hygiene hypothesis” originates in the observation of a lower prevalence of eczema and hay fever in large families [17], which was later shown for asthma prevalence as well [18]. The protective effect of being surrounded by other children was strengthened by data on day-care attendance, in particular attendance to day-care centres early in life [18, 19]. The beneficial effect of day-care has shown to be modified by variations of the Toll-like receptor 2 (TLR2) gene (TLR2/-16934 polymorphism), as children with the T allele, but not

the AA-homozygotes, were less likely to be sensitised if they attended to day-care, indicating complex gene-environment interactions [20].

The association between large families/attendance to day-care and reduced allergy prevalence led to the interpretation that infections and/or microbial exposure during childhood might protect against allergy. Studies on the effect of respiratory infections on allergy development are inconsistent, without any clear protection, while a substantial amount of findings indicate that microbial exposure is important [21-23]. A low diversity of the intestinal microbiota very early in life has been associated with development of sensitisation, AD, asthma and allergic rhinitis [24-26]. The moderate increased risk of allergy development in children delivered by caesarean section [27], might be associated with a lower total microbiota diversity in these children [28]. Many studies have shown that living on a farm is associated with a relatively low prevalence of allergic diseases, with ingestion of unpasteurised milk and exposure to livestock as identified protective factors [29-31]. A recent study found no clear evidence of synergy or saturation of the protective effects of sibling or farm exposure, suggesting that different mechanisms may underlie these protective factors [32].

Use of broad-spectrum antibiotics has been associated with allergy development [33]. An anthroposophic lifestyle, characterised by limited use of antibiotics and vaccines and a diet rich in *Lactobacilli*, has been associated with a reduced prevalence of atopy [34]. A lot of other factors, such as dietary changes with a decreased intake of omega 3 fatty acids, obesity, reduced breast-feeding, vaccinations, air pollution and smoking, have also been proposed to influence allergy development [35-37].

Allergen exposure

A repeated exposure to allergens is required for development of sensitisation and allergic symptoms. Low doses of allergens favour development of sensitisation and allergic symptoms while high doses of allergens may induce tolerance [38]. The use of immunotherapy as a treatment of allergic disease indicates that increasing doses of allergen can promote tolerance.

Exposure to farm animals early in life, in particular before birth, has shown a protective effect on allergies [39], but the effect of pet exposure is less clear. One should keep in mind that it is difficult to distinguish between the contribution of allergens and microbes when evaluating the effect of animal contact on allergy development. A meta-analysis from 2012 reported no associations at all between pet keeping early in life and asthma and ARC in

school age [40], while another meta-analysis from 2013 reported a decreased risk for AD in children exposed to pets during infancy [41]. Neonatal exposure to cats increased the risk for AD in children with mutations in the Filaggrin gene (associated with disease susceptibility), indicating that early pet exposure might be unfavourable for subgroups of children [42]. The possibility of allergen exposure and sensitisation *in utero*, will be discussed later, on page 32.

Immunological mechanisms

The main task of the immune system is to protect us against harmful microbes, such as certain bacteria, viruses, fungi and parasites. Thus, the ability to distinguish between self and non-self is obligatory in order to achieve optimal protection and minimal damage to the host. A plastic immune system has evolved to keep this balance, involving mechanisms for both up- and down-regulation of immune responses [1].

Brief overview of the innate and adaptive immune system

The innate immune system is our first line of defence, comprising epithelial barriers and specialised immune cells, namely granulocytes (neutrophils, eosinophils, basophils) mast cells, dendritic cells (DC), macrophages and Natural killer (NK) cells (Figure 1). Upon infection, the invader is recognised by innate immune cells, predominantly macrophages and neutrophils, bearing receptors for molecular structures on the surface of the microbe, leading to phagocytosis and/or secretion of inflammatory mediators. This is the initiation of the innate immune response. Secreted cytokines and chemokines contribute to the inflammation by increasing the permeability of blood vessels, regulating immune responses and recruiting other immune cells to the site of infection. The phagocytosis is further triggered by microbes coated with molecules acting like opsonins, originally generated by the complement system. Cells infected with viruses or intracellular bacteria are effectively killed by NK cells. Eosinophils and mast cells play an important role in the defence against helminths, by their ability to degranulate and release inflammatory mediators.

Figure 1. A brief overview of the innate and the adaptive immune system.

The DCs migrate to the lymph nodes and display the antigen generating activation, clonal expansion and differentiation of antigen-specific naïve T cells to effector T cells. T cells are not activated by soluble antigens that are not processed by APCs. Activated cytotoxic T cells promote destruction of infected cells and Th cells stimulate macrophages to clear their microbial load. This type of adaptive immune response is called cell-mediated immunity. The other type, humoral immunity, is mediated by antibodies secreted by B cells and plasma cells. B cells can be independently activated by antigen stimulation, but they usually need

assistance of Th cells, who interact with the antigen presented on the cell surface of the B cell. Cytokines secreted by Th cells are important for the function of the adaptive immune system *e.g.* T and B cell proliferation and differentiation, but one should keep in mind that many cytokines influence both innate and adaptive immune responses. Upon activation, B cells differentiate into antibody secreting plasma cells or memory B cells. The main assignment for the antibodies is to facilitate extracellular protection by binding to pathogens causing neutralisation, opsonisation and activation of the complement system.

The innate immune system is particularly important in the early stage of the infection as there is a delay of several days for the adaptive immune system to attain full function. The immune responses are down-regulated after successful clearance of the infection. The majority of the antigen-specific lymphocytes die by apoptosis, while the remaining make up a pool of long-lived memory cells. Memory cells can remain for decades and provide a fast immune response upon reinfection [1].

T helper cells

Activation of CD4+ T cells

T cell activation is initiated by interactions between an APC, predominantly DCs and T cells. Tissue resident immature DCs capture and process the antigen and present an antigen-derived peptide on the major histocompatibility complex (MHC) molecule class II on the cell surface. The DCs mature and migrate to the lymph nodes and activate naïve CD4+ T cells by interactions between the MHC-peptide complex and the T cell receptor (TCR). The first signal for T cell activation is antigen recognition and the second signal is co-stimulation. The binding of the cluster of differentiation 28 (CD28) receptor on the T cell and the CD80/86 complex expressed on the DC is the best characterised co-stimulatory pathway. Another pathway for co-stimulation is mediated by signaling through the inducible co-stimulator (ICOS) expressed on T cells and ICOS ligand expressed on DCs. The activated T cells also express CD40L, which interacts with CD40 on the DC, generating up-regulation of co-stimulatory molecules and cytokine secretion. Thus, like a positive feed-back loop, engagement of CD40L-CD40 “improve” the DC and promote T cell proliferation and differentiation.

Activation signals are counterbalanced by inhibitory signals. Suppressive effects are mediated by interactions between the inhibitory receptors cytotoxic T lymphocyte antigen 4

(CTLA-4) and programmed death 1 (PD-1) and their ligands; CD80/86 and PDL1/L2 [1]. Immune suppression of activated Th cells is also mediated by Interleukin-10 (IL-10) and transforming growth factor (TGF- β) secreting regulatory T cells (Treg).

Differentiation of CD4+ T cells

The naïve Th cells will differentiate into different subsets of effector cells depending on the immunological micro milieu at the time of activation, *i.e.* presence or absence of certain stimuli. Cytokines are secreted by the surrounding immune cells generating distinct subsets of Th cells, Th1, Th2 and Th17 for host defence and Treg for immune regulation/suppression. The effector functions of the subsets are markedly different, each one, except Tregs, adapted to combat a certain type of infection.

Th1 immunity is the appropriate defence against intracellular microbes. This defence is predominantly mediated by the signature cytokine for Th1 cells; Interferon- γ (IFN- γ), which promotes cytotoxic activity of cytotoxic T cells and pathogen killing by macrophages [43]. DCs, macrophages and NK cells stimulate Th1 differentiation by providing IL-12 and IFN- γ (Fig 2). IL-12 is considered to be the most potent inducer of Th1 differentiation [44]. T cell mediated IFN- γ secretion is induced by activation of the transcription factors signal transducer and activator of transcription 4 (STAT4), STAT1 and T-box expressed in T cells (Tbet). IFN- γ itself amplifies Th1 differentiation and stimulates production of the Th1-associated chemokines CXCL9, CXCL10 and CXCL11 [45-47]. These chemokines are predominantly secreted by macrophages and they attract CXCR3 expressing Th1 cells, B cells, mast cells and NKT cells to the site of inflammation [48].

Th2 immunity is mounted in response to helminth infections. IL-4 plays a major role in Th2 differentiation and for effector functions. Mast cells and eosinophils are thought to be the initial source of IL-4 in case of helminth infection, even though the Th2 cell itself is the major source of IL-4. It has been speculated that naïve Th cells default to the Th2 pathway in absence of innate stimuli [49]. IL-4 activates STAT6 and the master regulator of Th2 differentiation GATA binding protein 3 (GATA-3), inducing IL-4, IL-5 and IL-13 production in order to amplify the immune response and accomplish effector functions. IL-4 and IL-13 improve humoral immunity by inducing IgE class switch and they also up regulate MHC class II molecules on B cells. IL-5 is important for eosinophil growth, maturation, activation

and survival, and the eosinophils are mainly recruited by CCL11-CCR3 interactions. Mast cell activation is mediated by IL-5 and IL-13 [43]. Furthermore, the IL-4 and IL-13 induced chemokines CCL17 and CCL22 attract Th2 cells, DCs, basophils, mast cells, NK cells and NKT cells by interaction with the CCR4 receptor [48, 50, 51]. CCL18 is induced by IL-4, IL-13 and IL-10, suggesting that this chemokine has Th2-associated as well as anti-inflammatory features [52].

Figure 2. The figure shows a simplified overview of the cytokines needed for differentiation of the Th subsets, the main transcription factors, cytokine secretion and primary function for each Th subset.

Th17 cells are the third subset of Th cells essential for satisfactory host defence, namely for clearance of fungi and extracellular bacteria. Five cytokines are acknowledged for Th17 differentiation, TGF-β, IL-1β, IL-6, IL-21 and IL-23, leading to activation of STAT3 and the lineage specific transcription factor retinoic acid-related orphan receptor C (RORC) [53]. IL17A/F and IL-22 are the main effector cytokines, primarily involved in the maintenance of inflammation, neutrophil activation and epithelial barrier function [53, 54]. The chemokine CCL20 is secreted by Th17 cells and induces migration of Th cells, DCs and B cells via CCR6 [53].

Mechanisms for down-regulation of immune responses after successful clearance of the infection are essential in order to minimise tissue damage. Tregs are specialists of immune suppression, targeting T effector cells and other cell types, possessing cell contact dependent as well as independent mechanisms. These include (i) modulation of DC function mediated by CTLA-4 and lymphocyte-activation gene 3 (LAG3), (ii) metabolic disruption by IL-2 starvation, (iii) granzyme A/B and perforin induced cytolysis and (iv) secretion of anti-inflammatory cytokines such as TGF- β , IL-10 and IL-35 [55, 56].

In addition to the thymus-derived natural occurring CD4+CD25+Forkhead box p3 (Foxp3)+ Tregs, CD4+CD25- cells can differentiate into a regulatory subset, *i.e.* induced Tregs, when stimulated with antigen and an appropriate cytokine environment, *e.g.* TGF- β [54, 57].

The division of Th cells into subtypes generates a working model to help immunologists to understand the immune system, but it is important to remember that the model is a simplification. Other subpopulations of Th cells have been suggested as well, but their lineage specific transcription factors have not been identified yet, challenging their existence. IL-9 secreting Th9 cells probably involved in the fight against helminths and IL-22 secreting Th22 cells, possibly important for barrier function, are two examples [43, 54]. Another subpopulation is the follicular Th cells, which help the B cells to become activated in the germinal center reaction. The signature cytokine of follicular Th cells is IL-21 [1].

It is generally accepted that the Th1 and Th2 subsets cross-regulate each other at the transcriptional and cytokine level [54, 58-60]. Th1-associated Tbet and IFN- γ have inhibitory effects on Th2 differentiation and the Th2-associated GATA-3 and IL-4 exert inhibitory effects on Th1 differentiation [58, 59]. TGF- β inhibits development of Th1 and Th2 cells [1]. Th17 differentiation is down regulated by IFN- γ and IL-4, suggesting that suppressive actions of TGF- β are necessary to allow development of Th17 cells (reviewed in [53]).

The immune system allows short-term alterations in the Th1/Th2 balance, for example during infection, a normal pregnancy and very early in life [61, 62]. However, an extensive activation of Th1 or Th2 immunity can be pathological. Autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and type I diabetes are associated with a Th1 deviation, probably in combination with a Th17 deviation [63] and allergic diseases with a Th2 deviation [64].

B cells and antibodies

Naïve B cells are activated by antigen recognition, *i.e.* cross-linking of antigen receptors (T cell independent activation) or a cell-cell contact dependent pathway (T cell dependent activation). The naïve B cell antigen receptor comprises membrane bound IgD and IgM antibodies. Upon activation, antigen-specific B cells proliferate and differentiate into memory B cells or antibody secreting plasma cells. T cell independent B cell activation, with *e.g.* polysaccharides, results in IgM and IgG₂ antibody secretion, leading, for example, to activation of the complement system. Antibody production to protein antigens such as allergens, is dependent on the assistance of Th cells. The antigen-specific B cells take up the encountered antigen, process it and present a protein derived peptide on their MHC class II molecules. Antigen-specific Th cells interact with this MHC-peptide-complex and provide the B cells with the signals needed for antibody isotype switching *i.e.* CD40L-CD40 stimulation and the appropriate cytokines [1]. IL-4 and IL-13 induce IgE antibody production [65] in response to helminth infections or allergens. IgA is a neutralising antibody, mostly important at mucosal surfaces, and its isotype switching is predominantly stimulated by TGF- β [66]. IgG antibody synthesis seems to be promoted by Th1- and Th2-like immunity, but the regulation of antibody isotype switching is not completely elucidated. A presumed Th1 dominant situation such as Lyme Borreliosis has been associated with high IgG₁ and IgG₃ subclass antibody levels, suggesting that IFN- γ might stimulate the production of these subclasses [67]. In mice, Th1 immunity has been shown to be associated with production of complement activating and opsonising IgG_{2a}, and IgG_{2b} corresponding to human IgG₁ and IgG₃ [68, 69]. Allergic diseases, representing a Th2-biased situation, have been associated with high IgG₄ levels to allergens [70]. IL-4 has shown to induce IgG₄ switching, supporting the assumption of IgG₄ as a “Th2-associated antibody” [65], in addition to IgE.

