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Th1, Th2 and Treg associated factors in relation to allergy

Camilla Janefjord

Division of Paediatrics, Unit of Clinical and Experimental Research, Department of
Molecular and Clinical Medicine, Faculty of Health Sciences, Linköpings universitet,
SE-581 85 Linköping, Sweden



Linköpings universitet
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To my family

Abstract

Background: Immune responses are often divided into T helper 1 (Th1), Th2 and Treg like immunity. Allergy is associated with Th2 like responses to allergens and possibly to reduced regulatory functions. Activation via the CD2 receptor increases the production of the Th1 associated cytokine IFN- γ and enhances the responses of activated T cells to IL-12. This may be due to an up-regulation of the signal-transducing β_2 -chain of the IL-12 receptor. CD2 function may be impaired in allergic children. As IL-12 is a strong promotor of Th1 like responses, this may be one contributing factor to the Th2-skewed immune responses found in allergic children. IL-27 and its receptor component WSX-1 may also play a role in Th1 like responses. The transcription factors T-bet, GATA-3 and Foxp3 are associated with Th1, Th2 and Treg type of immune responses, respectively.

Aim: To investigate possible mechanisms behind the reduced Th1 and/or Treg associated immunity in relation to allergy by studying the CD2 induced regulation of IL-12R β_2 , WSX-1, T-bet, GATA-3 and Foxp3, as well as the production of different cytokines in children and adults. The aim was also to study the development of these factors during the first two years of life in relation to development of allergy in children from a country with high (Sweden) and low (Estonia) prevalence of allergy.

Material and methods: Four different study groups were included; 32 12-year-old children, 38 7-year-old children, 61 children followed from birth to two years of age and 20 adults. Peripheral blood mononuclear cells were cultured with PHA (which partly signals via CD2), IL-2 and IL-12 alone and in combination or with anti-CD2 alone or combined with anti-CD28 antibodies. mRNA expression of cytokine receptors and transcription factors was analysed with real-time PCR and production of Th1, Th2 and Treg associated cytokines with ELISA.

Results: We found lower PHA-induced IL-12R β_2 and IFN- γ production in 12-year-old children with positive skin prick tests (SPT), compared with SPT negative children. We also found lower IL-2 induced IL-12R β_2 in children with allergic airway symptoms and high IgE levels compared to children without a history of allergy and low IgE levels. This was accompanied with lower IL-2 and IL-12 induced IFN- γ . The spontaneous mRNA expression of IL-12R β_2 , WSX-1, T-bet, GATA-3 and Foxp3 was similar at birth and at 24 months. PHA induced up-regulation of all markers at all ages except for GATA-3, which was up-regulated in allergic children only at 6 and 12 months. PHA-induced T-bet and WSX-1 increased from birth to 24 months in non-allergic children. At a specific age, similar levels of all markers were found in allergic and non-allergic children, except for higher spontaneous IL-12R β_2 at 24 months and higher PHA-induced WSX-1 at birth in allergic children. All cytokines increased with age. No clear differences were found between Swedish and Estonian children. CD2 stimulation induced Foxp3 and IL-10, while CD2 together with CD28 stimulation induced both Th1 and Th2 related transcription factors and cytokines. The combination also hampered the CD2 induced expression of Foxp3.

Conclusions: The CD2 pathway and the response to IL-2 may be impaired in allergic children as lower IL-12R β_2 and IFN- γ were found in allergic, compared to non-allergic children. This difference was not found in adults. CD2 may be involved in induction of regulatory T cell responses as stimulation via CD2 in the absence of other co-stimulatory molecules induced Foxp3 and IL-10. Different developmental patterns of Th1 and Th2 associated factors may influence the development of allergic diseases in childhood.

Sammanfattning

Bakgrund: Olika ämnen framkallar olika typer av immunsvår, vilka brukar delas in i T-hjälpar 1 (Th1)-, Th2- och T-regulatoriskt (Treg) svar. Allergier är associerade med en övervikt av Th2-lik svar. Vilken typ av immunsvår som ska dominera styrs bl a av olika receptorer på cellernas yta samt olika signalämnen, s k cytokiner som immuncellerna producerar. CD2-receptoraktivering av T-celler kan vara involverad i induktionen av Th1-lik svar, genom att stimulering via denna receptor ökar T-cellernas förmåga att svara på den Th1-inducerande cytokinen IL-12. Detta kan bero på att en uppreglering av den signalöverförande β_2 -kedjan av IL-12 receptorn sker. CD2-vägens funktion kan vara minskad hos allergiska barn, vilket skulle kunna var en möjlig bakomliggande faktor till de allergiska barnens Th2-övervikt.

Mål: Att studera olika faktorer som kan ligga bakom det minskade Th1-lik svaret hos allergiker genom att studera CD2-receptor-stimulerat uttryck av IL-12 receptorns β_2 -kedja (IL-12R β_2), IL-27-receptorenheten WSX-1, transkriptionsfaktorerna T-bet (Th1), GATA-3 (Th2) och Foxp3 (Treg) samt produktionen av olika cytokiner, i relation till allergi. Att följa utvecklingen av uttrycket av dessa faktorer hos en grupp barn från ett land med hög (Sverige) och låg (Estland) allergiprevalens, och relatera detta uttryck till allergiutveckling under de första 2 levnadsåren.

Material och metoder: Fyra olika studiegrupper ingår i avhandlingen; 32 st 12-åringar, 38 st 7-åringar, 61 st nyfödda följda till 2 år samt 20 st vuxna. Perifera mononukleära celler odlades med mitogenet PHA (signalerar delvis via CD2), cytokinerna IL-2 och IL-12 enbart eller i kombination, antikroppar mot CD2-receptorn enbart eller i kombination med antikroppar mot CD28-receptorn. mRNA nivåerna av cytokinreceptorerna och transkriptionsfaktorerna analyserades med realtids PCR och cytokinproduktionen med ELISA.

Resultat: Allergiska barn hade lägre PHA-inducerat uttryck av IL-12R β_2 och lägre produktion av IFN- γ , jämfört med friska barn. Barn med luftvägssymtom och höga IgE nivåer hade lägre IL-2-inducerat uttryck av IL-12R β_2 , associerat med lägre IFN- γ produktion efter IL-2/IL-12 stimulering jämfört med friska barn med låga IgE nivåer. Det spontana mRNA uttrycket av de olika faktorerna var lika vid födseln och vid 2 år. PHA uppreglerade alla faktorer vid alla åldrar utom GATA-3, som bara uppreglerades hos de allergiska barnen vid 6 och 12 månader. PHA-inducerat uttryck av T-bet och WSX-1 ökade hos de icke-allergiska barnen från födseln till 2 års ålder. Vid en specifik ålder uttryckte de allergiska och icke-allergiska barnen liknande nivåer av de olika markörerna, med undantag för högre IL-12R β_2 vid 2 år och högre WSX-1 vid födseln hos allergiska barn. Alla analyserade cytokiner ökade med åldern och inga klara skillnader hittades mellan de svenska och de estniska barnen. Stimulering via CD2 inducerade Foxp3-mRNA och IL-10-produktion. Aktivering via CD2, vid samtidig stimulering via CD28, resulterade i uppreglering av både Th1- och Th2-relaterade faktorer och cytokiner. Kombinationen CD2/CD28 hämmade det CD2-inducerade Foxp3-uttrycket.

Slutsatser: Det Th1-lik svaret efter stimulering via CD2-receptorn eller IL-2 kan vara sämre hos allergiska barn, då lägre nivåer av IL-12R β_2 och IFN- γ hittades hos allergiska, jämfört med icke-allergiska barn. Denna skillnad hittades inte mellan vuxna allergiker och friska. CD2-receptor vägen kan även vara inblandad i induktionen av regulatoriska T-cellsvar, eftersom CD2-stimulering utan andra ko-stimulatoriska signaler resulterade i en uppreglering av Foxp3 och av IL-10-produktionen. Olika utveckling av Th1- och Th2-associerade faktorer kan påverka allergiutveckling under barnåren.

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Abbreviations

APC	antigen presenting cell
BAL	bronchoalveolar lavage
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
EBI	Epstein-Barr virus induced
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
Fliz	fetal liver zinc finger protein 1
FOG	friend of GATA
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-12R β_2	IL-12 receptor beta 2 chain
ISAAC	International Study of Asthma and Allergies in Childhood
LFA	lymphocyte function-associated antigen
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
NOD mice	non-obese diabetic mice
NTC	no template control
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
ROG	repressor of GATA
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
Tc cell	T cytotoxic cell
TGF- β	transforming growth factor β
Th cell	T helper cell
TNF	tumour necrosis factor
Treg	regulatory T cell

List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. *Camilla K Janefjord* & Maria C Jenmalm. PHA-induced IL-12R β_2 mRNA expression in atopic and non-atopic children. *Clinical and Experimental Allergy* 2001; 13: 1493-1500.
- II. Helena Aniansson-Zdolsek, *Camilla K Janefjord*, Karin Fälth-Magnusson & Maria C Jenmalm. Reduced IL-2-induced IL-12 responsiveness in atopic children. *Pediatric Allergy and Immunology* 2003; 14: 351-357.
- III. *Camilla K Janefjord*, Malin Fagerås-Böttcher, Tiia Voor, Kaja Julge, Bengt Björkstén & Maria C Jenmalm. Development of Th1, Th2 and Treg associated immunity during the first two years of life in relation to allergy. Manuscript.
- IV. *Camilla K Janefjord*, Magnus Grenegård, Karin Fälth-Magnusson, Maria C Jenmalm & Malin Fagerås-Böttcher. CD2 controlled expression of regulatory T cell associated Foxp3 and IL-10 in humans. Manuscript.

Introduction

History

The word immune originates from the Latin word *immunis*, which means exempt or safe. Immunology is the science of the immune system, including its structure and mechanisms used to induce protection against different infectious agents. The origin of immunology is often ascribed Edward Jenner, who in 1796 discovered that cowpox could induce protection from human smallpox. Robert Koch discovered that infectious diseases are caused by microorganisms in the late 19th century.

Basic immunology

The main task of the immune system is to recognise self and non-self and to eliminate any foreign invader, with minimal pathological consequences for the host. Many different mechanisms have evolved to deal with the vast range of intruders, *e g* viruses, bacteria, fungi, parasites and tumours. The immune responses are often divided into innate immunity, mediated by granulocytes, macrophages, dendritic cells and natural killer cells (NK cells), and adaptive immunity mediated by lymphocytes (Figure 1) [1, 2].