The allergic immune response

There are three phases of an allergic reaction; sensitisation, immediate hypersensitivity and the late phase reaction. The sensitisation phase begins with interactions between DCs and the encountered allergen. Allergens are in general distributed transmucosally, at very low doses, by diffusion through the epithelial layer. Specialised DCs capture and process the allergen and present an allergen derived peptide to the naïve T cells. They differentiate into Th2 cells, which help B cells to become activated and induce B cell IgE class switch by secretion of IL-

4 and IL-13. Furthermore, Th2 cells activate eosinophils by secretion of IL-5. The allergen-specific IgE antibodies bind to the high affinity Fc ϵ receptor type I (Fc ϵ RI) expressed on the cell surface of mast cells, basophils and to some extent on eosinophils. The cells are “sensitised” and an immediate hypersensitivity reaction is possible at the next encounter of the allergen.

The activation of mast cells is induced by binding of allergens to the IgE antibodies, mediating cross-linking of the Fc ϵ RI molecules and an immediate degranulation. A mixture of biogenic amines, *e.g.* histamine, enzymes such as tryptase and chymase, lipid mediators including prostaglandins, leukotrienes and platelets-activating factor and immune mediators, *e.g.* cytokines and chemokines, are released into the extra-cellular space, generating allergic symptoms. Histamine induces vasodilation, increased vascular permeability and plasma leakage. The enzymes cause tissue damage, the lipid mediators stimulate for example bronchoconstriction and cytokines such as IL-9 and IL-13 promote mucus secretion.

The allergic inflammation is maintained by actions of cytokines and chemokines secreted by APCs, mast cells, Th2 cells and surrounding epithelial cells. The immunological milieu in the inflamed tissues comprise pro-inflammatory cytokines such as TNF, IL-1 β , IL-6, eosinophil recruiting/promoting CCL11, IL-3, IL-5, GM-CSF as well as the Th2 cell recruiting/promoting CCL17, CCL22, IL-4, IL-13 [1, 71]. The inflamed tissue is infiltrated with eosinophils and basophils. This is the late phase reaction and this stage can become chronic in tissues frequently exposed to allergens.

Cytokines in allergic disease

It is generally accepted that established allergic disease is associated with increased Th2-deviated immune responses to allergens, shown as increased GATA-3 expression and IL-4, IL-5, IL-9, IL-13 secretion, both systemically [72, 73] as well as locally in the affected tissues [74, 75]. IL-31 was identified as a Th2-associated cytokine around a decade ago [76] and it has shown to be associated with pruritus and allergic manifestations, such as AD and allergic asthma [77, 78]. The epithelial derived cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) enhance Th2-like cytokine responses directly by influencing cytokine secreting immune cells and possibly indirectly by polarisation of DCs [77]. Accordingly, increased IL-25, IL-33 and TSLP expression has been reported in lesional AD skin, nasal mucosa and elevated levels in nasal secretions in subjects with allergic rhinitis [77, 79, 80]. A newly discovered cell type, type 2 innate lymphoid cells (ILC2), are activated by IL-25, IL-33 and TSLP and they produce the effector cytokines IL-5 and IL-13 [81]. Thus, they are expected to be involved in allergic diseases. The percentage of ILC2s in peripheral blood was increased after nasal allergen challenge in allergic subjects [82] and ILC2s were detected in the lung tissue and bronchoalveolar lavage fluid (BALF) in a murine ovalbumin-induced asthma model [81]. The role of ILC2s in allergic diseases needs further investigation. The ability of allergic individuals to secrete Th1-associated cytokines such as IFN- γ upon allergen stimulation is usually equal [72, 83] or diminished [84] as compared with controls. The Th2-associated immunity in allergic disease might be explained by an impaired frequency and/or capacity of Tregs to down regulate Th2-like immune responses, allowing a sustained Th2-driven allergic inflammation. Allergen-specific IFN- γ -, IL-4- and IL-10 secreting T cells, presumably representing Th1, Th2 and Tr1 cells, have been detected in allergic and healthy individuals but in different proportions [85]. Th2 cells were the predominant subset in the allergic group and the Tr1 cells in the non-allergic group, indicating an important role for IL-10 secreting Tr1 cells in the development of tolerance to allergens. Moreover, one of the immunological mechanisms behind the successful induction of allergen tolerance during allergen-specific immunotherapy could be induction of Tr1 cells, suppressing allergen-specific Th1- and Th2-responses by secretion of the anti-inflammatory cytokines IL-10 and TGF- β [86]. Th17 cells and their inflammatory mediators attract and promote neutrophil development, and are not known to drive Th2-responses, implying a role for Th17 cells in non-allergic asthma [87]. Lei *et al.* failed to report any differences in circulating IL-17A levels between allergic

asthmatics and healthy controls [78], while Ciprandi *et al.* revealed increased serum levels of the same cytokine in a study group with allergic rhinitis as compared with controls [88]. However, all patients in the latter study were previously treated with allergen-specific immunotherapy. In another study, elevated birch-induced mRNA expression of IL-17A after allergen-specific immunotherapy in sensitised children with allergic rhinitis was associated with poor therapeutic outcome [89]. Clearly, the role of Th17 cells in allergy is not settled and needs additional investigation.

Chemokines in allergic disease

Chemokines comprise a large protein family, with more than 50 members. They are divided into four groups depending on the location of two N-terminal cysteine residues. The two major groups are the CC ligands (CCL, cysteine residues are adjacent) and the CXC ligands (CXCL, cysteine residues separated by another amino acid). The two minor groups are represented by the C ligands with only one N-terminal cysteine residue and the CX3C ligands (CX3CL) with cysteine residues that are separated by three amino acids. In concurrence, the chemokine receptors are named CCR, CXCR, XCR and CX3CR, representing receptors for the corresponding chemokine groups.

Microbial recognition and cytokine stimulation induce chemokine production. Chemokines are secreted by leukocytes, predominantly macrophages, but also endothelial cells, epithelial cells and fibroblasts are important chemokine producers [1, 90].

Chemokines are crucial not only for recruitment of leukocytes from the circulation to the tissues but also in the regulation of leukocyte maturation. Leukocyte migration is mediated by a chemical gradient. The chemokine ligand-receptor interaction generates increased cell motility and integrin affinity promoting leukocyte migration. Thus, the composition of infiltrating leukocytes is organised by chemokines present at the site of inflammation.

Table 1. The chemokine ligands and the distribution of chemokine receptors on immune cells involved in allergic disease

Cell	Chemokine receptors	Chemokine ligands
Eosinophil	CXCR4 CCR1 CCR3	CXCL12 CCL3/5/7/9/10/14/15/16/23 CCL5/7/8/ 11 /13/15/24/26
Basophil	CXCR4 CCR1 CCR2 CCR3	CXCL12 CCL3/5/7/9/10/14/15/16/23 CCL2/7/12/13/16 CCL5/7/8/ 11 /13/15/24/26
Mast cell	CXCR4 CCR3 CCR4 CXCR3	CXCL12 CCL5/7/8/ 11 /13/15/24/26 CCL17/22 CXCL9/10/11
Dendritic cell	CXCR4 CCR1 CCR4	CXCL12 CCL3/5/7/9/10/14/15/16/23 CCL17/22
Monocyte	CXCR4 CCR1 CCR8	CXCL12 CCL3/5/7/9/10/14/15/16/23 CCL1
Th1 cell	CXCR4 CCR2 CCR5 CXCR3	CXCL12 CCL2/7/12/13/16 CCL3/4/5/8/14 CXCL9/10/11
Th2 cell	CXCR4 CCR3 CCR4 CCR8	CXCL12 CCL5/7/8/ 11 /13/15/24/26 CCL17/22 CCL1
NKT cell	CXCR4 CCR1 CCR2 CCR4 CCR5 CXCR3 CXCR6	CXCL12 CCL3/5/7/9/10/14/15/16/23 CCL2/7/12/13/16 CCL17/22 CCL3/4/5/8/14 CXCL9/10/11 CXCL16

Chemokines and receptors studied in this thesis are marked with bold. References [1, 48].

Th1-associated chemokines

The IFN- γ induced chemokines CXCL9, CXCL10 and CXCL11 [45, 46] attract CXCR3 expressing cells such as Th1 lymphocytes, NKT and mast cells [48] (Table 1). CXCL9, CXCL10 and CXCL11 are predominantly associated with Th1-like diseases, *e.g.* sarcoidosis, tuberculosis [91], Sjögren's syndrome [92], rheumatoid arthritis [93] and Crohn's disease [94]. A few studies have implicated a role for the CXCR3 ligands in allergic disease. Increased expression of CXCL10 has been reported in the airways of atopic asthmatics, but this increase was less pronounced as compared with CXCL10 expression in patients with sarcoidosis [91]. Moreover, the increased CXCL10 expression was accompanied with high

CCL11 expression in the atopic asthmatics, but not in the sarcoidosis or tuberculosis patients, indicating an up-regulation of both Th1- and Th2-associated chemokines locally in the asthmatic airways [91]. Furthermore, CXCL9 was elevated in the nasal lavage of allergic rhinitis patients and CXCL10 in BALF of asthmatics after allergen challenge [95, 96]. Acute asthma exacerbations were associated with increased CXCL9 and CXCL10 levels in the circulation in contrast to stable asthma [97].

A murine model of asthma implicated an important role for CXCL10 in airway hyperreactivity and Th2-associated inflammation [98]. Transgenic mice, overexpressing CXCL10 in the lung, experienced increased airway hyperreactivity and augmented recruitment of eosinophils and Th2 cells to the lung [98]. Conversely, CXCL9 inhibited migration and CCR3-mediated functional responses in murine eosinophils, indicating that CXCL9 negatively regulates Th2-associated allergic inflammation [99, 100]. More research is needed to elucidate if the CXCR3 ligands actively promote allergic inflammation, or if they represent a negative feedback mechanism leading to suppression of Th2-associated immune responses.

Th2-associated chemokines

There are plenty of studies suggesting a role for CCL11, CCL17, CCL18 and CCL22 in allergic disease [101-110]. These Th2-associated chemokines are predominantly induced by IL-4 and IL-13, but production of CCL18 is considerably enhanced in synergy with IL-10 [50-52, 111]. In contrast, the production of CCL17 and CCL22 is suppressed by IL-10 [50, 112]. The amplification of the allergic response is partly driven by these chemokines, as they influence the composition of infiltrating leukocytes at the site of the allergic inflammation. CCL11 recruits eosinophils, mast cells, basophils and Th2 cells via CCR3, while CCL17 and CCL22 induce migration of Th2 cells, DC, basophils, mast cells, NK cells and NKT cells by interaction with the CCR4 receptor [48]. CCL18 has shown to attract T cells [113]. The receptor for CCL18 is not yet determined, but CCR8 was recently suggested [114]. Increased levels of CCL11, CCL17, CCL18 and CCL22 in the circulation have been associated with established allergic disease, predominantly AD [101-105], but also with asthma [105, 106] and ARC [105]. Others have reported similar levels of these Th2-associated chemokines in ARC patients and controls [104, 115]. An increase in Th2-associated chemokines has also been reported in the affected tissues; CCL17, CCL18 and CCL22 were elevated in BALF of asthmatics [107, 108] and after allergen challenge [109].

Furthermore, epithelial cells in the nasal mucosa of ARC patients expressed more CCL17 than controls [110].

Chemokines have been used as markers for Th1/Th2 immunity in allergic diseases and other immune-mediated disorders, but little is known about the predictive value of circulating chemokines, before disease onset. Although established allergic disease is characterised by a Th2-skewed immunity, the timing of the development of this Th2 skewing is unknown.

Pregnancy

Pregnancy immunology

The maternal immune system tolerates the fetus during pregnancy, despite the fetal expression of paternal antigens, which are foreign to the mother. The maternal tolerance must be kept without compromising the protection against infections. Today, modulation of the maternal immune system to a Th2-/anti-inflammatory phenotype and appropriate interactions between maternal and fetal immune system are thought to be the key events in the maintenance of the feto-maternal tolerance [116].

The placenta and the amniotic sac comprise a physical barrier between the mother and the fetus, although there are several possibilities of immunological interactions between the maternal and fetal immune system *i.e.* a “local interaction” in the decidua and a “systemic interactions” in the intervillous space in the placenta [117].

In the first trimester of pregnancy, fetal extravillous trophoblasts migrate into the decidual tissue and establish contact with decidual stroma cells and maternal NK cells, macrophages and T cells. The extravillous trophoblasts express MHC class I products; human leukocyte antigen (HLA) class I molecules HLA-C, HLA-E and HLA-G but not HLA-A, HLA-B or MHC class II molecules. This specific expression profile is believed to protect the fetus, probably by prevention of T and NK cell activation [118].

The systemic interaction takes place in the intervillous space of the placenta. Villous trophoblasts, with fetal blood vessels, are bathing in maternal blood, allowing exchange of nutrients. Thus, the fetal and maternal blood streams are in very close proximity.

A normal pregnancy is traditionally described as a Th2-deviated condition [61]. Thus, an imbalance between Th1 and Th2 immunity in pregnancy, leading to increased Th1-like

immune responses has been associated with spontaneous abortions [119, 120], pre-eclampsia [121] and pre-term labour [122]. Similarly, Th17-like immunity has been associated with spontaneous abortions [123, 124] and might be detrimental for the pregnancy in combination with a Th1 deviated immunity. In addition to a Th2-deviated immunity, the suppressive mechanisms by Tregs and alternative activated macrophages are also considered to be important for the maintenance of the feto-maternal tolerance [125, 126].

Clinical data support the hypothesis of pregnancy as a Th2-deviated situation. Patients with Th1-like diseases such as rheumatoid arthritis and psoriasis experience a temporary reduction of symptoms during pregnancy [127, 128]. Similarly, the rate of relapse declines during pregnancy and increases postpartum in patients with multiple sclerosis [129].

The influence of pregnancy on the course of asthma and allergy are inconclusive.

Generally, asthma symptoms decrease in one-third, remains the same in one-third and increase in one-third of the asthmatic women during pregnancy (reviewed in [130]). Kircher *et al.* reported a concordance between symptoms of asthma and rhinitis during pregnancy, indicating that symptoms of rhinitis may undergo similar alterations as asthma [131]. The atopic status of the women was not assessed in that study, however.

Allergy and pregnancy

The immunological similarities between allergy and pregnancy, *i.e.* Th2-deviated immune responses to allergens [72, 132, 133] and at the feto-maternal interface [61] have raised the question whether maternal allergy is beneficial in a reproductive perspective. This assumption embraces an influence of allergic disease on the immune system, beside the allergen-specific immune responses. On one hand, the Th1/Th2 imbalance in allergic disease has shown to be highly antigen-specific, *e.g.* atopic children produced more Th2-like cytokines in response to the allergen they were sensitised to, but not to other allergens [72]. On the other hand, there are studies on unrelated antigens indicating generally altered immune responses in the allergic group. Allergic women had reduced IFN- γ and IL-10 production in response to fetal and paternal alloantigens during and after pregnancy [134-136]. Furthermore, low IFN- γ and high IL-4 and IL-5 production has been observed in allergic individuals after stimulation with bacterial antigens, *i.e.* a purified protein derivate from *Mycobacterium tuberculosis* [137, 138]. In addition, diminished IL-10 responses have been shown in allergic individuals in response to lipopolysaccharide [139] and viral antigen (Influenza A) [140]. Thus, the disturbance in immune regulation associated with allergic

disease might influence the immune system in different ways, beside the allergen-specific immune responses.

A few studies have indicated favorable effects of maternal allergy on becoming pregnant and on the maintenance of the pregnancy. Maternal allergy was associated with a shorter waiting time to pregnancy [141], longer gestational age, higher birth weight and length [142-144], and a lower risk of pre-term birth [145]. Furthermore, allergic mothers have shown to give birth to more children than non-allergic mothers [146] and a possible increased fertility rate in women with allergic rhinitis and eczema has been reported [147]. In contrast, others have shown an inverse relationship between maternal allergy and the number of children [148-150].

Quite a few studies have been conducted to explore the Th1/Th2 balance in allergic and non-allergic women during pregnancy. The idea of exaggerated Th2-responses during pregnancy in allergic women is supported by a study on allergen-induced cytokine secretion by PBMCs in allergic and non-allergic women during the second and third trimesters of pregnancy and 6 weeks postpartum [151]. The non-allergic women showed decreased allergen-induced IL-13 secretion in late gestation while the IL-13 secretion remained high in the allergic women [151]. Furthermore, allergic women had reduced IFN- γ and IL-10 production in response to fetal and paternal alloantigens during and after pregnancy [134-136].

Allergy and pregnancy are characterised by a Th2 deviation, but whether pregnancy magnifies the Th2-skewed immunity of allergic women needs further investigation.

Immunological interactions between mother and child during pregnancy

The developmental origins of allergic disease probably precede disease manifestation. The impact of early life events on the development of diseases later in life, *i.e.* “fetal programming of diseases” was highlighted in 1989 by D.J.P Barker. Inadequate nutrition *in utero* was found to be linked to development of coronary heart disease in adult life, indicating long-term effects of the intra-uterine environment [152].

Studies on the protective effect of farm exposure on childhood allergy development initially indicated the first year of life as a particular important time period [30], but more attention has been drawn to prenatal influences during the last years. Thus, in support of pregnancy as

an important “time window” for determination of future health and disease, maternal exposure to farms and stables during pregnancy protects against development of asthma symptoms, ARC, AD and allergic sensitisation in the offspring, whereas exposures during infancy had weaker effects [39, 153, 154]. Prenatal farm exposure was also associated with immunological modulation, as shown by increased number and improved suppressive effect of cord blood (CB) Tregs as well as increased expression of receptors of the innate immune system in children of mothers exposed to farm environment during pregnancy [39, 155]. An appropriate maternal (prenatal) exposure to microbes has been suggested to underlie the protective “farm effect”. Data from a mouse model of allergic asthma support this hypothesis, as asthma protection in the offspring was dependent on bacterial exposure and functional maternal TLR signalling [156]. Environmental exposures several years before pregnancy could also be important for the immune development in the offspring. Maternal exposure to cats during her first year of life predicted a positive maternal record for *Toxoplasma gondii*, and maternal immunity to *Toxoplasma gondii* was inversely related with CB allergen-specific IgE in her offspring [157]. Trans-generational effects of prenatal exposures have also been observed. The risk of childhood asthma was increased if the child’s maternal grandmother smoked during pregnancy, even if the child’s mother did not smoke during pregnancy with the index child, suggesting an inherited asthma susceptibility possibly mediated through epigenetic mechanisms [158].

The observation of maternal allergy as a more significant risk factor for allergy development in the offspring as compared with paternal allergy [159-161] has shed more light on the pregnancy as an important time period for fetal immune development [162]. The immunological mechanisms behind this phenomenon are not known, but indicate an influence of the maternal immunity on allergy development, besides the contribution of the genes. Fetuses of allergic mothers might be exposed to a particular strong Th2 environment, due to an exaggerated Th2 immunity of allergic mothers during pregnancy, possibly influencing the development of immune responses in the offspring, to an IgE favouring, Th2-like phenotype. Accordingly, higher CB IgE levels and higher percentages of IgE-coated CB basophils have been observed in children of allergic mothers as compared to children with paternal or no allergic history [159, 163-165]. Maternal allergic sensitisation was associated with elevated allergen-induced IL-13 in the human neonate [166] and a decreased production of mitogen-induced IFN- γ in newborn mice [167]. A diminished Th1 immunity has also been

observed in human neonates of allergic mothers, shown as lower numbers of IL-12-producing CBMCs as compared to the neonates of non-allergic mothers [168].

An impaired regulatory function in children of atopic mothers has also been suggested. Thus, a reduced number of Lipid A/peptidoglycan induced Tregs and a reduced suppressive capacity of mitogen induced T effector cells were reported at birth in children of atopic mothers [143].

It is not completely elucidated if allergic sensitisation can occur prenatally or not. The possibility of fetal allergen exposure is supported by the detection of house dust mite allergen in the amniotic fluid and in the fetal circulation, indicating a transamniotic and a transplacental transfer [169]. A maternal-fetal passage of β -lactoglobulin, ovalbumin and birch pollen was shown in dual placenta perfusion experiments, [170-172] but a lot of allergen was also retained in the placenta, predominantly localised in the syncytiotrophoblast cell layer of the villous trees [173]. Allergen-induced T cell responses have been shown in fetal blood during gestation, already in gestational week 22 and at birth, shown as a capability of PBMCs/CBMCs to proliferate and produce cytokines in response to allergens [62, 174-176]. On the other hand, the neonatal CD4⁺ T cell population has shown a typical phenotype of recent thymic emigrants, with receptors lacking the specificity of conventional T cells and possibility to interact with a multitude of antigens, *e.g.* allergens [177].

Allergen-specific IgE antibodies to food and inhalant allergens have been detected in CB indicating that the neonate is capable of producing IgE antibodies before birth [178, 179]. IgE antibodies are not believed to be transported across the placenta [180], but there might be an uncertainty regarding contamination of CB with maternal IgE [181].

The mechanism for uterine programming of the fetus is not known, but factors operating in the intrauterine milieu and/or epigenetic inheritance are possible routes. It is widely accepted that maternal IgG antibodies are transferred to the fetus over the placenta, probably in order to protect the neonate from infections [182]. High CB levels of IgG antibodies to inhalant allergens have been associated with less allergic symptoms during childhood, but the role of these antibodies in allergy development are not completely understood [183].

The bidirectional maternal-fetal trafficking of small numbers of cells during pregnancy, *i.e.* naturally acquired microchimerism, is another well-recognised immunological exchange between mother and child. A recent study indicated a protective effect of maternal microchimerism on asthma development. The rate of asthma was lower in children who

harboured maternal cells in the circulation as compared to the children who did not [184]. On the other hand, maternal microchimerism has been associated with autoimmune diseases indicating harmful effects of these cells on the offspring's health [185]. The immunological consequences of maternal cells in her offspring, pre- and postnatally, on the immune maturation and development of allergy need to be further investigated.

Similarly, little is known about the influence of maternally secreted immunological mediators such as cytokines and chemokines during pregnancy on allergy development in the offspring. In fact, there is an uncertainty with respect to *which* factors in the intrauterine milieu that contribute to programming of the fetal immune system, *when*, *how* and *where* these factors are operating.

In conclusion, the mother may influence the immune development of her offspring, genetically and immunologically. Little is known about possible mechanisms for fetal programming in allergic diseases, but factors operating in the intrauterine milieu, epigenetic inheritance and appropriate prenatal microbial stimulation are thought to be important.

Development of the immune system during childhood

Prenatal development of the immune system

The development of the fetal immune system shows temporal variations between species. In mice, for instance, mature α/β T cells are found in the periphery very late in gestation, while mature α/β T cells appear in peripheral tissues in the first trimester of human pregnancy [186]. Thus, the human fetal immune system seems to be relatively early developed (Fig 3).

“Macrophage-like cells” have been detected in the yolk sac and mesenchyme already in gestational week (gw) 4-6, in the liver in gw 9-14, in the bone marrow in gw 12-13, in thymus and the gut in gw 11-22. Moreover, the presence of “macrophage-like cells”, including a MHC class II positive subpopulation, was accompanied by CD3+ T cells and CD20+ B cells in the lymph nodes of 14½-15 weeks old fetuses [187].

T cell precursors, defined by their expression of CD7, have been detected in embryonic tissues *i.e.* liver, yolk sac and upper thorax regions as early as 7 weeks of fetal gestation.

Functional studies on these CD7+ cells, purified from fetal liver, revealed an ability to express markers of mature T cells, such as CD2, CD3, CD4, CD8 and CD25 after culture in T cell conditioned medium with IL-2 [188]. Furthermore, T cells expressing the TCR, CD2 and CD3 have been identified in thymus in gw 13-15 [189].

Figure 3. An overview of the development of the fetal immune system during early pregnancy.

Pro/pre B cells have been observed in the liver at gw 8 [190] and in the lymph nodes at gw 14½-15 [189]. Prenatal IgE production has been detected, by using VDJCε transcripts as a marker for IgE production, in the liver at gw 20 [191], indicating a fetal ability to produce IgE.

Postnatal development of the immune system

The increased susceptibility to infections and decreased immune responses to vaccines in newborns are probably explained by an impaired immune function at birth [192]. Neonates have inadequate cell-mediated immunity, inflammatory responses, antibody production and a poor defense against intra-cellular pathogens, indicating a reduced function of the innate as well as the adaptive immune system [193].

Functional impairments of the innate immune system are reflected by less NK cell cytotoxicity and reduced microbicidal activity of neonatal neutrophils as compared with their adult counterparts [194, 195]. Neonatal APC and monocytes have shown a general reduced ability to mount Th1-polarising immune responses. The expression of TLR and the downstream signaling molecules are similar in infants and adults, but TLR-mediated cytokine responses of monocytes and APC at birth indicate less TNF, IFN-α, IFN-γ, IL-1β and IL-12 production as compared with these cells in peripheral blood from adults. In contrast, the TLR-induced production of IL-6, IL-8, IL-10 and IL-23 has shown to be enhanced in newborns, indicating that the neonatal immunity may not always be diminished (summarised in [195, 196]).

The ability to produce antibodies is impaired in newborns, probably influenced by the immaturity of neonatal Th cells and B cells [197]. The mother provides her offspring with humoral protection by active transfer of IgG antibodies through the placenta and postnatally by IgA antibodies through breast-feeding [198].

The T cell population at birth consists of a high proportion of naïve T cells and a low proportion of memory T cells, probably as a result of the protective environment during gestation. The proportion of naïve/memory T cells is modified by the postnatal antigen exposure, reaching adult proportions at 12-18 years of age [199]. Neonatal CBMCs respond in a dampened fashion to mitogen and innate stimuli, *i.e.* with less proliferation, IFN- γ , IL-10 and IL-17 production and less suppressive capacity of isolated Tregs than adult cells [200]. The reduced ability of neonates to mount Th1-associated immune responses is particularly pronounced in children who develop allergic diseases later in life. Lower numbers of IL-12 and IFN- γ -producing CBMCs and lower IFN- γ secretion after allergen stimulation have been associated with development of allergic symptoms and sensitisation later in life [174, 175, 201]. The discrepant immune response at birth, might be associated with exposure to a strong Th2 environment *in utero*. Accordingly, Th2-associated cytokines are readily produced by CBMCs after mitogen and allergen stimulation [62, 200]. The neonatal Th2-deviated allergen-specific immune responses are down regulated with age and the Th1-like immune responses are up regulated. A delayed down-regulation of the neonatal Th2-like immunity during childhood has been observed in children developing allergic disease [62, 202].