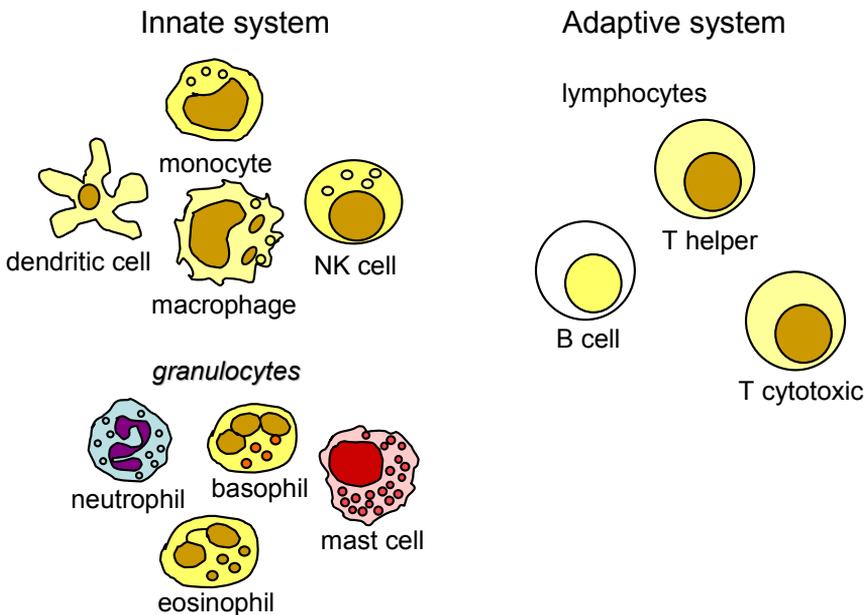


Figure 1. Leukocytes of different kinds constitute the cells of the immune system.

Innate immunity provides a first line of defence, including physical barriers and cells responding immediately with phagocytosis of microorganisms, extinction of infected cells and cooperation with adaptive immunity [3]. The components of innate immunity are present prior to exposure and do not differ between different infectious agents, but rather recognise common structures of the pathogens. Innate immunity is especially important early in infections since there is a delay of full function of adaptive immunity of 4-7 days.

Following antigen exposure, the adaptive immunity provides highly specific and long-lived responses against the specific immune trigger. Repeated encounters with the same antigen will result in a faster and even more effective adaptive response. The main mechanisms used by adaptive immunity are cell-to-cell contact (cell-mediated immunity), *e g* cytotoxicity, and production of soluble factors (humoral immunity), *e g* antibodies. The cells involved are B lymphocytes (B cells), which upon activation differentiate into antibody-producing plasma cells, and T lymphocytes (T cells), which can be further divided into the two main groups T helper (Th) and T cytotoxic (Tc) cells. The cytotoxic T cells are involved in the defence against intracellular pathogens due to their ability of killing virus-infected cells. The T helper cells are important in directing the immune responses and activation of other immune cells. The innate and adaptive responses should not be considered as two separate systems, but as closely integrated in all immune reactions (reviewed in [4]).

Initiation of immune responses

When a foreign pathogen enters the body, it is engulfed by professional antigen presenting cells (APC), *e g* macrophages and dendritic cells, which degrade the pathogen and present the resulting peptides to T cells. During antigen presentation, the peptide (antigen) is bound to major histocompatibility complex (MHC) molecules on the cell surface of the APC. MHC molecules are of two classes; class I generally presents intracellular antigens and class II extracellular antigens. When the APC meets a T cell equipped with a T cell receptor able to bind the antigen-MHC complex, that T cell receives a first signal of activation. A second signal is provided by co-stimulatory and adhesion molecules such as cluster of differentiation (CD) 58 (lymphocyte function-associated antigen 3, LFA-3), intercellular adhesion molecule 1 (ICAM-1) and CD80/CD86 on APC and their corresponding ligands CD2, LFA-1 and CD28 on the T cell. Both the first and the second signal are required for activation of the T cells (Figure 2).

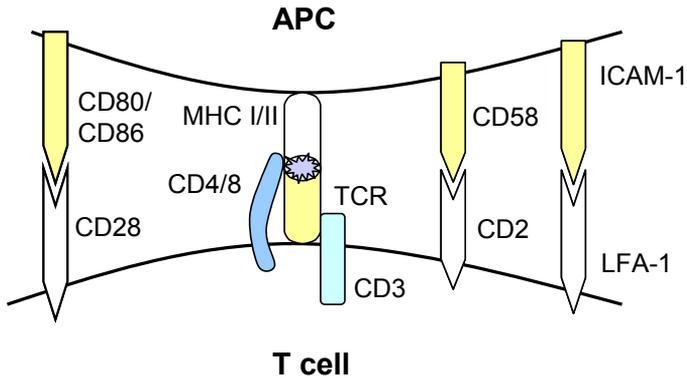


Figure 2. Antigen presentation. The antigen presenting cell presents the antigen bound to MHC molecules. The antigen-MHC complex is recognised by the T cell receptor and CD4/8. Adhesion and co-stimulatory molecules also signal to the T cell.

The cells of the immune system do not only communicate by physical interactions but also via soluble proteins, *i.e.* cytokines. The cytokines act via specific cytokine receptors and may have both stimulatory and inhibiting properties, for summary see Table 1. Activated T helper cells are involved in almost all activities of the adaptive response, and they also influence the innate response (Figure 3).

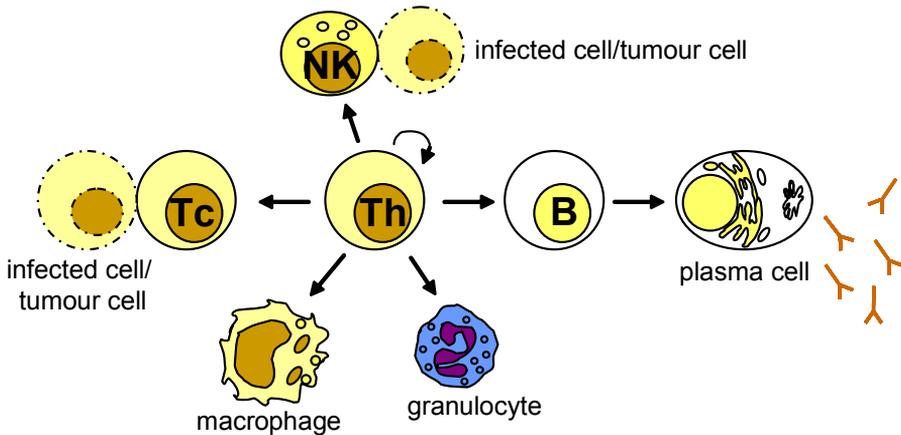


Figure 3. T helper cells may influence a vast variety of cells in the immune system.

Table 1. Summary of cytokines referred to in this thesis, modified from Janeway *et al* [1]. IL=interleukin, IFN=interferon, TGF=transforming growth factor, TNF=tumour necrosis factor, Th=T helper, Treg= regulatory T cells and NK=natural killer.

Cytokine	Produced by	Effects	Associated with
IFN- γ	T cells, NK cells	Activation of macrophages, increased expression of MHC and antigen processing components, Ig class switching, suppresses Th2	Th1
IL-2	T cells	T cell proliferation	Proliferation
IL-4	T cells, mast cells	B cell activation, IgE switch, promotes Th2 differentiation	Th2
IL-5	T cells, mast cells	Eosinophil growth, differentiation	Th2
IL-9	T cells	Mast cell enhancing activity, stimulates Th2	Th2
IL-10	T cells, macrophages	Suppression of macrophage function	Treg, anti- inflammation
IL-12	macrophages, dendritic cells	Activation of NK cells, induces differentiation to Th1	Th1
IL-13	T cells	B cell growth and differentiation, inhibits macrophage inflammatory cytokine production and Th1 cells, IgE switch, induces allergy/asthma	Th2
IL-15	Many non T cells	IL-2 like	Th1
IL-18	Activated macrophages	Induces IFN- γ by T and NK cells, favours Th1 and later Th2 response	Th1
IL-21	Activated Th cells	Induces proliferation of B, T and NK cells	Th1
IL-27	Monocytes, macrophages, dendritic cells	Induces IL-12 receptor via T-bet	Th1
TGF- β	T, monocytes, chondrocytes	Inhibition of cell growth, induces switch to IgA	Treg, anti- inflammation
TNF- α	Macrophages, NK and T cells	Local inflammation, endothelial activation	Pro- inflammation
Lymphotoxin (TNF- β)	T cells, B cells	Killing, endothelial activation	Th1

Tolerance and down-regulation of responses

Immunological tolerance has evolved to allow discrimination between self and non-self. In the thymus, potentially self-reactive T cells are eliminated in a process called negative selection. Cells escaping this central tolerance are ignored, deleted or rendered anergic in the periphery (peripheral tolerance) [5].

The immune responses must be down-regulated after successful clearance, to avoid harmful damage by the immune cells. One such function is the antigen-dependence of effector function. This means that lymphocytes, in the absence of antigen, will lose their effector functions within a few days, and either die or become relatively inactive as memory cells [6]. Three major pathways for active termination of immune responses have been described; cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) mediated T cell inhibition, Fas-mediated activation-induced cell death and interleukin (IL)-2-mediated feedback regulation [5].

In addition, several subsets of T cells exhibiting regulatory properties have been described and proposed to have a role in the maintenance of self-tolerance and in the suppression of immune responses [7, 8].

T cells

T lymphocytes mature in the thymus, where they develop to recognise self MHC molecules presenting foreign antigens. All T cells express CD3 and a unique T cell receptor. T helper cells also express CD4 while cytotoxic T cells express CD8, both of which are involved in antigen recognition by the T cell (Figure 2). Thus, CD4 binds the MHC class II molecules restricting Th cells to recognise peptides presented by MHC class II, *i e* of extracellular origin such as fragments of engulfed micro-organisms. CD8 on cytotoxic T cells binds MHC class I, presenting peptides of intracellular origin resulting in killing only those cells infected with intracellular pathogens or tumour cells. MHC class II is present on APC such as macrophages, dendritic cells and B cells, while MHC class I is expressed by all nucleated cells [1, 2].