In summary, the development of the immune system starts early in fetal life, implying a possibility for the maternal immune system to interact with the developing fetal immune system from early to late gestation. It is unknown if a certain time period during gestation is particularly important for the shaping of the immune responses in the offspring. The neonatal immune system is considered to be immature and a delayed maturation of the immune system might be associated with development of allergic disease.

AIMS OF THE THESIS

The overall aim of this thesis was to explore the Th1/Th2 balance in allergic and non-allergic women during pregnancy and its influence on the shaping of the Th1/Th2 profile in the neonate and the development of allergic diseases in the offspring. We hypothesised that the immune profile would be biased towards Th2 during pregnancy in allergic and non-allergic women, with a more pronounced deviation in the allergic group, and that allergy development in the offspring would be preceded by a pronounced Th2 profile at birth.

The specific aims of each individual paper were:

- I To study the Th1/Th2 balance in allergic and non-allergic women by measuring specific and total IgE antibody levels during pregnancy and after delivery.
- II To investigate if CB Th1- and Th2-like chemokine levels are associated with allergy development during the first 6 years of life.
- III To determine (i) if the pregnancy magnifies the Th2 immunity in allergic and non-allergic women, (ii) if the maternal chemokine levels during pregnancy influences the offspring's chemokine levels during childhood and (iii) to evaluate the relationship between circulating Th1/Th2-associated chemokines and allergy in mothers and children.
- IV To explore (i) if maternal allergy influences the gene expression locally in the placenta, systemically in PBMC and fetally in CBMC, (ii) if the gene expression in the placenta and PBMC influences the gene expression in CBMC and (iii) how the gene expression at birth relates to allergy development during childhood.

MATERIAL AND METHODS

Design and study population

The study participants were recruited at the maternity health care clinic in Linköping, County of Östergötland, Sweden between January 2000 and April 2002. Eighty-six women agreed to participate and gave their informed consent. Of these 86 women, 25 declined participation at various time points during pregnancy and 5 women had spontaneous abortions. Thus, the remaining 56 women had a normal pregnancy, gave birth to one healthy child and followed the research schedule (Fig 4).

The allergic status of the pregnant women, the father and siblings were elucidated by interviews. An experienced allergy research nurse interviewed the women using structured questionnaires. Present and former symptoms of allergic disease *i.e.* asthma, ARC and AD was recorded. Maternal autoimmune diseases were an exclusion criteria. Seventeen mothers had ARC, 4 had asthma (of whom 1 also had ARC) and 2 had AD (both of them also had ARC). The allergic status of the women were further determined using the Phadiatop® test; 13 women were sensitised (according to Phadiatop testing) with allergic symptoms and 30 women were non-sensitised without allergic symptoms (Fig 4). Maternal and neonatal characteristics at the inclusion and delivery are presented in paper I, in Table 1.

The women were followed with sample collections at 5 occasions (gestational week, gw 10-12, 15-16, 25, 35 and 39) during pregnancy, at delivery and 2 occasions postpartum (2 and 12 months, Table 2). The sample taken 12 months postpartum was considered as a reference point, reflecting a non-pregnant state. Human chorionic gonadotropin (hCG) levels was quantified (hCG + beta, Cobas e 602, Roche Diagnostics Scandinavia AB, Stockholm, Sweden) in these samples in order to reveal a new pregnancy. One woman was assumed to be pregnant again as the hCG levels was considerably increased (13 490 U/L). This sample was excluded in paper III, but not in paper I, which was already published when the hCG analysis was performed.

Figure 4. Flow-chart of the study population. Eighty-six women were included in the study, 25 women dropped out during pregnancy, 5 women had spontaneous abortions and 56 women had a normal pregnancy and followed the research schedule. Twenty women reported allergic symptoms of whom 13 were also sensitised whereas 36 women reported no allergic symptoms of whom 30 were non-sensitised. Fifty-six children was born, 19 experienced allergic symptoms during the first 6 years of life and 27 children had no symptoms of allergic disease. Ten children declined participation at various time points during childhood and 9 of the remaining 46 children choose to participate with questionnaires only at the 6 year follow up (marked with Q in the figure). Of the 19 children with allergic symptoms, 11 were also sensitised and 15 of the 27 children without allergic symptoms were non-sensitised. Two of the 3 children with allergic symptoms who participated with questionnaires only at the 6 year follow up are included in the group of sensitised children with allergic symptoms as well. These children visited the allergy clinic very often. The diagnosis of these 2 children were based on notes in the medical records and SPT:s performed within the clinical practice. Abbreviations used, all symp: allergic symptoms, no all symp: no allergic symptoms, sens: sensitised, not sens: not sensitised.

Table 2. The research schedule for the mothers.

	Time point							
	gw 10-12	gw 15-16	gw 25	gw 35	gw 39	Delivery	2 m pp	12 m pp
Interview (nurse)	x							
Plasma	x	x	x	x	x	x	x	x
PBMC	x	x	x	x	x	x	x	x
Placenta						x		
Breast milk (colostrum)						x		
Faeces				x				

Samples used in this thesis are marked with bold. Abbreviations used, gw = gestational week, m pp = months postpartum, PBMC = peripheral blood mononuclear cells

The children were examined by an allergy research nurse at 6 and 12 months of age and by a paediatric allergologist at 24 months and 6 years of age. Samples were collected, and the parents answered questionnaires regarding environmental factors and allergic symptoms in the children (Table 3).

Table 3. The research schedule for the children.

	Time point						
	Birth	3 m	6 m	12 m	18 m	24 m	6 yr
Examination (nurse)			x (n=53)	x (n=53)			
Examination (physician)						x (n=47)	x (n=37)
Questionnaires		x	x	x	x	x	x
SPT			x egg, milk PhInf	x egg, milk, cat, PhInf		x egg, milk, cat, birch, timothy, PhInf	x egg, milk, cat, birch, timothy, PhInf, Phad
Plasma	x (CB)		x	x		x	x
PBMC	x (CB)		x	x		x	x
Saliva			x	x		x	x
Faeces	x (1 w)	x	x	x		x	x

Samples used in this thesis are marked with bold. The number of children attending to the clinical examinations are indicated in parenthesis. Furthermore, 9 children participated with questionnaires only at the 6 year follow up. Abbreviations used, CB = cord blood, w = week, yr = years, PhInf = Phadiatop Infant, Phad = Phadiatop

Study subjects

Paper I. Circulating allergen-specific and total IgE levels were measured in 20 women with and 36 women without allergic symptoms during pregnancy and after delivery. Maternal and neonatal characteristics at the inclusion and delivery are included in this paper, in Table 1. The number of available plasma samples were; gw 10-12 (n=47), gw 15-16 (n=54), gw 25 (n=51), gw 35 (n=54), gw 39 (n=39), 2 months postpartum (n=50), 12 months postpartum (n=48).

Paper II. This paper includes clinical data on development of allergic symptoms in the children during the first 6 years of life. The levels of cytokines, chemokines and total IgE were quantified in 46 CB plasma/serum samples in the children of the 56 women described in paper I. The total and allergen-specific IgE levels were measured during childhood. The number of available plasma samples were; 6 months (n=48), 12 months (n=46), 24 months (n=41), 6 years (n=36).

Paper III. Chemokine analysis were performed in plasma samples from 56 women during pregnancy and postpartum (described in paper I) and in their children during childhood (described in paper II).

Paper IV. This paper includes data on gene expression in placenta, PBMCs (gw 39) and CBMCs from 19 women. Selection of the 19 pregnant women were performed based on (i), allergic status (allergic symptoms combined with sensitisation or no allergic symptoms without sensitisation) and (ii), availability of matched PBMC (gw 39), placenta and CBMC samples. Twenty-seven children, of whom 24 CBMC samples were available (13 from the original cohort and 11 from the placebo group of a randomised placebo-controlled allergy prevention study [203]), were also included to allow assessment of gene expression of some selected genes in relation to allergy development during childhood. The selection of the additional 11 children was done based on (i) attendance to the clinical examination at 8 years of age and (ii) availability of CBMCs. The clinical examinations took place at 6 years of age for the children in the original cohort and at 8 years of age for the children participating in the allergy prevention study.

Clinical methodology

Clinical definitions, paper II-IV

AD was defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical morphology and distribution. The criteria suggested by Hanifin and Rajka [204] was used for assessment of AD diagnosis.

Asthma, at 6 years of age, was defined as ≥ 1 episodes of bronchial obstruction after two years of age, at least once verified by a physician. At 2 years of age, asthma was defined as ≥ 3 episodes of bronchial obstruction since birth, at least once verified by a physician or two episodes of bronchial obstruction combined with AD or food allergy.

ARC was defined as rhinitis and conjunctivitis, *i.e.* seasonal itching with watery discharge from nose and eyes, appearing at least twice after exposure of an inhalant allergen and not related to infection.

Urticaria was defined as allergic if it appeared within one hour after exposure to a particular allergen, at least at two separate occasions.

Food allergy, paper II-III

Symptoms of food allergy were defined as vomiting and/or diarrhoea on at least two separate occasions after intake of certain offending food.

Food allergy, paper IV*

A diagnosis of food allergy required vomiting and/or diarrhoea, hives, aggravated eczema or wheezing on at least two separate occasions after ingestion of certain offending food.

Oral allergy syndrome was defined as allergic if it appeared at least at two separate occasions after intake of certain offending food.

*The diagnosis of food allergy was changed in paper IV, to achieve common clinical definitions for the merged cohorts. The clinical definitions for asthma, AD, ARC, urticaria and oral allergy syndrome were already the same for the 2 cohorts.

Sensitisation

Allergic sensitisation was determined by SPT and quantification of allergen-specific IgE antibodies in the circulation. The allergens used for SPT and the Phadiatop Infant®/Phadiatop® tests used at the different ages during childhood are presented in Table 3. The procedures are described in detail in paper II.

Laboratory methodology

Collection of blood and placenta samples (Paper I-IV)

Venous blood from mothers and children was drawn in heparin treated tubes (Vacuette, Greiner Labortechnik, Kremsmünster, Austria). The CB samples were collected by cutting the umbilical cord at the placental site, carefully cleaning it to avoid contamination of maternal blood, and then squeezing it to collect the CB in heparin-treated tubes (Vacuette). The plasma samples were collected and frozen in -70° C (maternal samples) or -20° C (children's samples). The placenta samples were collected immediately after delivery by cutting a piece, approximately 1x1x1 cm in size on the maternal side of the placenta, using a scalpel. The placenta tissue was transferred to a sterile tube, frozen and stored at -70° C.

Isolation of CBMCs/PBMCs (Paper IV)

The maternal PBMCs were separated and frozen as described in detail elsewhere [205]. Briefly, Lymphoprep (Nycomed Pharma AB, Oslo, Norway) was used to isolate the PBMC population, followed by 2 washes with Hank's balanced salt solution (HBSS; Life Technologies, Paisley, Scotland). The PBMCs were resuspended and immediately frozen in tissue culture medium with 10% dimethyl sulphoxide (DMSO, Sigma, St Louis, MO, USA) and 50% fetal calf serum (FCS, Flow Laboratories, Irvine, Scotland).

The CBMCs and PBMCs from the children were isolated and frozen as previously described [206]. Briefly, 1 volume of RPMI-1640 (Life Technologies) was added to the cells after plasma removal for the PBMC preparations and 2 volumes of RPMI-1640 for the CBMC preparations. The PBMCs/CBMCs were isolated on Ficoll-Paque density gradient (GE Healthcare Bio-Sciences, Uppsala, Sweden), washed 3 times with RPMI-1640 supplemented with 2 % FCS (Life Technologies). The PBMCs were resuspended and subsequently frozen in a solution of 40% RPMI-1640, 10 % DMSO and 50% FCS. The PBMC samples from mothers and children were frozen with a rate of -1° C/minute to -70° C, stored for

approximately 24 hours and then transferred to liquid nitrogen for long-term storage. The CBMCs/PBMCs were isolated within 24 hours.

Cell cultures (Additional data)

After thawing the PBMCs in a water bath (37°), the viability of the cells was checked in a Bürker chamber after staining with trypan blue (Merck AB, Darmstadt, Germany). The mean viability of the thawed PBMCs was 89%. Aliquots with 1 million viable PBMCs were cultured in 1 ml AIM-V serum free medium (Life Technologies) with 20 µM β-mercaptoethanol (Sigma) at 37°C with 5 % CO₂ (Forma CO₂-incubator model 3862, Forma Scientific Inc., Marietta, Ohio, USA). The PBMCs were stimulated, based on the availability of cells, as presented in Table 4.

Table 4. Protocol used for *in vitro* stimulation

Priority	Stimulation	Time (days)	Analytes
1	Control (medium)	6	IL-5, IL-13, IFN-γ, CXCL10, CCL17
2	Birch (10 kSU/ml)	6	IL-5, IL-13, IFN-γ, CXCL10, CCL17
3	PHA (2 µg/ml)	1	IL-10, IL-5, IL-13, IFN-γ, CXCL10, CCL17
4	Cat (10 kSU/ml)	6	IL-5, IL-13, IFN-γ, CXCL10, CCL17
5	Birch (10 kSU/ml) α-IL-4R (2 µg/ml)	6	IL-4
6	Cat (10 kSU/ml) α-IL-4R (2 µg/ml)	6	IL-4
7	Control (medium)	1	IL-10
8	Birch (10 kSU/ml)	1	IL-10
9	Cat (10 kSU/ml)	1	IL-10
10	Tetanus toxoid (100 ng/ml)	6	IL-5, IL-13, IFN-γ, CXCL10, CCL17
11	Tetanus toxoid (100 ng/ml)	1	IL-10
12	Tetanus toxoid (100 ng/ml) α-IL-4R (2 µg/ml)	6	IL-4

Abbreviations used: PHA = phytohemagglutinin, α-IL-4R = anti-IL-4 receptor

The birch and cat allergen extracts were purchased from Allergologisk Laboratorium A/S, (ALK, Hørsholm, Denmark), the PHA from Sigma, the tetanus toxoid and the α-IL-4R from Calbiochem, VWR International AB, Stockholm, Sweden. Optimal doses and time points for cytokine secretion were evaluated as described in detail elsewhere [72]. The supernatants

were collected in two aliquots after centrifugation (400 g, 10 minutes) and stored in -70°C until analysis. The cells were lysed in Buffer RLT (Qiagen, Hilden, Germany) and the total ribonucleic acid (RNA) was collected and stored in -70°C.

Total and allergen-specific IgE levels (paper I-III)

The allergen-specific and total IgE levels in plasma samples from the mothers and their children were analysed by ImmunoCAP technology (Pharmacia Diagnostics, Uppsala, Sweden). The procedure is described in detail in paper I and paper II.

Enzyme-linked immunosorbent assay (ELISA, paper II)

ELISA was used for quantification of CXCL11, CCL17 and CCL18 in the CB samples in paper II and IL-4 and IL-10 in cell supernatants from allergen-stimulated PBMCs (additional data). Double-antibody sandwich ELISAs were developed and optimised for quantification of CXCL11, CCL17 and CCL18. Commercially available ELISA kits (Peli-pair, Sanquin, Amsterdam, The Netherlands) were used for the IL-4 (Cat no. M9314) and IL-10 (Cat no. M9310) assays.

Costar 3690-plates (Costar Inc., Corning, NY, USA) were coated with 1 µg/ml monoclonal anti-human CXCL11 (clone 87328, cat no MAB672, R&D Systems, Abingdon, UK) / CCL17 (clone 54026, cat no MAB364, R&D Systems) / 0.5 µg/ml CCL18 (clone 64507, MAB394, R&D Systems) or coating antibodies for IL-4/IL-10 diluted 1/100 in 50 µl carbonate buffer pH 9.6 per well. The concentration of the coating antibody for IL-4 and IL-10 was not provided by the manufacturer. Three different concentrations of the coating antibody were tested: 1, 2, 4 µg/ml for CXCL11 and CCL17 and 0.5, 1 and 2 µg/ml for CCL18. The plates were incubated overnight, washed 4 times with phosphate buffered saline (PBS) with 0.05% Tween (PBT) using a microplate washer (Anthos microplate washer Fluido, Salzburg, Austria). The wells were supplied with 100 µl prewarmed (37°) PBS with 2 % low-fat milk, incubated for 1 hour on a plate shaker, followed by 4 washes.

A 7-point standard curve with 2-fold dilutions in PBS with 1 % bovine serum albumin (BSA, Sigma) for detection of the chemokines in plasma or in AIM-V serum free medium (Life Technologies) with 20 µM β-mercaptoethanol for detection of the cytokines in supernatants was constructed using recombinant human CXCL11 / CCL17 / CCL18 (cat no 672-IT, 364-DN, 394-PA, R&D Systems) / IL-4 / IL-10 (Sanquin). The standard curve, a negative control

(medium only) and the samples were added in duplicates, (50 µl/well) to the plate and incubated on a plate shaker for 1 hour. The CB samples were diluted in a range of 1:2 to 1:600 in PBS with 1 % BSA (PBSB) and the cell supernatants were assayed undiluted. After washing 4 times, 50 µl biotinylated anti-human CXCL11 / CCL17 / CCL18 Antibody (cat no BAF672, BAF364, BAF394, R&D Systems), diluted to a concentration of 200 ng/ml in high-performance ELISA dilution buffer (Sanquin) were added to the wells. Three different concentrations of the detection antibody were tested: 50, 100, 200 ng/ml. The biotinylated detection antibodies for IL-4/IL-10 were diluted 1/100 in HPE-buffer. The concentration of the detection antibody for IL-4 and IL-10 was not given by the manufacturer. After a 1 hour incubation with shaking, the plates were washed 4 times and streptavidin-poly-horse radish peroxidase (Sanquin), diluted 1/10 000 in HPE-buffer, was added to the wells in 50 µl aliquots. After 30 minutes incubation with shaking, the plates were washed 4 times and 50 µl 3,3',5,5'-tetramethylbenzidine liquid substrate system (Sigma-Aldrich) was added to each well and incubated as before (30 minutes) but in the dark. The reaction was stopped with 1.8 M H₂SO₄. The optical densities were read at 450 nm in a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA) and the data were acquired using SOFTmaxPRO Version 3.1.2 computer software (Molecular Devices). All steps were performed at room temperature. The lower detection limits are presented in Table 5.

Luminex (paper II-III)

CB cytokines and chemokines were quantified with a Luminex kit (Beadlyte® Human Multi-Cytokine Beadmaster™ Kit, Upstate, CA, USA) in paper II. An in-house Luminex assay was developed for quantification of chemokines in plasma samples (paper III) and allergen-induced cytokine and chemokine secretion from PBMCs (additional data). All Luminex assays were performed at room temperature, unless something else is stated, and the beads were always protected from light.

The analysis of cytokines and chemokines in CB plasma/serum with the Luminex kit (see above) was done according the guidelines by the manufacturer and is described in detail elsewhere [207]. Briefly, Luminex® beads with monoclonal antibodies for detection of IL-4, IL-5, IL-9, IL-10, IL-12(p70), IL-13, IFN-γ, CCL11, CXCL10 and CCL22 were mixed with a 7-point standard curve with 3-fold dilutions, a blank and the samples (final dilution 1:2). The

plate was incubated over night at 4°C, washed and incubated with biotinylated detection antibodies for 1.5 hour, followed by washes and an incubation with Streptavidin Phycoerythrin for 30 minutes. The reaction was stopped, the beads were washed and analysed on a Luminex¹⁰⁰ instrument (Biosource, Nivelles, Belgium). The data were acquired using the StarStation 2.0 software (Applied cytometry systems, Sheffield, UK).

The sensitivity limits of the assays represent the lowest concentration (of each analyte) needed to produce a signal that is clearly distinguished from the blank (Table 5).

Table 5. Sensitivity limits for the cytokines and chemokines analysed by ELISA, a commercial Luminex kit* and an in-house Luminex assay.

Cytokine/chemokine	ELISA	Luminex kit	In-house Luminex
CXCL9	-	-	41
CXCL10	-	5	3
CXCL11	4	-	17
CCL11	-	10	-
CCL17	8	-	2
CCL18	8	-	-
CCL22	-	12	2
IFN- γ	-	4	14
IL-4	4	4	-
IL-5	-	8	14
IL-9	-	3	-
IL-10	5	1	-
IL-12(p70)	-	8	-
IL-13	-	2	41

* = Beadlyte® Human Multi-Cytokine Beadmaster™ Kit, Upstate. The cut off levels are shown in pg/ml.

Development of an in-house Luminex assay (paper III)

An in-house Luminex assay was developed for quantification of CXCL9, CXCL10, CXCL11, CCL17 and CCL22 in plasma samples from mothers and children in paper III. CCL18 was not selected to the panel, as CCL18 is present in the circulation at substantially higher levels as compared with the other selected chemokines. The protocol was modified from de Jager *et al.* [93].

Coupling of antibodies to microspheres

Carboxylated microspheres were bought from Luminex Corporation (Austin, TX, USA) and

the coupling reaction was performed according to the manufacturer's protocol with some modifications. The microspheres were resuspended by gentle inversion for 1 minute, dispensed by sonication for approximately 20 seconds and washed once in milliQ-H₂O. Aliquots of 17.5×10^6 to 30×10^6 microspheres were activated in 500 μ l 100 mM monobasic sodium phosphate, pH 6.2, supplemented with 5 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce, Rockford, IL, USA) and 5 mg/ml N-hydroxysuccinimide (Pierce) in Eppendorf low protein binding tubes (Eppendorf AG, Hamburg, Germany) for 20 minutes. The activated microspheres were washed twice in 50 mM 2-morpholinoethanesulfone acid (Sigma), pH 5.0 and resuspended in 1.5 ml 50 mM 2-morpholinoethanesulfone acid, pH 5.0 containing 5 μ g antibody/ 10^6 microspheres. The following capture antibodies were used in the coupling reaction, monoclonal anti-human CXCL9 (clone no 49106, cat no MAB392, R&D Systems), CXCL10 (clone no 4D5/A7/C5, cat no 555046, BD Biosciences, Stockholm, Sweden), CXCL11 (clone no 87328, cat no MAB672, R&D Systems), CCL17 (clone no 54026, cat no MAB364, R&D Systems) CCL22 (clone no 57226, cat no MAB336, R&D Systems). The mixture was incubated with mixing by rotation on a multiple tube mixer (ELMI, Riga, Latvia). After 2 hours, the coupled microspheres were incubated with mixing by rotation in PBT with 0.1% BSA and 0.05% NaN₃ (PBS-TBN, Merck). After 30 minutes, the microspheres were washed twice in PBT, counted by a hemacytometer and stored in 1 ml PBS-TBN in the dark at 4°C.

Luminex assay for detection of chemokines

The assay was performed according to the procedure recommended by Luminex with some modifications. The final protocol is presented below.

In detail, 2000 coupled microspheres of each set dissolved in 50 μ l PBSB were added to each well of a 1.2 μ m pore-size filter plate (Millipore multiscreen, Millipore Corporation, Bedford, USA) prewetted with PBSB. A 7-point standard curve with 3-fold dilutions in PBSB was constructed using recombinant human CXCL9, CXCL10, CXCL11, CCL17, CCL22 (cat no 392-MG, 266-IP, 672-IT, 364-DN, 336-MD, R&D Systems) and added in 50 μ l aliquots to the wells. 50 μ l blank (PBSB) and undiluted samples (final dilution in the well 1:2) were added to the microspheres as well and incubated for one hour on a plate shaker (Orbital Shaker SO3; Stuart Scientific, UK) and then over night at 4°C. The liquid was removed by vacuum filtration (Multiscreen® Vacuum Manifold, Millipore) and the remaining microspheres were washed 2 times with 100 μ l PBSB. The microspheres were resuspended in

100 µl biotinylated anti-human CXCL10 (clone no 6D4/D6/G2, cat no 555048, BD Biosciences) CXCL9 (BAF392), CXCL11 (BAF672), CCL17 (BAF364), CCL22 (BAF336) Antibody (R&D Systems) solution, diluted to a concentration of 200 ng/ml for CCL22, 500 ng/ml for CXCL9, CXCL11, CCL17 and 1000 ng/ml for CXCL10 on a plate shaker. After an 1 hour incubation, the microspheres were washed 2 times, resuspended and incubated in 100 µl of 1 µg/ml Streptavidin R-phycoerythrin conjugate (SA-PE, Molecular Probes, Eugene, USA) for 30 minutes on a plate shaker. The microspheres were washed twice and resuspended in 75 µl PBSB by shaking for approximately 5 minutes. The samples were analysed on a Luminex¹⁰⁰ instrument and the data were acquired using the StarStation 2.3 software (Applied cytometry systems, Sheffield, UK). The sensitivity limits are shown in Table 5. The optimisation and evaluation of this in-house Luminex assay is presented in the result section on page 55.

A second in-house Luminex assay has been developed in our laboratory, for simultaneous detection of IL-5, IL-10, IL-13 and IFN- γ separately and in combination with the chemokines described above. IL-5, IL-13, IFN- γ , CXCL10 and CCL17 were chosen for detection of allergen-induced responses in supernatants from the women in the study during pregnancy and postpartum. CXCL9, CXCL11 and CCL22 were not selected to the panel, as the spontaneous and stimulated secretion were very high for CCL22, thus requiring dilution of the supernatants and CXCL11 was not secreted at all. CXCL9 was not prioritised as it was not associated with allergy in the women or in the children (paper III, paper SII [208]). The protocol is the same as described above, but AIM-V was used as blank and for dilution of the standard curves. Recombinant human CXCL10, CCL17, IFN- γ , IL-13 (266-IP, 364-DN, 285-IF, 213-IL, R&D Systems), IL-5 (554606, BD Biosciences) were used. The supernatants were undiluted. The following coating antibodies were used: IL-5 (clone JES1-39D0, BD Biosciences), IL-13 (Ref: M191302, Sanquin), IFN- γ (clone NIB42, BD Biosciences) with a coupling concentration of 5 µg antibody/10⁶ microspheres. Biotinylated anti-human CXCL10 (1000 ng/ml, clone no 6D4/D6/G2, cat no 555048, BD Biosciences), CCL17 (500 ng/ml, BAF364, R&D Systems), IFN- γ (500 ng/ml, clone 4S.B3, cat no 554550 BD Biosciences), IL-5 (500 ng/ml, clone JES1-5A10, cat no 554491, BD Biosciences), IL-13 (200 ng/ml Ref: M191304, Sanquin) were used for detection. A Luminex²⁰⁰ instrument was used for analysis and the data were acquired using the xPONENT 3.1TM software.

Real-time PCR

The procedures for cell preparation, tissue homogenisation, RNA extraction, reverse transcription polymerase chain reaction (PCR), real-time PCR and the PCR arrays are described in detail in paper IV.

Data handling and statistics

The analysis of cytokines (IL-4, IL-10) and chemokines (CXCL11, CCL17, CCL18) with ELISA were always performed in duplicates and the samples were re-analysed if the coefficient of variance (CV) was >15%.