Cytotoxic T cells

The Tc cells accomplish their killing process mainly by the release of perforin and granzyme, two preformed cytotoxic proteins. Perforin is a pore-forming protein which induces pores in the cell membrane, through which granzyme can enter and induce apoptosis (programmed cell death). The membrane bound Fas ligand, expressed on CD8 positive cells, is also able to induce apoptosis by binding to Fas on the target cell.

All cells are susceptible to lysis by Tc cells, but only infected cells and tumour cells, which present foreign antigens on their MHC-molecules, are killed due to the specific recognition of those cells by the TCR [1, 2].

Helper T cells

Upon activation, naïve T helper cells can differentiate along the Th1 or the Th2 pathway, which differ in cytokine pattern and thus in function [9]. Lately, another T cell development has also been emphasised, namely the regulatory pathway described below. The Th1/Th2 concept was originally described in mice [10], where Th1 cells were shown to produce interferon (IFN)- γ , tumour necrosis factor (TNF)- α and IL-2 and Th2 cells produced IL-4, IL-5, IL-6, IL-9 and IL-13 (reviewed in [9]). In humans, Th1 cells produce IFN- γ and lymphotoxin, while Th2 cells secrete IL-4, IL-5 and IL-9 [11]. Although not as distinctly applicable in humans as in mice, the Th1/Th2 concept has been a working model for allergy research [12]. Due to the unclear distinction between Th1 and Th2 responses in humans, these are often referred to as Th1/Th2 like responses/immunity.

Several factors influence the development of the T helper cells towards Th1 or Th2 type. These factors include type of antigen presenting cell, intensity and nature of TCR and co-stimulatory signals, the cytokines present and the genetic background of the naïve T cell [13]. The cytokines present in the environment, especially IL-12 and IL-4, are considered to be the most important factor, however [14-16]. The cytokines promoting one response are also able to down-regulate the other. Thus, IL-12 promotes Th1 like responses and inhibits Th2 like responses [17], and IL-4 promotes Th2 like responses and inhibits Th1 like responses [18]. During recent years the signalling pathways, *i e* IL-12/signal transducer and activator of transcription (STAT) 4 and IL-4/STAT6 as well as the role of Th1 and Th2 associated transcription factors, especially T-bet and GATA-3, have been emphasised in the Th1/Th2 commitment process (for review see [19]).

Th1 and Th2 like immunity have been associated with different functions, largely depending on their cytokine profile. Thus, Th1 like responses are associated with production of IFN- γ and cell-mediated inflammatory reactions by activation of macrophages and cytotoxic cells for eradication of intracellular pathogens. This also generates delayed type hypersensitivity reactions [9, 11]. Th1 like cells may also contribute to humoral immunity by inducing production of strongly complement activating and opsonising antibodies, which synergise in macrophage activation [11]. Clinically, Th1 type responses are found in chronic inflammation and certain

autoimmune diseases.

Th2 like responses are associated with high production of IL-4, IL-5, IL-9 and IL-13 and are involved in humoral immunity, which is important in the battle against extracellular pathogens and helminths. Thus, Th2 like responses are involved in the activation of B cells, mast cells and eosinophils. Naïve B cells receive a first stimulatory signal when encountering an antigen able to bind the specific B cell receptor. To become fully activated, the B cell requires T cell help. This is provided by the T cell via specific recognition of the MHC-peptide on the surface of the B cell, by CD40-CD40L interaction, as well as other co-stimulatory interactions and by production of cytokines [1]. Activation of the B cell results in proliferation and isotype switch and affinity maturation of the antibody being produced. The cytokine environment seems to influence the isotype switch, for example class switching to immunoglobulin (Ig) E occurs in the presence of IL-4 [20]. Due to production of IL-5, Th2 like responses are also involved in eosinophilia [21]. Thus, Th2 like responses are involved in allergic reactions.

Regulatory T cells

Several subsets of T cells exhibiting regulatory properties have been described including Tr1 cells, Th3 cells and naturally occurring CD4⁺CD25⁺ regulatory T cells (Treg) [22]. Tr1 and Th3 cells are induced in the periphery in an antigen-dependent manner and mediate their immuno-suppressive effects mainly via IL-10 and TGF- β dependent mechanisms (reviewed in [22-24]).

In contrast, the naturally occurring CD4⁺CD25⁺ regulatory T cells are derived from the thymus [25], and mediate suppressive effects through ligation of the T cell receptor and cell-cell contact [26]. This cell subset, which constitutes 5-15% of peripheral CD4 T cells, was first identified in mice in 1995 by Sakaguchi *et al* [26], but later also in humans [27]. The key factor in controlling CD4⁺CD25⁺ Treg development and function seems to be the transcription factor Foxp3 [28-30]. Treg cells constitutively express high levels of CD25 (IL-2 receptor α -subunit), CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor (GITR), all of which are also up-regulated upon stimulation of effector CD4⁺ T cells [8, 31]. Peripheral CD4⁺CD25⁺ Tregs may also be generated through up-regulation of Foxp3 [32].

Antigen-specific or polyclonal TCR stimulation activates CD25 positive Treg cells and induces suppressive functions *in vitro*, but Treg cells do not proliferate or produce cytokines in response to conventional T cell stimuli (*e g* anti-CD3 and ConA)

[8, 31]. In addition, IL-2 produced by activated T cells seems essential for Treg activation and survival [8, 31]. Once activated, Treg cells inhibit IL-2 production of responder cells in an antigen non-specific manner [8]. Thus, IL-2 produced by responder T cells maintains and activates Treg, which in turn inhibit IL-2 production of responder T cells, with immuno-suppression as a consequence. The mechanisms used by Treg to induce suppression of effector cells are not entirely known, but it has been hypothesised that expression of CTLA-4 or membrane-bound TGF- β is involved. *In vivo*, several mechanisms are probably used, and cell-contact dependent suppression has been suggested to work together with the cytokines IL-10 and TGF- β [8].

The importance of functional Treg cells has been stressed in several immunological disorders including autoimmunity, chronic infections and allergy [8]. Mutations in the key Treg marker Foxp3 has been shown to cause the severe syndrome immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), characterised by multiple organ autoimmunity as well as allergic manifestations [33, 34].

Allergen specific T cells exist not only in allergic individuals but also in non-allergic individuals, thus active suppression by Treg cells may be important in inducing and maintaining peripheral tolerance to allergens [35]. Treg cells have been proposed to be involved in the suppression of allergen-specific responses in several ways, *e g* suppression of APC, Th1 and Th2 effector cells, regulation of B cells resulting in reduced IgE and increased IgG4 and IgA, suppression of mast cells, basophils and eosinophils and involvement in airway remodelling [35].

General aspects of allergy

“Allergy is a hypersensitivity reaction initiated by immunologic mechanisms” as defined by the EAACI nomenclature task force [36]. It is the outcome when the immune system reacts towards harmless antigens (called allergens) that normally are tolerated. Typical allergic symptoms are asthma, rhinitis, conjunctivitis, eczema and gastrointestinal reactions. These symptoms typically vary with age, often referred to as the atopic march [37, 38]. Eczema and gastrointestinal problems often caused by food allergens dominate in the first years of life, whereas asthma and rhino-conjunctivitis to inhalant allergens, such as birch pollen and pet allergens often debut in pre-school ages. Accordingly, children with atopic eczema early in life are more prone to develop asthma and/or rhinitis than children in the general population [37, 39].

Atopy is personal or familiar tendency to produce IgE antibodies and to

develop typical symptoms such as asthma, rhino-conjunctivitis or eczema/dermatitis [36]. Thus, the term atopic should only be used when the presence of IgE antibodies has been verified.

During the last decades, the prevalence of allergic diseases has increased dramatically in the industrialised high-income countries [40, 41]. Although more recent studies report stabilising or even decreasing rates [42-44], allergic diseases are common. The prevalence of asthma, allergic rhino-conjunctivitis and atopic eczema is 10-15% in Swedish 13-14 year olds according to the International Study of Asthma and Allergies in Childhood (ISAAC) [45]. Up to one third of all children have or used to have some allergic symptoms during childhood. However, the prevalence of allergic diseases in the former socialist countries in Europe, with a life style similar to that prevailing in the Western Europe 30-40 years ago, is still low [45].

Both genetic and environmental factors have been suggested to influence the development of allergic diseases. Indeed, allergic diseases in parents and siblings are strongly associated with development of allergic disease in the child [46]. However, the increased prevalence found in the Western world during the last 30-40 years can not be explained by genetic factors, since the time elapsed is too short for the human genotype to change appreciably. Instead, the increased prevalence is often ascribed different environmental factors usually related to a change in lifestyle leading to less exposure to microbes [47]. Exposure to allergens and tobacco smoke, day care, farming, animals (domestic and pets), endotoxin (microbial pressure), childhood infections, vaccinations and use of antibiotics are some factors that have been suggested to influence the development of allergic diseases [48, 49]. During the last years, the focus has shifted from finding risk factors triggering allergic diseases to finding health promoting factors that are lacking, thereby rendering the immune system prone to develop allergic diseases.

Mechanisms of allergy

The classical allergic reaction involves the production of IgE antibodies to allergens. Both allergic and non-allergic individuals produce allergen specific antibodies but the dominating isotype differ. Thus non-allergic individuals produce mainly antibodies of the IgG isotype whereas the allergic individuals also produce antibodies of the IgE isotype [50].

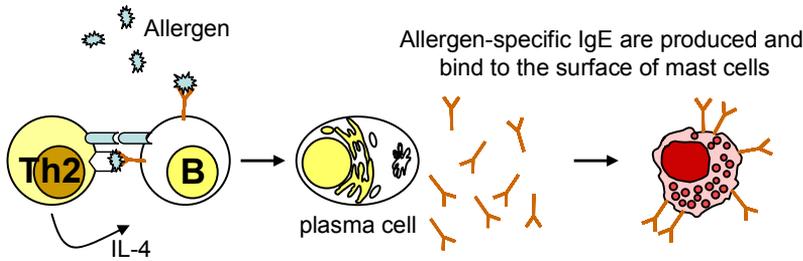
Upon the first exposure to an allergen, specific IgE antibodies are produced by plasma cells with the help from Th2 like cells, a process known as sensitisation

(Figure 4). The majority of the IgE antibodies produced are found on the surface of mast cells, where they are bound to high affinity FcεRI receptors. Basophils and activated eosinophils also express FcεRI receptors and bind IgE [51].