For the Luminex assay in paper II, all CB samples were analysed in one run, 34 samples were analysed in duplicates and 12 samples as singles. The mean CV of the duplicates were 17 %.

For the Luminex assay in paper III, all samples from the sensitised women with allergic symptoms and the non-sensitised women without allergic symptoms and all samples from the children were analysed in duplicates. Of the samples from the non-sensitised women with allergic symptoms (n=7) and the sensitised women without allergic symptoms (n=6), 31 % of the samples were analysed in duplicates and 69% as singles. All cytokine and chemokine analysis in the cell supernatants on the Luminex platform were performed in singles (additional data).

5-parametric curve fitting was always used and in case of very low levels of cytokine/chemokine in the majority of the samples, a weighting of 1/y was applied (only available for the Luminex data). The curve fitting with/without weighting was always the same for all maternal/children's samples.

Mean values of the duplicates, after subtraction of the blanks, were used. Undetectable levels were given a value corresponding to half the cut-off value. To achieve stimulated amounts of cytokine/chemokines, unstimulated responses were withdrawn from the stimulated responses. If the difference became smaller than half the cut off value, they were given the half of the cut off value.

All samples from the same individual were always analysed on the same plate to allow assessment of longitudinal changes in cytokine and chemokine levels. Furthermore, samples from both allergic and non-allergic subjects were included on each plate to reduce the impact of the inter-assay variation when comparing the groups.

The gene expression was analysed in singles for the PCR arrays and in duplicates for the real-time PCR assays. Matched placenta and PBMC samples were analysed on the same PCR array plate. The comparative threshold cycle (Ct) method was used for normalisation, *i.e.* the Ct value of the house-keeping gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) was subtracted from the Ct value of the target gene. Samples with Ct values >35 were given a Δ Ct value of 16 for genes from the PCR array and a value of 9 for genes from the real-time PCR assays.

The statistical package IBM SPSS Statistics 14.0 (paper I) / 15.0 (paper II) / 21.0 (paper III, IV) for Windows (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Non-parametric tests, corrected for ties, were used. The probability level of $p < 0.05$ was considered as significant in all papers.

In paper I, Friedman's test was used to determine if there were any longitudinal changes in the IgE levels during pregnancy and the year after delivery. Wilcoxon's signed rank test was used to investigate if there were any differences in the IgE levels between different time points. Mann-Whitney *U*-test was used for comparisons between unpaired groups. The Chi-squared test was used for categorical variables and Fisher's exact test was used when the expected frequency for any cell was less than 5 (Stat View for Windows Version 5.9, SAS Institute Inc., Cary, North Carolina, USA).

To verify our findings, we also used a non-parametric approach, which makes it possible to take the missing values into account. This approach was used to investigate if the fluorescence intensities / IgE levels changed between gestational week 10-12 and 39, during and after pregnancy and if there was any difference in the levels between pregnancy and postpartum (5 and 2 time points combined, respectively).

The observed data were ranked in the same way as in Friedman's test and the ranks were adjusted to get the same mean rank value for each individual. To determine if there was any inclination in the fluorescence intensity / IgE concentration during gestational week 10-12 to 39 and week 10-12 to 12 months after delivery a regression model with the mean rank values as the response variable (Y) and the time points as the X variable was used. Furthermore, we explored if there was any difference in the mean fluorescence intensity / IgE level between pregnancy and after delivery (mean values of 5 and 2 time points, respectively).

In order to determine the p-values, we needed the distributions for the regression coefficients and mean rank difference given that there was no difference between the time points. These distributions were approximately studied by simulation. In each simulation step, the adjusted ranked values were randomly rearranged within the same individual without any change in the positions of the missing values, and the regression coefficients and mean rank difference were recalculated. A total of 20000 simulations were made and $p < 0.05$ was considered to be significant. This analysis was conducted and performed by a statistician, Olle Eriksson, Department of Mathematics, Division of Statistics, Linköping University, Sweden.

In paper II-IV, correlations between chemokines / IgE / mRNA levels were analysed with Spearman's rank order correlation coefficient test. Actions were taken to deal with the issue of multiple correlations. In paper III, clear patterns were required for presentation *i.e.* repeated statistical significant correlations between several time points. In paper IV, only strong relationships, defined as $Rho \geq \pm 0.8$ and $p < 0.001$ were reported. Friedman's test was used for evaluation of alterations in chemokine / IgE levels during the analysed time period, and Wilcoxon's signed rank test for evaluation of possible differences between the time points (paper III). Wilcoxon's signed rank test was used to compare gene expression in placenta and PBMCs in paper IV. Comparisons between unpaired groups were done with the Mann-Whitney *U*-test (paper I-IV). In paper II, logistic regression (Minitab 15, Minitab, Inc., State College, PA) was used to elucidate if CB chemokines / IgE predicted allergic symptoms and sensitisation during the first 6 years of life.

Ethics

This study was approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping (approval number: Dr 99184 and complementary Dr: M98-06). The allergy prevention study, described in paper IV, was also approved by the same Regional Ethics Committee (approval number: Dr 01-284 and complementary Dr: M206-04). All families gave their informed consent.

RESULTS AND DISCUSSION

Clinical diagnosis and sensitisation

Nineteen children reported allergic symptoms during the first 6 years of life, 27 children reported no symptoms of allergic disease and 10 children dropped out of the study during childhood (Fig 4). The children were also divided into groups with more strict definitions of allergic disease, *i.e.* allergic symptoms and accompanying sensitisation (n=11) and no allergic symptoms without any accompanying sensitisation (n=15). The distribution of allergic manifestations in the 19 children who developed allergic symptoms during childhood is presented in paper II in Table 1.

Methodological aspects

Optimisation and evaluation of the Luminex assay

The optimal concentrations of the reagents presented in the final protocol were determined by titration of the capture- and detection antibodies and the fluorofor SA-PE.

Five µg coating antibodies per 10⁶ microspheres was used for all chemokines according to the recommendation by the manufacturer (Targeted Assay Development Workshops, Planet xMAP Europe 2006, Luminex Corporation). Ten and 25µg antibodies/10⁶ microspheres were also tested for CXCL9 as an attempt to achieve higher assay sensitivity. Several different concentrations of the detection antibody were tested: 25, 50, 100, 200, 500, 1000 ng/ml for CXCL9, 250, 1000, 2000 ng/ml for CXCL10, 50, 100, 200, 500 ng/ml for CXCL11 and CCL17 and 25, 50, 100, 200, 500 ng/ml for CCL22. Three different concentrations of SA-PE were tested, 1, 2 and 4 µg/ml. The optimal concentration of the capture- and detection antibodies and SA-PE was determined by selecting conditions providing the highest assay sensitivity.

A high photomultiplier tube (PMT) setting, *i.e.* increasing the gain on the PMT to 770 Volts, was tested in order to increase the assay sensitivity, particularly needed for CXCL9 (Table 5). High PMT (770 Volts) increased the overall signal and the assay sensitivity for some chemokines as compared to the original setting of 640 Volts. The sensitivity limit for CXCL11 was increased (from 17 pg/ml to 5 pg/ml) with the high PMT setting, but the sensitivity limit for CXCL9 was not affected. The main drawback with the increased PMT

was the reduction in dynamic range. An example is shown in figure 5. The original setting of 640 Volts was used, as we judged that the disadvantages with the high PMT setting outweighed the advantages.

Figure 5. High PMT (770 Volts) increased the overall signal and the assay sensitivity as compared with the original setting (640 Volts), but decreased the dynamic range. Abbreviations used: MFI = median fluorescence intensity, V = Volt

The possibility of cross-reactivity between the different antibodies was examined by comparing the median fluorescence intensity (MFI) generated from a standard curve of the monoplex assay with the MFI generated from the corresponding standard curve of the pentaplex assay. The MFI generated from the pentaplex assay was similar to those generated from the monoplex assays (Figure 6a-e).

Figure 6. The median fluorescence intensity (MFI) was similar between the monoplex and pentaplex assays, indicating that no cross-reactivity occurs between the included antibodies.

The specificity of the assay was investigated by “spiking-experiments”. The microsphere sets were mixed and a single chemokine standard (in the middle part of the standard curve) was added to each well. The selected concentrations were 2500 pg/ml for CXCL9, 2000 pg/ml for CXCL11, 500 pg/ml for CCL17 and 1000 pg/ml for CXCL10 and CCL22. A mixture of all biotinylated detection antibodies was added to the wells. Positive readings were achieved for the microspheres coupled with the specific capture antibody for the current chemokine, whereas irrelevant chemokines did not show any readings above background for any microsphere sets (Figure 7a-e). Addition of reagents for detection of IL-5, IL-10, IL-13 and IFN- γ to the pentaplex Luminex assay was evaluated by the same tests, *i.e.* the performance of the antibodies together/separated and detection of chemokine standards separately, as described above. In agreement with de Jager *et al.* [93] no cross-reactivity between the assay

components was shown for the pentaplex (chemokine detection) or the nonaplex assay (cytokine and chemokine detection).

Figure 7. The figure shows the MFI generated from each microsphere sets (x-axis) after addition of a single chemokine standard (heading). This test indicates that only the relevant chemokine is detected on each microsphere sets, and not anyone of the other 4 chemokines included in this multiplex Luminex assay.

The reproducibility of the assay was evaluated by assessment of intra and inter-assay variation. The intra-assay variation was $\leq 10\%$, evaluated by analysing one internal control sample in 12 wells on the same plate. The inter-assay variation was determined during the analysis of the chemokines in mothers and children in the study (paper III), by including two

internal control samples on each plate. The inter-assay variation was 26% for CXCL9, 12% for CXCL10, 10% for CXCL11, 17% for CCL17 and 12% for CCL22.

The effect of repeated “freezing and thawing” cycles on chemokine levels in plasma was evaluated in 2 samples by comparing the chemokine levels in aliquots which has been thawed 1, 2 or 3 times (Fig 8). This test indicates that chemokines are not very likely to degrade during the first 2 cycles of freezing and thawing.

Figure 8. The figure shows the CXCL9, CXCL10, CXCL11, CCL17, CCL22 and CCL18 levels in aliquots of 2 samples, thawed 1, 2 or 3 times, respectively. S1 = sample 1, S2 = sample 2

In paper II and III, all plasma/serum samples were thawed for the first time upon chemokine analysis on the Luminex platform. The chemokine analysis using ELISA and the re-analysed samples (due to CV > 15%) were performed on previously thawed samples.

Both CB plasma and serum samples were included in paper II. We have previously measured CXCL9, CXCL10, CXCL11, CCL17, CCL18 and CCL22 in plasma and serum samples from the same individual at the same time point (n=11). The chemokine levels in plasma and serum correlated positively with each other [208].

Th1/Th2 immunity during pregnancy in allergic and non-allergic women

We characterised the peripheral balance between Th1- and Th2-associated immunity during pregnancy and postpartum in allergic and non-allergic women by quantifying IgE antibodies (paper I), chemokines (paper III) and secreted cytokines and chemokines after allergen stimulation (additional data). In paper IV, gene expression profiles comprising Th1-, Th2-Treg- and Th17-associated genes were assessed systemically in PBMC and locally in the placenta.

Pregnancy modulated the Th1/Th2 balance in women with allergic symptoms and in sensitised women with allergic symptoms, when total IgE antibodies were used as a marker for Th2 immunity. The levels of total IgE were increased at gestational week 10-12 as compared to 12 months after delivery in the sensitised women with allergic symptoms, but not in the non-sensitised women without allergic symptoms (Fig 9). Similarly, the total IgE levels decreased from 10-12 weeks of pregnancy to 12 months postpartum in the women with allergic symptoms (regardless of sensitisation, $p < 0.01$) but this pattern was not observed in the women without allergic symptoms (Fig 10).

Figure 9. The total IgE levels were increased during early pregnancy (gw 10-12) as compared to 12 months postpartum, in sensitised women with allergic symptoms (A), but not in non-sensitised women without allergic symptoms (B). ** = $p < 0.01$, Wilcoxon's signed rank test

Thus, our IgE data do not indicate a Th2 shift during pregnancy in the non-sensitised women without allergic symptoms, but support the idea of an association of allergy with an enhanced

Th2 deviation during pregnancy. Amoudruz *et al.* also observed increased total IgE levels during pregnancy as compared with 2 years postpartum in a group of allergic pregnant women [209], while this relationship was not noted by others [210].

The allergen-specific IgE levels were measured to investigate if the observed increase in total IgE levels during pregnancy in the sensitised women with allergic symptoms is caused by increased responses to allergens. The allergen-specific IgE levels did not change during pregnancy and after delivery for any of the studied groups, *i.e.* (i) women with allergic symptoms, (ii) women without allergic symptoms, (iii) sensitised women with allergic symptoms and (iv) non-sensitised women without allergic symptoms (Fig 10). Thus, the pronounced Th2 shift in early pregnancy might reflect a stronger general Th2 shift during pregnancy in allergic individuals rather than increased immune responses to allergens.

Figure 10. The figure shows a summary of the changes and absence of changes in specific IgE and total IgE levels during and after pregnancy in the studied groups.

All women were not able to donate blood at all study follow-ups, thus generating missing values in our statistical analysis. To verify our main findings in paper I, we also used a non-parametric approach, which makes it possible to take these missing values into account. This approach was used to investigate if the fluorescence intensities/IgE levels changed between gestational week 10-12 and 39, during and after pregnancy and if there was any difference in the levels between pregnancy and postpartum (5 and 2 time points combined, respectively).