First encounter with allergen

Allergen activation of B cells

Sensitisation



Re-exposure to allergen

Early reaction

Late phase reaction

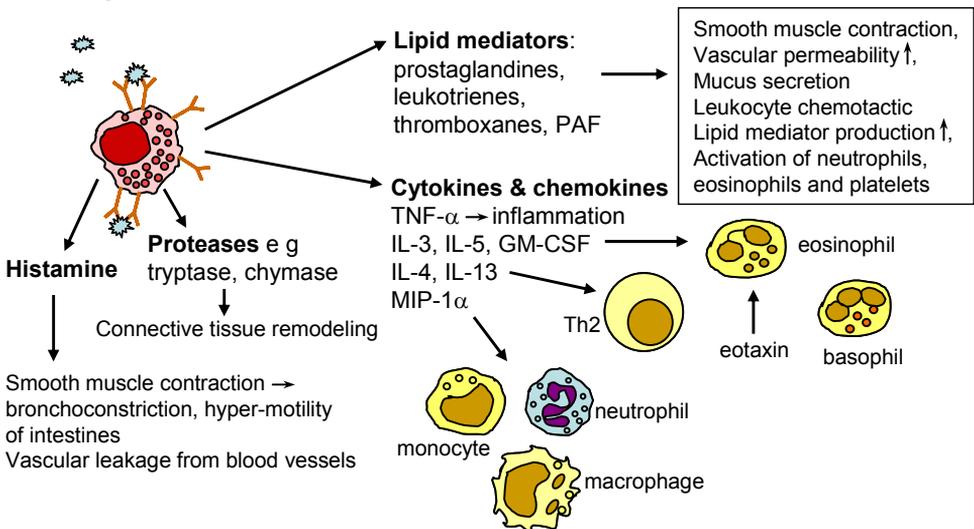


Figure 4. Schematic overview of allergic sensitisation and inflammatory responses. Upon the first exposure to allergen, specific IgE antibodies are produced and bind to receptors on the cell surface of mast cells, *i e* sensitisation. The next time allergen encounter occurs, the allergen can bind and cross-link the surface bound IgE antibodies. This results in activation of the mast cells with release and production of inflammatory mediators as a consequence. These mediators cause a rapid reaction due to release of histamine and preformed proteases and also contribute to a later response with secretion of newly synthesised mediators. This late response causes a more sustained inflammation with recruitment and activation of a wide range of cells.

After re-exposure to the same allergen, the allergen can bind and cross-link the specific IgE antibodies on the surface of the mast cells, resulting in release of preformed inflammatory mediators such as histamine, tryptase and chymase [52, 53]. This takes place within minutes after allergen exposure. Different responses such as broncho-constriction, vascular leakage from blood vessels and hyper-motility of the intestines are then triggered [51]. Activated mast cells can also rapidly synthesise and release prostaglandins, leukotrienes, thromboxanes and platelet-activating factor as well as several growth factors, chemokines and cytokines (TNF- α , IL-4, IL-5, IL-9, IL-13 etc) [51-53]. These mediators act to induce a more sustained inflammation (late phase reaction), which include recruitment of T cells, particularly Th2 cells, eosinophils and basophils, and may lead to chronic inflammation in tissues often exposed to allergens [52] (Figure 4).

Allergic diseases are associated with Th2 like immunity to allergens in affected tissues [54, 55]. The Th2 associated cytokine IL-4 promotes B cell isotype switch to IgE, recruits basophils, eosinophils and monocytes and is involved in up-regulation of Fc ϵ RI. IL-5 promotes eosinophil cell survival and activation [21]. IL-9 and IL-13 are also involved in the allergic response promoting mucus secretion, airway inflammation, airway hyperresponsiveness and tissue fibrosis [56, 57]. Whereas IL-9 primarily enhances the allergic inflammation induced by other Th2 cytokines, IL-13 may induce all pathological features of murine asthma even without traditional effector cells such as mast cells and eosinophils. IL-13 has also been proposed to be involved in the chronicity of allergic inflammation by regulation of several factors promoting its own production, keeping a Th2 positive feed-back loop [57].

Besides the pro-inflammatory actions of mast cells, basophils, eosinophils and Th2 cells, impaired suppression of the inflammatory responses mediated by Treg cells has recently been suggested to contribute to the allergic inflammation. The inhibitory properties of CD4⁺CD25⁺ cells may be reduced in allergic individuals or may be overcome by strong activation signals, both of which may contribute to unbalanced allergen activation of Treg and effector Th2 cells (reviewed in [58]). Contradictory results showing similar suppressive effects in allergic and non-allergic individuals of the CD4⁺CD25⁺ cells have also been reported, however [58]. There are several potential ways that Treg may inhibit the allergic responses, including suppression of effector cells involved in allergic inflammation and reduction of the production of IgE [35].

Development of the immune system

Infancy and early childhood are associated with increased susceptibility to infections partly due to an immature immune system. Indeed, the immune responses at birth and in early childhood are immature in several aspects, but the immune system should not be considered totally naïve [59].

Infants have higher proportion of naïve T cells and lower proportion of memory cells than adults [60], and this probably accounts for some of the impaired responses seen in infants. During the first three months of life, the naïve phenotype is found on about 90% of the T cells, but the percentage decreases steadily until reaching adult levels (about 50%) between 12 and 18 years of age [60]. In contrast, the memory phenotype is found on about 10% of the T cells during the first three months and increases thereafter to about 30% by the age of 12-18 years [60].

Several functions of the T cells are reduced in neonates, *e g* proliferation and cytokine production [59]. Both Th1 and Th2 associated cytokines have been shown to increase with age in response to different stimuli [61-64]. The lower cytokine production at younger ages probably accounts for a considerable part of the reduced responses seen in neonates, since cytokines are important in T helper cell function and thereby also involve other immune cells and their responses. For instance, impaired IFN- γ production contributes to lower NK cell cytotoxicity and lower IL-4 contributes to lower IgE production by B cells [59]. In addition, the expression of and responses to several surface molecules, *i e* CD3, CD2 and CD28 is also lower on T cells in children than adults [65-67].

In early life, the immune system is Th2-deviated, possibly due to the potential harm Th1 like responses may cause during pregnancy [68]. After priming in the presence of IL-12, neonatal, but not adult, CD45R0- CD4 T cells produce IL-4 in addition to IFN- γ [69]. Impaired Th1 induction and responsiveness have been reported for several, but not all infectious pathogens and vaccine antigens studied (reviewed in [70]). Both CD4 and CD8 T cells from cord blood produce lower IFN- γ levels in response to PMA/ionomycin than cells from adults, with the greatest reduction found in CD4 T cells [71]. This reduction may partly be explained by the hyper-methylated CpG and non-CpG sites found in the IFN- γ promoter of cord blood cells [71]. The production of IFN- γ increases from birth and reaches adult levels around five years of age [59, 63, 64]. However, although Th1 like responses are often impaired or partially skewed to Th2, the poor cytokine production by human neonatal T cells is not restricted to Th1 associated cytokines [70, 72].

The antigen presenting cells are central in induction of antigen specific responses, and consequently their function contributes to the overall effectiveness of the immune responses. Phagocytosis and antigen presentation are comparable in cord blood and adult APC, whereas chemotaxis and production of TNF- α and IL-12 are reduced [59, 70], indicating that neonatal APC seem to have a reduced Th1 inducing capacity. In response to LPS, cord dendritic cells fail to produce IL-12 and are less effective in stimulating CD4 positive T cells to produce IFN- γ than their adult counterparts [73]. The production of the strong Th1 inducer IL-12 does not reach adult levels until after the age of 12 years [74]. The IL-10/IL-12 ratio is high at birth, while the opposite is true after the age of 5 years [74]. However, similar levels of IL-12 production in neonates and adults have also been reported, when highly purified dendritic cells derived from CD14⁺ monocytes have been used [70, 72]. The lower production of the pro-inflammatory cytokine TNF- α , together with the lower IFN- γ production by neonate T cells, may diminish the overall acute inflammatory responses in neonates.

The antibody responses in neonates show delayed onset, reach lower peak levels, last for shorter time and are of lower average affinity than adult responses [72]. Neonate serum has low levels of IgM and even lower IgA and IgE, while high levels of maternally transferred IgG are found [59]. In response to antigens, the neonates produce mostly IgM of low affinity. Even though the B cells are immature they are capable of IgE switching if IL-4 is present. Thus, the low production of IgE is not only due to impaired B cell function but also to the low IL-4 levels produced by neonate T helper cells [59].

With age, the immune system matures in response to stimuli mainly from the microbial environment, including the microflora of the gut and different infectious agents. Since the Th1 responses are hampered during pregnancy, postnatal immune development includes up-regulation of Th1 like immunity.

Little is known about the cytokine receptors and transcription factors studied in this thesis (reviewed later), regarding the development and expression in infants and children. One study reported similar up-regulation of the IL-12 receptor β_2 -chain (IL-12R β_2) in naïve T cells from cord and adult blood after CD3/CD28 stimulation [75]. Whereas the adults also up-regulated IL-12R β_2 on memory cells, the levels were undetectable on cord memory cells, probably due to the small number of memory cells in cord blood [75]. Similar proportions of regulatory T cells (CD4⁺CD25⁺), as well as expression of the transcription factor Foxp3 have been observed in peripheral blood

from adults and cord blood from term newborns [76]. Preterm newborns showed a higher proportion of CD4⁺CD25⁺ T cells which declined with gestational age to the level of adults [76]. Lower spontaneous levels of the transcription factor T-bet has been found in cord compared to adult CD4 positive T cells [77]. One study investigated the regulation of T-bet and GATA-3 in response to *Varicella zoster virus* (VZV) and *Dermatophagoides pteronyssinus* (*Der p*) in cord and adult peripheral blood [78]. Adult peripheral blood mononuclear cells were shown to up-regulate more T-bet with later down-regulation of GATA-3 after addition of VZV, whereas lower T-bet and no GATA-3 down-regulation were observed in cord blood. *Der p* on the other hand, induced similar amounts of GATA-3 in both cord and adult cells, *Der p* also induced later up-regulation of T-bet in cord blood, which was higher than in adult blood [78].

Development of immunity in allergic children

The postnatal development of immune functions may be slower in allergic compared to non-allergic children [79]. Cells from children with atopic heredity need higher doses of anti-CD3 to induce maximum proliferation [67]. Children with atopic eczema have lower numbers of lymphocytes forming rosettes with sheep erythrocytes [80] and a lower proportion of cells expressing CD2, the sheep erythrocyte receptor [81]. This might be a primary defect since lower E-rosetting cell numbers also have been found in newborns with, as compared to without, atopic heredity [82]. The responsiveness to the mitogen phytohaemagglutinin (PHA), which signals partly via CD2, is also lower in allergic children [80] even before the first allergic symptoms [83]. A reduced function of the CD2 pathway may have important consequences in allergy development, since CD2 increases the IL-12 responsiveness and subsequent IFN- γ production by T cells [84, 85]. In accordance, the reduced neonatal IFN- γ production is particularly pronounced in atopic children. Mononuclear cells in cord blood from children with heredity for allergy have, in several studies, been shown to produce lower IFN- γ levels in response to mitogens, than cells from children without heredity (reviewed in [86]).

Surface receptors

The cell surface receptors CD2, CD28, IL-12R β_2 and WSX-1 (IL-27 receptor subunit) have been studied more closely in this thesis and information about them is therefore reviewed in the following section.

CD2

CD2 (sheep erythrocyte receptor), is a transmembrane glycoprotein belonging to the immunoglobulin superfamily. In humans, it is expressed on mature T cells, most NK cells and thymocytes [87]. Antigen presenting cells express the CD2-ligand known as CD58 (or LFA-3) in humans and CD48 in rodents (reviewed in [88]). The interaction between CD2 and CD58/CD48 is highly specific, although it occurs with low affinity and rapid kinetics [88]. Ligation of CD2 induces increased intracellular free calcium levels and mitogen-activated protein kinase (MAPK) and nuclear factor of activated T cells (NFAT) activation [89, 90]. Blocking of the calcium-calmodulin dependent phosphatase calcineurin inhibits the cytokine production induced by co-stimulation via CD2 [91].

The CD2 receptor has been described to provide an alternative pathway of T cell stimulation [92], as well as being an adhesion molecule [93] and a co-stimulator for T cells [87, 91, 94]. The dimension of the CD2-CD58 and the TCR-peptide-MHC complexes are similar (reviewed in [87]). Thus, CD2 may enhance antigen recognition by bringing the plasma membranes of the T cell and of the APC to an optimal distance for TCR-peptide-MHC interactions. Indeed, Bachmann *et al* confirmed that CD2 enhances the interaction of MHC and the T cell receptor, facilitating interactions at low antigen concentrations [89]. Supporting the co-stimulatory role of CD2, signalling via this receptor enhances CD3 induced proliferation as well as IL-2 and IFN- γ mRNA and protein expression in purified T cells from mice [94]. Human T cells stimulated with anti-CD3 antibodies have been shown to elevate the production of several cytokines, *i e* IFN- γ , IL-2, IL-4, IL-5 and IL-10 in response to stimulatory CD2 antibodies [95]. Another study on human T cells, using anti-CD3 antibodies and CD58 transfected cells (instead of anti-CD2 antibodies), reported enhanced production of TGF- β , IL-10, IFN- γ , IL-5 and TNF- α but not of IL-2, IL-4 or IL-13 [91].

CD2 signalling may be more important in induction of Th1, as compared to Th2 responses, since it interacts with the response to IL-12, *i e* stimulation via CD2 enhances the responsiveness of activated T cells to IL-12 [84, 85]. This may be caused by an up-regulation of the β_2 -chain of the IL-12 receptor, which is induced during development according to the Th1 pathway [96]. In addition, a tight interaction between MHC and the T cell receptor, partly mediated by CD2, favours Th1 responses [97]. In support of that, higher expression of CD2 is found in Th1 as compared to Th2 cells [98]. The monocyte-stimulated IFN- γ production by T cells is also CD2 dependent [99]. This may be due to activation of STAT1 protein following CD2 signalling [100, 101], which in turn may have an influence on the IFN- γ promoter.

A defect in the CD2 function may be present in allergic diseases, since atopic children have a lower proportion of CD2 positive lymphocytes [81, 102]. They also have a reduced responsiveness to PHA [80, 83], which activates the T cells via CD2 [103] and the T cell receptor [104]. This could be a primary defect, since this phenomenon has been seen already at 1 month of age in children with atopic heredity [82]. A reduced function of the CD2 pathway could have important consequences for the development of allergy, since a decreased IL-12 responsiveness would result in difficulties to induce Th1 type immune responses [105, 106].

A role of CD2 has also been implicated in the induction of T cell anergy [107, 108]. Co-stimulation via CD2 alone, *i e* in the absence of co-stimulation of *e g* CD28 and LFA-1, induced T cell anergy and differentiation of IL-10 producing CD4⁺CD25⁺ regulatory T cells able to suppress proliferation of bystander cells [108].

CD28

The CD28 receptor is one of the most studied and best known co-stimulatory molecules. Its co-stimulatory effects were originally discovered due to the enhancing effect on T cell proliferation monoclonal antibodies that bound to this protein exerted [109]. CD28, like CD2, is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. It is expressed on almost all CD4 T cells, about half of the CD8 T cells and on developing thymocytes (reviewed in [110]).

The CD28 ligands, CD80 (B7-1) and CD86 (B7-2) are expressed on antigen presenting cells including dendritic cells, Langerhans' cells, macrophages and B cells [110, 111]. CD86 is constitutively expressed at low levels and rapidly up-regulated upon activation, while CD80 is inducibly expressed later than CD86. A clear distinction in functions between CD80 and CD86 has not yet been described, although CD86 may be more important early in responses [111]. The same ligand pair, CD80/CD86, is also used by the inhibitory receptor CTLA-4 involved in inhibition of T cell responses and regulation of peripheral tolerance [111]. CTLA-4 is rapidly up-regulated upon T cell activation and has higher affinity for both CD80 and CD86 compared to CD28 [112].

Ligation of CD28 to either of its ligands provides potent co-stimulatory signals that augment and sustain T cell effector functions, especially in conjunction with T cell receptor stimulation. This includes promoting clonal expansion and differentiation, production of high levels of IL-2, prevention of anergy and induction of critical survival signals via the anti-apoptotic factor Bcl-xL pathway (reviewed in

[110, 111, 113]).

Co-stimulation via CD28 enhances the production of both Th1 and Th2 like cytokines and may thus influence the differentiation of naïve T helper cells to both Th1 and Th2 cells [97, 110]. However, CD28 may be more important in induction of Th2 than of Th1 responses, especially in the absence of IL-2. Several studies using blockade of CD28 or deficient CD28 expression, have revealed impaired Th2, but not Th1 like responses [97, 110]. In addition, in allergic inflammation in mice, CD28 mediated signals seem essential for induction of Th2 type cytokines and IgE, recruitment of eosinophils into the airways and for the establishment of airway hyper-responsiveness [114, 115].

Besides its role in co-stimulation of naïve conventional T cells, more recent studies have suggested that the CD28 pathway may also be involved in T cell tolerance and the function of Treg. CD28 deficient mice have lower percentages of CD4⁺CD25⁺ T cells [116, 117], and non-obese diabetic (NOD) mice lacking CD28 or CD80/CD86 develop more severe and accelerated diabetes than wild-type NOD mice [116]. Further, co-stimulation via CD28 is involved in Treg proliferation and CD25 expression and may indirectly regulate Treg survival, by promoting IL-2 production by conventional T cells [117]. In contrast, mice CD28^{+/+} T cells down-regulate Foxp3, whereas CD28^{-/-} T cells up-regulate Foxp3 in response to antigen [118].

IL-12 and the IL-12 receptor

The cytokine IL-12 is mainly produced by antigen presenting cells [119, 120] and is a strong promoter of Th1 like immune responses [15, 121, 122]. The biological effects of IL-12 includes induction of IFN- γ production in NK, T, B and antigen presenting cells, activation of macrophages, induction of T cell proliferation, enhancement of NK and T cell cytolytic activity and as mentioned previously regulation of Th1 like differentiation [123]. Thus, IL-12 is important in resistance to intracellular pathogens, but may also be involved in uncontrolled inflammation and autoimmunity. IL-12 was originally discovered as a soluble factor able to induce IFN- γ production, augment NK cell mediated cytotoxicity and enhance the mitogenic response in resting peripheral blood lymphocytes [124].

IL-12 is a heterodimeric cytokine composed of p35, which shows homology to other single-chain cytokines, and of p40, showing homology to the extracellular domain of members of the haematopoietic (four helix bundles) cytokine receptor family [124]. The p40 unit is expressed at higher levels and co-expression of both

subunits is necessary for secretion of biologically active IL-12 (p70) [125]. IL-12 and its receptor belong to the class I cytokine/cytokine receptor family, and are grouped in the same subfamily as *e.g.* IL-6, IL-23 and IL-27 (IL-6/IL-12 cytokine family and gp130 receptor family) (Figure 5) [126].

The cellular responses to IL-12 are mediated by the IL-12 receptor (Figure 5). It consists of β_1 and β_2 subunits, both of which are necessary for high affinity for IL-12 [127]. The β_2 -chain, in contrast to β_1 , contains tyrosine residues in the cytoplasmic domain and is considered to be the signal-transducing component [127, 128]. The IL-12 receptor is mainly expressed on activated T and NK cells [129]. Thus, mRNA expression of the IL-12 receptor subunits is neither detected in naïve CD4 T cells from mice [130] nor in human freshly isolated PBMC [131] or in naïve human T cell clones [96]. Although the β_1 -chain protein has been found on resting purified T cells from human peripheral blood, the signal transducing β_2 -chain remained undetectable [132]. Following T cell activation, both the IL-12 receptor chains are up-regulated. The expression of the β_2 -chain is more regulated than that of the β_1 -chain, and β_2 is only expressed when naïve T cells develop according to the Th1, but not the Th2, pathway [96, 130, 131]. Thus, Th1 cells respond to IL-12, whereas Th2 cells do not.

The expression and regulation of the IL-12R β_2 -chain has been investigated in a number of studies, using different cells and culture conditions, and varying results have been reported. However, the expression in human T cells has been reported to be enhanced by IL-12 itself [96, 131, 133], IFN- α [96, 131, 133, 134], IFN- γ [133], IL-15, IL-21 [135] and IL-27 [134] as well as by PHA [132] and simultaneous stimulation via CD3 and CD28 [75]. In the mouse, anti-CD3 [136, 137], IFN- γ [130], IL-12, IL-2 [137], IL-18 [138, 139], IL-12 combined with TNF- α (at low antigen dose) [136] and co-stimulation via CD28 [137, 140, 141] has been shown to enhance the IL-12R β_2 expression. In addition, the transcription factor T-bet can induce IL-12R β_2 -chain expression [142, 143]. The Th2 associated IL-4 can down-regulate the expression of the IL-12 receptor in both mice and human cells [96, 130, 144].