The significant change in IgE levels during the analysed time period in the sensitised women with allergic symptoms was confirmed by the non-parametric method taking missing values into account. In the sensitised women with allergic symptoms, IgE levels changed between gestational week 10-12 and 39 ($p<0.02$) and 12 months after delivery ($p<0.0001$), respectively. The IgE concentration was increased at gestational week 10-12, decreased during pregnancy and continued to decrease postpartum. Furthermore, a significant difference in the mean IgE concentration during pregnancy compared to after delivery was shown in the sensitised women with allergic symptoms ($p<0.0005$), and the mean level during pregnancy was increased compared to the mean level after delivery. None of these changes were observed in the non-sensitised women without allergic symptoms.

The non-parametric method taking missing values into account showed that the fluorescence intensities (from the Phadiatop test) changed over the analysed time period ($p<0.002$), but not between gestational week 10-12 and 39 ($p=0.13$), in the women with allergic symptoms. A significant difference in the mean fluorescence intensity during pregnancy compared to postpartum was shown ($p<0.002$), and the mean level was higher during pregnancy than postpartum. Women without allergic symptoms showed no significant changes in fluorescence intensity during and after pregnancy. Thus, we cannot completely exclude an effect of enhanced IgE responses to allergens during pregnancy, even though the conventional statistics (Friedman's test), based on existing data, did not indicate such relationship.

Allergen stimulations of PBMCs were performed to further explore the impact of allergens on the Th1, Th2 and Treg/anti-inflammatory cytokine and chemokine production during pregnancy and after delivery in relation to maternal allergy. PBMCs from gw 10-12, 15-16, 25, 35 and 2 and 12 months postpartum were stimulated with birch and cat allergen extracts, PHA and tetanus toxoid. Results are presented for allergen-induced IL-4, IL-5, IL-10, IL-13, IFN- γ and CCL17 secretion of the sensitised women with allergic symptoms and non-sensitised women without allergic symptoms. The CXCL10 secretion was higher than expected and the supernatants need to be diluted and reanalysed.

As expected, sensitised women with allergic symptoms produced higher levels of birch-induced IL-4 at gw 25, IL-5 at gw 10-12, 25 and 35, and IL-13 at gw 15-16 and 35 as compared with the non-sensitised women without allergic symptoms ($p=0.04-0.008$).

Similarly, sensitised women with allergic symptoms had higher cat-induced IL-4 secretion at gw 15, 25, 35 and 2 and 12 months after delivery, IL-5 at gw 10-12 and 2 months postpartum and IL-13 at gw 10-12 than the non-sensitised women without allergic symptoms ($p=0.04-0.001$). Thus, our findings support that allergy is associated with increased Th2-deviated immune responses to allergens. Birch- and cat-induced IL-10, IFN- γ and CCL17 levels did not differ between the two groups at any occasion.

The allergen-induced levels of IL-5, IL-13, CCL17 and IFN- γ were modulated by the pregnancy in both groups. In the sensitised women with allergic symptoms, birch-induced IL-5 and IL-13 levels were increased at gw 15-16, 25 and 35 as compared with 2 months postpartum (Fig 11).

Figure 11. Birch-induced IL-5 (A) and IL-13 (B) secretion in sensitised women with allergic symptoms and non-sensitised women without allergic symptoms during pregnancy and postpartum. The total number of samples and the number of positive responders are indicated below the x-axis. * = $p < 0.05$, ** = $p < 0.01$, Wilcoxon's signed rank test. The box indicates the 25th, 50th and 75th percentiles and the whiskers indicate minimum and maximum values.

The birch-induced secretion of IL-5 and IL-13 did not change during pregnancy and after delivery for the non-sensitised women without allergic symptoms. In contrast, IFN- γ responses to birch were similar during pregnancy and postpartum in the sensitised women with allergic symptoms but, in fact, increased at gw 10-12 as compared with 12 months postpartum and decreased at gw 15-16, 25, 35 as compared with 2 months postpartum in the the non-sensitised women without allergic symptoms (Fig 12).

Figure 12. Birch-induced IFN- γ secretion in sensitised women with allergic symptoms (**A**) and non-sensitised women without allergic symptoms (**B**) during pregnancy and postpartum. The total number of samples and the number of positive responders are indicated below the x-axis. * = $p < 0.05$, ** = $p < 0.01$, Wilcoxon's signed rank test. The box indicates the 25th, 50th and 75th percentiles and the whiskers indicate minimum and maximum values.

The cat-induced CCL17 levels were higher at gw 25 than 2 months after delivery in the sensitised women with allergic symptoms ($p = 0.01$). The non-sensitised women without allergic symptoms had higher IL-5 responses to cat at gw 25 and 35 than 2 months postpartum ($p = 0.03$, $p = 0.047$ respectively), higher IL-13 at gw 10-12 as compared to 12 months postpartum ($p = 0.04$) and lower IFN- γ secretion at gw 35 than 2 months after delivery ($p = 0.002$). The allergen-induced levels of IL-4 and IL-10 were similar during pregnancy and after delivery in both groups.

Thus, our data on allergen-induced cytokine and chemokine secretion during and after pregnancy indicate that certain allergen-specific responses are magnified during pregnancy in sensitised women with allergic symptoms as well as in the non-sensitised women without allergic symptoms. It is notably that enhanced Th2-like immune responses during pregnancy predominantly occurred in response to birch in the allergic group and in response to cat in the non-allergic group. Cat-ownership was recorded in the questionnaires. Eight non-sensitised women without allergic symptoms owned a cat but none of the sensitised women with allergic symptoms did, possibly indicating a more persistent and a higher exposure to cat allergen during pregnancy in the non-allergic group.

The birch- and cat-induced IFN- γ responses were modified by pregnancy in the non-allergic group. The IFN- γ secretion in response to birch was actually increased at gw 10-12 as compared with 12 months postpartum. IFN- γ might be necessary during implantation [211], indicating that Th1-like immune responses might be important in early pregnancy. The allergen-induced IFN- γ secretion was down-regulated in the second and third trimesters of pregnancy, which has also been reported in another study [151]. In contrast, the IFN- γ responses was not altered in the allergic group. We have previously reported a decreased capacity of the allergic women in this cohort to produce IFN- γ and IL-10 to paternal antigens and pooled unrelated antigens during pregnancy (paper SIII, [134]). Thus, certain antigens may modulate Th1- and Th2-responses during pregnancy whereas other antigens do not. The levels of the regulatory/anti-inflammatory cytokine IL-10 were not increased during pregnancy as compared with postpartum in any of the groups. This was a little bit unexpected, but others have also failed to reveal increased IL-10 levels systemically during pregnancy, *i.e.* after stimulation with cat and house dust mite allergen [151].

Antigen-induced cytokine secretion is widely used to characterise immune regulation in various diseases, but it has been difficult to use circulating cytokines as markers for Th1 and Th2 immunity. Th1- and Th2-like cytokines are in general undetectable or only sporadically detectable in peripheral blood [207]. Chemokines, on the other hand, are easily detected in the circulation, making them attractive as markers for Th1 and Th2 immunity. Thus, the Th1-associated chemokines CXCL9, CXCL10 and CXCL11, the Th2-associated chemokines CCL17 and CCL22 and the Th2/anti-inflammatory chemokine CCL18 were quantified in peripheral blood, to further study the Th1/Th2 balance during pregnancy in relation to maternal allergy.

The chemokine levels were not associated with maternal allergic disease, *i.e.* similar levels were observed at all time-points during pregnancy and postpartum when comparing women with *versus* without allergic symptoms as well as when comparing sensitised women with allergic symptoms *versus* non-sensitised women without allergic symptoms.

The pregnancy modified the chemokine levels with the same pattern in women with and without allergic symptoms (Friedman's test, $p=0.02$ - <0.001). Thus, the maternal chemokine data is presented in relation to a normal pregnancy, *i.e.* we have combined the groups of women with and without allergic symptoms, as they showed similar levels and longitudinal changes. The Th1-associated chemokines CXCL10 and CXCL11 were increased at gw 39 as

compared with the first and second trimesters as well as compared with 2 and 12 months after delivery (Figure 13).

Figure 13. The figure shows median (closes circles, solid line) an range (broken lines) for the Th1-associated chemokines (A) CXCL9, (B) CXCL10 and (C) CXCL11 in 56 women with a normal pregnancy. Wilcoxon's signed rank test was used for comparisons between time points.

The levels of the Th2-like chemokines were not magnified by pregnancy, in fact the CCL17, CCL18 and CCL22 levels were decreased during pregnancy as compared with 2 and 12 months postpartum. The Th1-associated chemokine CXCL9 levels were also decreased as compared with 2 months postpartum (Fig 13A). The levels of CCL17 and CCL22 gradually decreased during pregnancy, with the highest levels at gw 10-12 and the lowest levels in the third trimester (gw 35 and 39).

Figure 14. The figure shows median (closes circles, solid line) an range (broken lines) for the Th2-associated chemokines (A) CCL17, (B) CCL18 and (C) CCL22 in 56 women with a normal pregnancy. Wilcoxon's signed rank test was used for comparisons between time points.

Five women, originally included in the study, had spontaneous abortions between gw 10-12 and 15-16. This group of women showed increased levels of total IgE and CXCL10 and decreased levels of CCL18 before the spontaneous abortion as compared with the 56 women with a normal pregnancy.

Figure 15A. The total IgE and (B) CXCL10 levels were increased and the (C) CCL18 levels were decreased at gw 10-12 in the 5 women who later had spontaneous abortions as compared with the 56 women with a normal pregnancy (available samples at gw 10-12, n=47). * = $p < 0.05$, Mann-Whitney *U*-test. The box indicates the 25th, 50th and 75th percentiles and the whiskers indicate minimum and maximum values.

The regulatory function of chemokines in successful pregnancies is poorly understood. Our additional observation of increased CXCL10, total IgE and decreased CCL18 levels in women with spontaneous abortions indicate an imbalance between Th1/Th2/anti-inflammatory immunity in this group. It is tempting to speculate that CXCL10 and CCL18 could serve as markers for spontaneous abortion risk, but these findings must be interpreted with caution as only five cases of spontaneous abortions were included and none of these pregnancies were verified by ultra sound. It would be interesting to analyse the Th1- and Th2-associated chemokines, studied in this thesis, as markers for spontaneous abortion risk, in a larger material with ultra sound verified pregnancies.

We were not able to reveal any differences in chemokine levels between allergic and non-allergic women, which might be related to the importance of a strict regulation between Th1/Th2/anti-inflammatory immunity for the maintenance of pregnancy. Others have also failed to reveal differences in chemokine levels in adults with ARC and healthy controls [104, 115], indicating that chemokines might be inappropriate markers for Th1- and Th2 immunity in an adult population with ARC as the main allergic manifestation.

The chemokine levels were modified by the pregnancy with the same pattern regardless of the allergic status of the mother. The increased CXCL10 and CXCL11 levels in late gestation

probably reflect the normal course of pregnancy with a strong pro-inflammatory response close to delivery, possibly related to the onset of labour. A recent study supported a role for CXCL10 in parturition, as mRNA and protein levels were elevated in choriodecidual tissue from women who delivered at term with labour as compared to women who delivered at term without labour (caesarean section) [212]. Furthermore, studies on pre-term deliveries revealed increased levels of CXCL10 and CXCL11 in serum [213], supernatants from fetal membrane extracts [214] and amniotic fluid [215].

Our chemokine data do not support the idea of an enhanced Th2-shift during pregnancy, with a more pronounced Th2-shift in the allergic women. Decreased CCL22 levels locally in the cervico-vaginal mucosa in pregnant women have also been reported, supporting our findings of reduced CCL22 levels during pregnancy [216]. The decreased levels of the Th2-like chemokines CCL17, CCL18 and CCL22 during pregnancy could be influenced by pregnancy hormones, or be related to a peripheral consumption of these chemokines, but these speculations need to be investigated in detail.

It is also important to keep in mind that the immunological milieu systemically does not always reflect the milieu locally, during pregnancy. In paper IV, mRNA expression of Th1-, Th2-, Treg- and Th17-associated genes was assessed with matched PBMC (gw 39) and placenta samples. The 40 genes included in the PCR array are presented in Table 2, in paper IV. The gene expression profile in the placenta was dominated by an enhanced expression of genes associated with Th2- and regulatory immune responses. mRNA expression of GATA-3, IL-5, IL-10, IL-33, CCL18, Epstein-Barr virus induced 3 (EBI3), indoleamine 2,3-dioxygenase 1 (IDO1), triggering receptor expressed on myeloid cells 2 (TREM-2) and p40 was increased in placenta tissue as compared with mononuclear cells from peripheral blood (please see figure 2 in paper IV). To the best of our knowledge, this Th2-/anti-inflammatory environment in the placenta tissue, including cells of fetal origin, has not been described before.

The gene expression systemically and locally during pregnancy was also related to maternal allergy. The sensitised women with allergic symptoms showed higher expression of p40 in placenta ($p=0.01$) and p35 in PBMCs ($p=0.02$) than the non-sensitised women without

allergic symptoms. These differences indicate either increased pro-inflammatory- (IL-12, IL-23) or regulatory (IL-35) immune responses in the allergic group.

Thus, we were not able to reveal a pronounced Th2 deviation during pregnancy by studying mRNA expression in PBMC and placenta, but one should keep in mind that many important Th2 markers (IL-4, IL-9, IL-13, CCL11, CCL17, CCL22) were undetectable in the majority of samples. Thus, expression of Th2-like genes could be increased in allergic women, but due to methodological limitations, impossible to detect.

Immunological interactions between mother and child during pregnancy

We have investigated the immunological interactions between mother and child during pregnancy by analysing gene expression in PBMC, placenta and CBMC (paper IV), circulating IgE antibodies and Th1- and Th2-associated chemokines in the mothers during pregnancy and postpartum and their children at birth and during childhood (paper II, III, SI).

The maternal IgE and chemokine levels during pregnancy and postpartum correlated with the offspring's IgE and chemokine levels at birth and during childhood (Table 6).