Signal transduction through the IL-12 receptor involves binding of Tyk2 (member of the Janus kinase (Jak) family) to the β_1 -chain and of Jak2 to the β_2 -chain. These receptor associated Jaks become phosphorylated and in turn phosphorylate tyrosine residues of the IL-12 receptor. These residues then serve as binding sites for signal transducers and activation of transcription, *i.e.* STATs (reviewed in [123]). One of the most important STATs in IL-12 signalling is STAT4, as demonstrated by the impaired IL-12 responses in STAT4 deficient mice [145, 146], and the rapid

phosphorylation of STAT4 following addition of IL-12 in human T cells [147]. Besides STAT4, IL-12 may also activate STAT1, STAT3 and STAT5 [123]. There are also other pathways than Jak/STAT involved in optimal IL-12 signalling, for instance activation of p38 MAPK (reviewed in [123]).

Altered expression of the IL-12R β_2 -chain has been associated with Th1 and Th2 related diseases. Thus, lower expression has been found in peripheral T cells from patients with allergic asthma. The levels did not reach those of the controls despite addition of IL-12 [144]. Lower expression has also been found in nasal biopsies from patients with allergic rhinitis [148]. In addition, mutations in the IL-12R β_2 gene have been associated with atopy [149]. In contrast, patients with Crohn's disease display elevated IL-12R β_2 expression, especially in inflamed areas [150, 151]. Compared to control subjects, elevated levels of both IL-12 receptor chains in the lung (BAL fluid) have also been reported in patients with active pulmonary tuberculosis or active sarcoidosis whereas the levels were lower in patients with asthma [152].

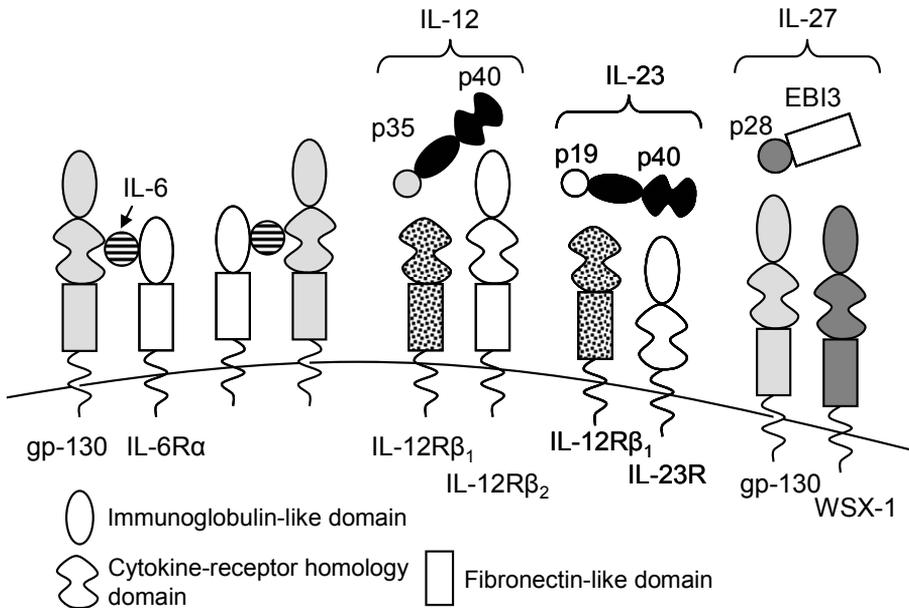


Figure 5. The IL-6/IL-12 cytokine family and their corresponding receptors belonging to the gp130 subfamily of the class I cytokine receptor family. IL-6 is a monomeric cytokine that forms a symmetrical complex with gp130 and the IL-6 receptor α -chain. IL-12 is a covalently linked heterodimer composed of p35 and p40 and the IL-12 receptor consists of IL-12R β_1 and β_2 , which both show homology to gp130. IL-12 p40 can also form IL-23 together with p19 and the corresponding receptor is a heterodimer between IL-12R β_1 and IL-23R. IL-27 is composed of p28 and EBI3 and binds a receptor formed by gp130 and WSX-1.

WSX-1

WSX-1 or T cell cytokine receptor (TCCR) is a novel member of the class I cytokine receptor family, showing structural similarities to members of the gp130 subfamily, to which the IL-6 and IL-12 receptors also belong (Figure 5) [153, 154]. High levels of WSX-1 are expressed in thymus, spleen, lymph nodes and on T and NK cells [153, 154]. Together with gp130, WSX-1 forms a signal-transducing receptor for IL-27 [155]. IL-27 is a newly identified heterodimeric cytokine composed of Epstein-Barr virus induced gene 3 (EBI3) and p28, both of which are related to the subunits of IL-12; EBI3 to p40 and p28 to p35 [156].

Signalling via WSX-1 activates STAT1 [134, 157] and STAT3 [134] and promotes the expression of the Th1 associated transcription factor T-bet, which in turn induces IL-12R β_2 expression [134, 157]. These findings indicate that WSX-1 acts before IL-12 in promoting IL-12 responsiveness during Th1 commitment.

WSX-1 was first reported to be important in mounting strong Th1 like responses early in infections, as WSX-1 deficient mice were shown to have impaired IFN- γ production associated with increased susceptibility to the intracellular pathogens *Listeria monocytogenes* [154] and *Leishmania major* [158]. Later it was proposed that the role of WSX-1 in *L major* infection mainly is to suppress the early IL-4 responses and thereby enable protective Th1 like responses [159].

Recently, several studies have indicated WSX-1 as an important negative regulator of inflammatory T cell responses (reviewed in [160]). WSX-1 deficient mice infected with *Toxoplasma gondii* produce normal levels of IFN- γ early in infection, but later they suffer from lethal CD4 T cell dependent inflammatory disease due to failure in down-regulating the response [161]. WSX-1 deficient mice also develop exaggerated T cell responses as well as increased production of inflammatory cytokines during infection with *Trypanosoma cruzi* [162]. Similarly, WSX-1 deficient mice were resistant to *L donovani* with rapid control of parasite growth in the organs. However, their wild-type littermates showed less severe immune-mediated liver damage during the eradication of parasites [163].

Supporting the role of WSX-1 as a suppressor of responses, it was recently demonstrated that the highest expression of WSX-1 was found on effector and memory T cells, while naïve cells expressed lower levels [164]. Infection with *T gondii* was also demonstrated to enhance the WSX-1 expression on CD4 and CD8 positive T cells during the first proliferative cycles. However, the expression on NK

cells was reduced, showing both positive and negative regulation of this receptor during activation [164].

In humans, IFN- α and IL-12 have been shown to down-regulate WSX-1 mRNA in primary NK cells [165]. The ligand IL-27 has been shown to be expressed in granulomas from patients with tuberculosis, sarcoidosis and Crohn's disease [166].

Little is known about WSX-1 in relation to allergic diseases and the only report so far used a murine model of asthma [167]. When challenged with OVA, WSX-1 deficient mice suffered from enhanced airway hyper-responsiveness and lung inflammation as well as increased production of several cytokines, including both IL-4 and IFN- γ . These results support an inhibitory role for WSX-1 not only in infections but also in allergic conditions, possibly by suppressive effects on Th2 cytokine production.

Taken together, WSX-1 and its ligand IL-27 are involved in the early induction of Th1 like responses, but they may have a more profound role in down-regulating immune responses protecting the host from immune mediated damage.

Transcription factors

In the following section, I review studies regarding the transcription factors T-bet, GATA-3 and Foxp3, which have been studied in this thesis.

T-bet

T-box expressed in T cells (T-bet, also known as TBX21) is a transcription factor expressed in Th1 but not Th2 cells, and it is required for Th1 lineage commitment [168, 169]. It belongs to the T-box family of transcription factors, containing a T-box DNA-binding domain of 189 amino acids [168].

T-bet is induced by stimulation via TCR and by IFN- γ , via the STAT1 signalling pathway [143, 170]. In addition, the recently described Th1 driving cytokines IL-15, IL-21 and IL-27 can induce T-bet expression [134, 135]. T-bet transactivates the IFN- γ gene resulting in production of IFN- γ . Accordingly, T-bet expression correlates with induction of IFN- γ and is detected in a variety of IFN- γ -producing cells, including T, B and NK cells [168]. T-bet expression is also induced by IFN- γ in antigen presenting cells [170].

T-bet can induce the expression of the IL-12R β_2 -chain [142, 143] and thereby

enable STAT4 activation by IL-12, which in turn induces IFN- γ and IL-18 receptor expression [139, 171]. Both IL-12 and IL-18 enhance IFN- γ [172], and can thereby amplify the effector mechanisms initiated by T-bet. Moreover, T-bet can redirect effector type 2 cells into type 1 cells, and reduce the IL-4 and IL-5 production in type 2 clones [168], possibly by a kinase-mediated interaction of T-bet with GATA-3 [173]. Interestingly, although T-bet is necessary for IFN- γ production by CD4 T cells and NK cells, it is not required for IFN- γ production by cytotoxic CD8 T cells [174]. Pearce *et al* showed that CD8 cells are also regulated by the transcription factor eomesodermin, which restores effector functions, *i e* IFN- γ production and expression of perforin and granzyme B, in T-bet deficient CD8 T cells [175].

In accordance with the role of T-bet in Th1 differentiation, CD4 T cells from T-bet deficient mice show impaired IFN- γ production, despite addition of IL-12, and they produce lower levels of Th1 associated IgG2a in response to protein antigen immunisation [174]. When infected with *L major*, they produce only small amounts of IFN- γ and they suffer from increased footpad swelling compared to their wild-type littermates [174]. Further, T-bet deficient mice spontaneously develop airway hyper-responsiveness and other features related to asthma [176].

Few reports are available on T-bet regulation in humans. Recently, Ylikoski *et al* demonstrated T-bet up-regulation in CD4 T cells after stimulation via CD3 and CD28, with further enhanced expression after addition of IL-12, IFN- α or IFN- γ [177]. Both cells cultured under Th1 and Th2 polarising conditions were shown to up-regulate T-bet within 24 h, although the levels were higher in Th1 cells. After 48 h, T-bet expression was stabilised in Th1, and decreased to undetectable levels in Th2 polarised cells [177]. Another group reported higher T-bet expression in human Th1, as compared to Th2 clones [178]. Consistent with that, higher T-bet levels have been reported in CD4 positive lamina propria mononuclear cells in the Th1 associated Crohn's disease [179]. The expression was similar in CD4 positive PBMC from Crohn's patients and controls, even after stimulation with anti-CD3, anti-CD28 and IL-12, however [179]. Lower T-bet expression has been reported in the airways of allergic asthma patients as compared to non-asthmatic controls [176]. In contrast, T-bet expression has been shown to be similar in patients with atopic eczema and healthy controls [180].

GATA-3

The T cell transcription factor GATA-3 was originally cloned in humans in 1991 [181-184], and is considered to be the key transcription factor co-ordinating Th2

polarisation [185]. GATA-3 contains two highly conserved zinc finger DNA binding domains, encoded by two separate exons [186]. GATA-3 expression is required both in embryonic and in T cell development [187, 188].

Naïve CD4 cells express GATA-3, which is down-regulated during Th1 commitment, but remains high or increases during Th2 differentiation, both in mice [189, 190] and in humans [191]. GATA-3 expression is enhanced by IL-4 via STAT6 and reduced by IFN- γ and IL-12 via STAT1 and STAT4 [192]. GATA-3 may also ensure Th2 commitment via a STAT6-independent auto-activation resulting in a positive feedback loop [193]. The expression of GATA-3 is also positively regulated by Mel-18, and negatively regulated by Fetal liver zinc finger protein 1 (Fliz-1), Friend of GATA 1 (FOG-1) and repressor of GATA (ROG) (reviewed in [19]). Fliz-1 represses GATA-3 at the transcriptional level, FOG-1 blocks GATA-3 activity and ROG interferes with GATA-3 DNA binding [19]. GATA-3 has also been suggested to be inhibited via interaction with T-bet [173].

The mechanisms used by GATA-3 to exert Th2 polarisation may be different for various cytokines. Hence, GATA-3 may induce optimal IL-5 production through transactivation of the IL-5 promoter [194, 195]. In contrast, it seems to have a moderate effect on the IL-4 promoter [194, 195], although it is involved in chromatin remodelling of the IL-4/IL-13 locus [196]. It has been suggested that the selective use of the GATA-3 N-finger in transactivation of IL-5, but not in chromatin remodelling of the IL-4/IL-13 locus, may cause the differential role of GATA-3 in IL-4, IL-13 and IL-5 induction [196]. To further support Th2 development, GATA-3 inhibits the production of IFN- γ [192, 197] and down-regulates the expression of IL-12R β_2 , even in the absence of IL-4 [192]. GATA-3 has also been suggested to down-regulate Th1 development through inhibition of STAT4, irrespectively of expression of IL-12R β_2 and T-bet [198].

In a murine model of asthma, T cell specific expression of a dominant negative GATA-3 reduced the allergic inflammation with decrease of eosinophilia, mucus production and IgE levels as well as lower production of Th2 associated cytokines [199]. Local intranasal administration of antisense DNA to GATA-3 has also been shown to reduce asthma symptoms in a murine model of asthma [200].

Human Th2 clones up-regulate GATA-3 mRNA expression upon stimulation, whereas Th1 clones do not [178]. Further, higher GATA-3 mRNA levels have been reported in atopic eczema [180], and the number of GATA-3 positive cells in sputum

[201] and in bronchial biopsies [202, 203] have been shown to be higher in asthmatic, as compared to healthy individuals. GATA-3 expression correlated positively with IL-5 expression and negatively with FEV₁ (forced expiratory volume in one second) [202]. In contrast, Caramori *et al* did not find any difference in GATA-3 protein expression in bronchial biopsies from asthmatic subjects as compared to controls, although they did report higher GATA-3 protein levels in T cells [204]. In contrast, a recent study reported no difference in GATA-3 mRNA expression in CD4 T cells from patients with asthma and controls [205].

Foxp3

Foxp3 belongs to a large family of transcription factors characterised by the winged helix/forkhead DNA-binding domain (Forkhead box (Fox)) [206].

The importance of Foxp3 was clearly demonstrated both in mice and humans when mutations in the Foxp3 gene were found to cause the severe immunological disorders seen in the scurfy mouse [207] and in humans with immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX) [33, 34]. Scurfy is a spontaneous, X-linked recessive mutation in the mouse Foxp3 gene, resulting in multiorgan autoimmune disease leading to death within 3-4 weeks of age [208]. The human counterpart, IPEX, is a rare recessive disorder characterised by multiple autoimmune manifestations usually resulting in death during infancy or childhood. Early onset of insulin-dependent diabetes mellitus, severe enteropathy, eczema, anaemia and massive lymphoproliferation are some clinical features found in IPEX [209].

In 2003, Foxp3 was shown to be predominantly expressed in Treg cells (CD4⁺CD25⁺), and to be both necessary and sufficient for their development and function [28-30]. This was supported by several findings in mice, *i e* Treg cells express Foxp3 whereas other T cells do not, expression of Foxp3 (mediated via retrovirus or transgenes) converted conventional T cells to Treg-like phenotype with suppressive properties, and lack of Foxp3 correlated with lack of Treg (for review see [210]). Similar to what has been found in mice, human CD4⁺CD25⁺ T cells exclusively express Foxp3 and the level of Foxp3 expression correlates with suppressive activity [32, 211]. Also similar to mice, retroviral Foxp3 gene transfer was shown to convert human CD4⁺CD25⁻ T cells to a regulatory phenotype with suppressive properties *in vitro* [211]. Besides expression in CD4⁺CD25⁺ T cells, Foxp3 has also been found in mouse [29] and human thymocytes [212].

Stimulation of human CD4⁺CD25⁻ T cells via anti-CD3/anti-CD28 induced Foxp3 expression, correlating to the expression of CD25 [32]. These cells exhibited suppressive properties shown to be cell-contact dependent and cytokine independent, similar to freshly isolated CD4⁺CD25⁺ Treg [32]. Thus, in humans, Treg cells expressing Foxp3 may be generated in the periphery. This was not reported by Yagi *et al* [211], although a recent study confirmed elevated Foxp3 expression after stimulation and also demonstrated that in humans not only CD4 but also CD8 cells up-regulate Foxp3 upon stimulation [213]. This is in contrast to what has been reported in mice, where Foxp3 was not induced upon stimulation of naïve T cells despite up-regulation of CD25 [28, 30], and suggests different regulation of Foxp3 and induction of Treg cells in mice and men. Studying the regulation of Foxp3 by different factors has been hampered by the tight relationship between expression of Foxp3 and survival and expansion of Treg cells. However, increasing evidence suggests that signalling via IL-2, CD28 and TGF- β may have an effect on the expression of Foxp3 (reviewed in [210]).

Even though it is quite clear that Foxp3 is involved in the regulatory functions of Treg cells, little is known about the molecular mechanisms behind this. A role for Foxp3 in transcriptional regulation of cytokines has been suggested. This is supported by the findings of consensus forkhead binding sites in the promoters of several cytokine genes, as well as the ability of Foxp3 to localise to the nucleus [214].

Little is known regarding Foxp3 in relation to allergy. In a murine model of asthma, increased numbers of Foxp3 expressing lung CD4⁺CD25⁺ T cells with suppressive activities were found upon blocking of the pro-inflammatory cytokine IL-6, proposing that IL-6 may be involved in the local presence of Treg cells. Low levels of Foxp3 mRNA and protein have been reported in human atopic dermatitis skin, despite high amounts of CD25⁺ cells [215]. Glucocorticoid treatment in asthmatic patients has been demonstrated to up-regulate Foxp3 mRNA expression and increase the number of regulatory T cells [216]. Thus, glucocorticoid treatment was proposed to promote regulatory cells, besides being immuno-suppressive and anti-inflammatory. The same study reported similar levels of Foxp3 expression in unstimulated CD4 T cells in healthy individuals and in patients with moderate asthma without corticosteroid treatment.

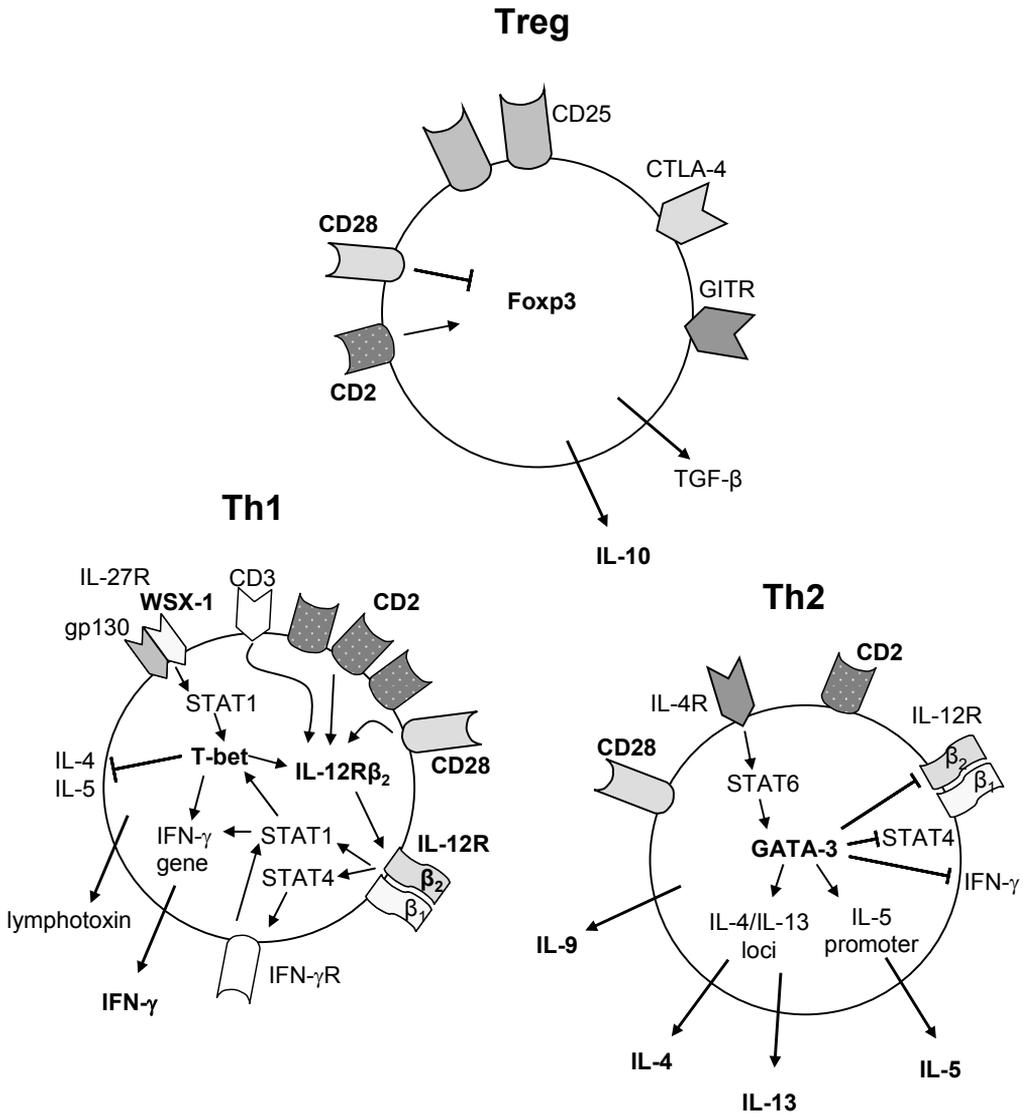


Figure 6. Summary of Th1, Th2 and Treg cells and some of their associated factors. Receptors, transcription factors and cytokines studied in this thesis are indicated in bold.