Table 6. Correlations between the maternal IgE/chemokine levels during and after pregnancy and the offspring's IgE/chemokine levels at birth and during childhood (Spearman's rank order correlation coefficient test, Rho, p-value)

Mother	Child	gw 15-16	gw 25	gw 35	gw 39	2 m pp	12 m pp
<i>IgE – IgE</i> Birth		0.34*			0.35*		0.36*
<i>IgE – CCL22</i> Birth		0.35*	0.36*	0.31*		0.40*	0.38*
<i>CXCL11 – CXCL11</i> 6 m 12 m 6 yr		0.40 **	0.48 **	0.37 *		0.49 **	0.53 *** 0.36 *
<i>CCL18 – CCL18</i> Birth 24 m 6 yr			0.44 **			0.51 ** 0.41 *	
<i>CCL22 – CCL22</i> 6 yr		0.42 *	0.46 **	0.37 *		0.41 *	0.37 *

gw = gestational week, m pp = months postpartum, m = months, yr = years, * = $p < 0.05$, ** = $p < 0.01$

*** = $p < 0.001$

The relationship between the maternal immunity during pregnancy and the prenatal synthesis of IgE, CCL18 and CCL22, supports the idea of prenatal priming of the immune system.

Immune mediators in the periphery may alter the immunological milieu at the maternal-fetal interface contributing to the shaping of the Th1/Th2 profile in the neonate. Our gene expression analyses, at the placental level, showed a negative correlation between placental p35 expression and fetal Tbx21 expression ($Rho = -0.88$, $p < 0.001$) indicating suppressive effects of placental p35, speculatively by IL-35, on the fetal Th1-like immunity. Maternal IL-5 expression in PBMC correlated with fetal Galectin-1 ($Rho = 0.91$, $p < 0.001$) expression, supporting our previous observations of an interplay between the systemic maternal Th2-like

immunity and the fetal immunity at birth. Galectin-1 has been suggested to be important for immune regulation including apoptosis and immune suppression [217].

An influence of the maternal immunity during pregnancy on the developing immune system of the offspring was observed at birth, but also later during childhood. The maternal CXCL11, CCL18 and CCL22 levels correlated with the corresponding chemokine levels in the offspring at various time points during the first 6 years of age, indicating long-term effects of the gestational environment. The idea of “fetal programming of diseases” suggests even longer effects of the intra-uterine environment, *i.e.* a link between events *in utero* and development of diseases in adult life [152].

Th1- and Th2-like immunity at birth and during childhood in allergic and non-allergic children

It is generally accepted that established allergic disease is characterised by a Th2 dominant immunity, but the timing of the development of this Th2 skewing is not known. The Th2 deviation, preceding allergic disease, might develop in very early life. We have measured mRNA expression of Tbx21, GATA-3, Foxp3, RORC and CCL22 in CBMC (paper IV) and CB IgE, cytokine and chemokine levels (paper II) and analysed in relation to future allergy development. The development of Th1- and Th2 immunity during childhood was assessed in paper III, by measurement of total IgE and chemokines in peripheral blood.

Increased gene expression of CCL22 was observed in CBMC in children later developing allergic symptoms and sensitisation as compared with children who did not (Fig 16A), while gene expression of the 4 transcription factors Tbx21, GATA-3, Foxp3 and RORC was not related to allergy development. In agreement with the gene expression data, circulating protein levels of CCL22 were also associated with allergy development later in life (Fig 16B).

Figure 16A. mRNA expression of CCL22 in CBMCs and **(B)** the CCL22 levels in CB from children later developing sensitisation and allergic symptoms and children who did not. The median is represented by a black line. * = $p < 0.05$, Mann-Whitney U -test

The mRNA- and protein levels of CCL22 did not correlate with each other, suggesting that other cells, possibly tissue macrophages, contribute to the CCL22 levels observed in peripheral blood.

Increased CB CCL22 levels were associated with development of allergic sensitisation (Fig 17A) and increased CCL17 levels with development of allergic symptoms (Fig 17B). Furthermore, CB CCL22 levels predicted development of allergic sensitisation, and CB CCL17 predicted development of allergic symptoms during the first 6 years of life. The Odds Ratio for CCL22 was 1.14, 95% confidence interval (CI) 1.03-1.26, $p=0.02$, and 1.27, (95% CI 1.01-1.59) $p=0.04$ for CCL17, based on 100-pg/ml intervals of CCL17/CCL22. Children who developed allergic symptoms and sensitisation later in life had higher CB CCL17 ($p=0.02$) and CCL22 levels (Fig 16B) and a tendency towards increased IgE levels ($p=0.09$) as compared with the children who did not develop allergic symptoms and sensitisation. Furthermore, increased ratios between the Th2-associated chemokines CCL17 and CCL22 and the Th1-associated chemokine CXCL10 ($p=0.04$ and $p=0.005$, respectively) were also associated with development of allergic symptoms and sensitisation.

Figure 17A. Increased CB CCL22 levels were observed in the children who developed allergic sensitisation during the first 6 years of life as compared with the children who did not. **(B)** Increased CB CCL17 levels were shown in the children who developed allergic symptoms compared with the children who did not develop allergic symptoms. The median is represented by a black line. * = $p<0.05$, ** = $p<0.01$, Mann-Whitney *U*-test

These patterns remained when studying the chemokines longitudinally during childhood. The levels of CCL22 were still higher at 24 months ($p=0.005$) and the CXCL10 levels were lower at 12 months of age ($p=0.007$), in the children who developed sensitisation as compared with the children who did not develop sensitisation. Development of allergic symptoms was associated with increased CCL17 levels later during childhood, namely, at 6 and 24 months ($p=0.03$ and $p=0.009$) as well as increased total IgE levels at 12 months of age ($p=0.03$). Allergic symptoms combined with sensitisation were associated with increased

total IgE levels at 12 months ($p=0.009$), CCL17 and CCL22 levels at 24 months ($p=0.02$ and $p=0.01$) and CCL18 levels at 6 years of age ($p=0.04$).

The high levels of CCL17 and CCL22 at birth, may reflect an enhanced maternal Th2 shift during pregnancy, influencing offspring immune development. We did observe positive correlations between maternal IgE and fetal IgE and CCL22 levels at birth (Table 7).

The CCL17 and CCL22 production is suppressed by IL-10 [50, 112], possibly indicating that the increased Th2-shift at birth actually reflect an impaired immunoregulatory capacity *in utero*.

High CCL17 and CCL22 levels at birth might affect the offspring postnatally, possibly promoting allergy development. If CCL17 and CCL22 are actively involved in the initiation of the disease, or if increased CCL17 and CCL22 levels are markers for a general, stronger Th2 shift at birth in these children, remains to be established.

Our chemokine data suggest that children with a more marked Th2 deviation at birth experience difficulties in the down-regulation of the neonatal Th2 immunity later in life, in combination with a (less marked) delayed up-regulation of the Th1 immunity. A continued Th2 dominance in the immune responses to allergens during infancy has also been associated with allergy development [62, 202]. A sustained strong Th2 immunity during the first years of life might stimulate IgE synthesis and promote allergy development.

It is tempting to speculate that CCL17 and CCL22 contribute to allergy development through different mechanisms, as increased CCL22 levels were related to development of sensitisation, while increased CCL17 levels were associated with development of allergic symptoms, but not sensitisation alone.

CCL22 might contribute to allergy development by an immunological mechanism causing elevated total IgE levels later in life. We did observe positive correlations between neonatal IgE, CCL17 and, in particular, CCL22 levels, and the total IgE levels later in life (Table 7).

Table 7. Correlations between CB IgE, CCL17, CCL22 levels and total IgE levels at 6, 12, 24 months and 6 years of age (Spearman's rank order correlation coefficient test, Rho, p).

	Total IgE 6 m	Total IgE 12 m	Total IgE 24 m	Total IgE 6 yr
CB IgE	0.49 **	0.28 ‡	0.22 NS	0.15 NS
CB CCL17	0.38 *	0.13 NS	0.02 NS	0.09 NS
CB CCL22	0.43 **	0.28 ‡	0.46 **	0.34 ‡

Definition of abbreviations: m=months, yr=years, ‡=p<0.1 *=p<0.05, **=p<0.01 NS=not significant

The association between high CB CCL22 levels and high total IgE levels during the first 6 years of age was confirmed in a larger study comprising 223 CB samples. This study did not support a relationship between CCL22 at birth and development of allergic sensitisation or allergic symptoms later in life. The authors suggest that CCL22 might be involved in an immunological mechanism only causing elevated total IgE levels, but not specific allergic sensitisation and allergic symptoms [218]. It should be noted that all children in the cohort had mothers with a history of asthma, demanding replication in a unselected population.

CCL17, but not CCL22, is expressed on dermal vascular endothelial cells in inflamed skin [219, 220] suggesting that increased CCL17 levels might be more closely associated with skin symptoms. Our chemokine data revealed an association between increased CCL17 levels at birth and at 24 months of age and development of AD (described in paper II and III). The relationship between elevated CCL17 levels and AD development during infancy, regardless of sensitisation, has been confirmed in 2 other cohorts of children (paper SII, [208, 221]).

It is tempting to speculate that CB CCL17 and CCL22 could be used as predictors of elevated IgE levels and future allergy development. CB IgE has been evaluated as a potential predictor of elevated IgE levels and allergy development, but the use of CB IgE as a predictor has been limited, due to poor sensitivity [222-225]. Total IgE levels are present at very low concentrations in CB, only 12 (26%) of the 46 CB samples in this cohort had detectable levels of total IgE, while CCL17 and CCL22 are easily detected in CB. The CB levels of CCL17 and CCL22 are markedly higher than adult levels (unpublished data), thereby reducing the impact of contamination of the CB samples with maternal blood, which is a concern for the quantification of CB IgE levels.

Further studies by our research group are underway to analyse chemokines as predictive markers for elevated IgE levels and future allergy development in a larger material using an unsupervised approach of machine learning.

SUMMARY AND CONCLUDING REMARKS

In this thesis, we aimed to investigate (i) the Th1/Th2 balance in allergic and non-allergic women during pregnancy, (ii) if the maternal immunity during pregnancy influences the offspring's immunity at birth and during childhood and (iii) if allergy development in the offspring is preceded by a pronounced Th2 profile at birth. Our main findings are illustrated in figure 18.

Th1/Th2 immunity during pregnancy in allergic and non-allergic women

We have found that pregnancy exaggerated the Th2 immunity in sensitised women with allergic symptoms and non-sensitised women without allergic symptoms (Fig 18A). This pattern was predominantly observed in the allergic group. The sensitised women with allergic symptoms had increased total IgE levels and birch- and cat-induced IL-5, IL-13 and CCL17 responses during pregnancy as compared with postpartum. The non-sensitised women without allergic symptoms had enhanced cat-induced IL-5 and IL-13 responses during pregnancy, but similar IgE levels as compared with postpartum. The decreased allergen-upinduced IFN- γ responses during pregnancy in the non-sensitised women without allergic symptoms also support the idea of a normal pregnancy as a shift towards Th2-associated immunity. Furthermore, the gene expression profile in the placenta revealed an enhanced Th2-/anti-inflammatory environment as compared with gene expression peripherally in PBMC. However, our results were conflicting, possibly due to the choice of markers for Th1/Th2 immunity (cytokines/antibodies *versus* chemokines) and laboratory methodology (allergen stimulations of isolated PBMCs *versus* plasma/mRNA levels). The Th2-associated chemokines CCL17, CCL18 and CCL22 were, in fact, lower during pregnancy than after delivery in both groups, regardless of presence or absence of maternal allergy. Furthermore, our gene expressions analyses failed to reveal a pronounced Th2 deviation in the placenta or PBMCs of sensitised women with allergic symptoms. Many important Th2 markers were undetectable in the majority of placenta/PBMC samples, however.

In conclusion, maternal allergy was associated with a pronounced Th2 deviation during pregnancy, reflected as an up-regulation of their (already higher) IgE and allergen-induced IL-5 and IL-13 levels during pregnancy, possibly exposing their fetuses to a particular strong Th2 environment during gestation.

Figure 18A. Th2-like immune responses are magnified during pregnancy in both groups, with more pronounced Th2 deviation in the allergic group. **(B)** The maternal immunity during pregnancy influenced the offspring's immune system at birth and during childhood. **(C)** Allergy development was associated with an enhanced Th2 profile at birth and a consolidation of a Th2-skewed immunity during childhood.

Immunological interactions between mother and child during pregnancy

Maternal total IgE levels during and after pregnancy correlated with CB IgE and CCL22 levels (Table 6, Fig 18B). Circulating CXCL11, CCL18 and CCL22 levels during pregnancy and postpartum correlated with the corresponding chemokine levels in the offspring's at various time points during childhood. At the mRNA level, maternal IL-5 expression in PBMCs was associated with neonatal Galectin-1, and placental p35 was negatively associated with neonatal Tbx21 expression.

Taken together, our data indicate an influence of the maternal immunity during pregnancy on the shaping of the offspring's immune profile at birth, but also later during childhood.

Th1- and Th2-like immunity at birth and during childhood in allergic and non-allergic children

Increased mRNA expression of CCL22 was observed in CBMC in children later developing allergic symptoms and sensitisation as compared with children who did not (Fig 18C). High CB levels of CCL17 and CCL22 were also associated with development of allergic symptoms and sensitisation during the first 6 years of life. A consolidation of an enhanced Th2-like immunity during childhood, showed as increased circulating CCL17, CCL18 and CCL22 levels, was observed in the children developing allergic symptoms and sensitisation. Our data indicate that the Th2 deviation preceding established allergy takes place very early in life. The consolidation of a high Th2-like immunity during childhood in children developing allergy, indicate a delayed down-regulation of the neonatal Th2-like immunity.

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