In conclusion, IL-12R β_2 , WSX-1 and T-bet are associated with Th1 like immunity, GATA-3 with Th2 like immunity and Foxp3 with Treg cells (Figure 6). Little is known of their role in allergic disease, however, particularly in relation to CD2 function and development of the immune system.

Aim of the thesis

The general aim of this thesis was to identify possible mechanisms behind the reduced Th1 like immunity and/or the reduced function of regulatory T cells in relation to allergy. The specific aims of the individual papers were:

- I. Study the mRNA expression of the IL-12R β_2 -chain in peripheral blood mononuclear cells (PBMC) stimulated via the CD2-receptor pathway (PHA), and to relate the expression of IL-12R β_2 to the production of Th1 and Th2 associated cytokines, in allergic and non-allergic children.
- II. Study the IL-2 and IL-12 induced mRNA expression of the IL-12R β_2 -chain in PBMC from allergic and non-allergic children, and to relate the IL-12R β_2 expression to the production of cytokines.
- III. Study the development of spontaneous and PHA-induced expression of transcription factors (T-bet, GATA-3 and Foxp3), cytokine receptors (IL-12R β_2 and WSX-1) and cytokine production in children from Sweden and Estonia, followed prospectively from birth up to two years of age with regard to development of allergic disease.
- IV. Study the role of CD2 more closely by the use of stimulatory CD2 antibodies alone or in combination with CD28 antibodies. Investigate the calcium response, the mRNA expression of transcription factors (Foxp3, GATA-3 and T-bet), cytokine receptors (IL-12R β_2 and WSX-1) and cytokine production (IFN- γ , IL-5 and IL-10) in relation to allergic signs and symptoms in adults.

Material and methods

Study groups

Four different study groups are included in this thesis.

Paper I

The study group of paper I consisted of 32, 12-year-old children identified from the International Study of Asthma and Allergies in Childhood (ISAAC), phase II, Linköping, Sweden. The original ISAAC cohort in Linköping comprised 911 children from a random sample of 16 schools in Linköping [217]. Sixty of those were asked to participate with their parents in a study investigating allergen induced Th1 and Th2 responses [218].

For the present study, we included children from whom peripheral blood mononuclear cells (PBMC), which had been cultured with PHA or medium alone, were available. Allergic symptoms were based on the ISAAC phase II questionnaire, skin prick tests (SPT) and a clinical examination by an experienced research nurse. Nine children were SPT negative and 23 were positive for one or several of the allergens tested. In the SPT negative group one child had allergic symptoms (allergic rhino-conjunctivitis and eczema) and in the SPT positive group all, but one, had or had previously had symptoms. Of the symptomatic children, 21 had allergic rhino-conjunctivitis (ARC), 6 suffered from asthma and 12 had allergic eczema (several children had more than one symptom). Nineteen of the children had current symptoms, 18 had ARC, 2 had asthma and 7 had allergic eczema.

Paper II

Thirty-eight, 7-year-old children comprised the study group for paper II. They had previously participated in a prospective study (from birth to 18 months of age), on T cell function in Linköping, Sweden. That study comprised 54 children selected from an original cohort of 172 children based on the availability of separated PBMC and a clear allergic (n=25) or non-allergic (n=29) cumulative history of allergic disease at the age of 18 months, as defined in [219]. All 54 children were asked to take part in the 7-year follow-up and 38 agreed to participate.

From birth up to 7 years of age, 21 children had a cumulative history of allergic disease, whereas 17 had not (Table 2). 12 children were SPT positive for one or several allergens tested and 25 were SPT negative (one child declined SPT at 7

years but had been positive at 6 years and had high total serum IgE level). Five of the SPT negative children had current allergic symptoms (four had eczema, one of those also ARC and one had asthma) and all of the SPT positive children had or used to have allergic symptoms.

Table 2. Clinical data on allergic symptoms among the children in study II. Cumulative (0-7 years) and current (within the last year) allergic symptoms and SPT results. Airway=AB and/or ARC, AB=asthma bronchiale, ARC=allergic rhino-conjunctivitis, AE=atopic eczema sy=symptoms and HDM=house dust mite.

Allergic symptoms	Cumulative 0-7 years	Current	SPT+		SPT+ and current symptoms	
None	17	17	None	25	None	17
Any	21	16	Any	12	Both	11
Airways	11	10	Birch	4	sy, no SPT+	5
AB	8	6	Timothy	4	no sy, SPT+	1
ARC	9	8	Cat	7		
AE	15	12	Dog	2		
Urticaria		1	Horse	3		
			HDM	2		
			Mugwort	1		

Paper III

In paper III, we used samples from 35 Swedish and 26 Estonian infants from Linköping, Sweden and Tartu, Estonia. An original cohort of 123 Swedish and 110 Estonian children were recruited with their families to participate in a prospective study regarding the influence of the environment on development of allergic diseases, described in detail elsewhere [220]. The children were followed prospectively from birth up to 24 months of age. SPT were performed and the parents filled out questionnaires concerning allergic diseases at 3 and/or 6, 12 and 24 months of age. Venous blood was drawn at birth (cord blood), 3 and/or 6, 12 and 24 months of age. PBMC were stimulated from 70 Swedish and 30 Estonian children. For this study, Swedish children with samples from at least three different time-points and all the available samples from the Estonian children were included. The Estonian group is smaller due to an unfortunate incident with the customs causing delayed delivery of one batch of the cells, making them unfit for further studies. In the Swedish group 13 infants developed allergic symptoms, 20 did not and 2 had uncertain diagnosis (Table 3). Nine of the infants with and four of those without allergic symptoms were SPT positive for one or several of the allergens tested. In the Estonian group, three children had allergic symptoms and three were SPT positive.

Table 3. Data on allergic symptoms and SPT in the participating children in study III. AE=atopic eczema, AB=asthma bronchiale and FA=food allergy. Two Swedish and three Estonian children are omitted in the symptoms column due to uncertain diagnosis.

Symptom	Swe	Est	SPT+	Swe	Est
None	20	20	None	21	22
Any	13	3	Any	14	3
AE	12	2	Egg	10	3
AB	2	2	Milk	2	1
FA	1		Birch	4	
			Cat	3	1

Paper IV

Study IV included 20 volunteers, aged 18-57 years. Ten were allergic based on questionnaires regarding allergic symptoms strengthened by positive IgE levels (Phadiatop analysis) and positive skin prick tests. The symptoms present were asthma 8/10, ARC 10/10, food allergy 5/10, eczema 2/10 and urticaria 4/10. Skin prick tests were done in those individuals reporting allergic symptoms and 8/10 were positive for birch, 7/10 for cat, 5/10 for dog, 8/10 for timothy and 1 for house dust mite. Ten adults with a negative history of allergic disease displayed negative Phadiatop and were not subjected to SPT.

Diagnostic criteria

In paper I-III, the children were examined for allergic symptoms by a paediatrician (III) or by an experienced research nurse (I, II), SPT were done and the parents answered questionnaires regarding allergic symptoms. The definitions are described in each paper, except in paper I where definitions are described in [218]. In brief, asthma was defined as four or more yearly episodes of bronchial obstruction, allergic rhinoconjunctivitis as sneezing or a runny or blocked nose in the absence of a cold or the flu, accompanied by itchy-watery eyes, and atopic eczema as an itchy rash, coming and going for at least 6 months. In paper IV, questionnaires, SPT and Phadiatop analyses were used to classify the individuals.

In paper I, the children are referred to as allergic or non-allergic based on skin prick test positivity. In paper II, children without allergy (no history of allergic symptoms, negative SPT) were compared with children having a history of cumulative allergic symptoms (0-7 years) or with children having current symptoms (within the last year), or with children having current airway symptoms (AB and/or ARC). In addition non-allergic children with low IgE levels were compared with allergic

children with high IgE levels. In paper III, the children are referred to as allergic or non-allergic based on cumulative allergic symptoms or not within the first two years of life. In paper IV, the adults are grouped as allergic or not based on questionnaires revealing presence of allergic symptoms, positive Phadiatop and positive SPT. Non-allergic adults had no allergic symptoms and negative Phadiatop.

Skin prick tests

Single (paper II and IV) or duplicate (paper I and III) skin prick tests were performed on the volar aspects of the forearms using standardised extracts of birch, timothy, cat, dog (I-IV), the house dust mites *Dermatophagoides pteronyssinus* and *D. farinae* (I, II IV), horse, mugwort and the moulds *Cladosporium herbarum* and *Alternaria* (II) as well as thawed egg white and fresh skimmed cow's milk (lipid concentration 0.5%) (III). All extracts were standardised allergen extracts from ALK (Soluprick®, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/ml) was used as a positive control and albumin diluent or glycerol as negative control. The wheal size was marked with a filter pen after 15 min and transferred onto a micropore tape for measuring. The tests were regarded as positive when the mean wheal diameter was ≥ 3 mm.

Total IgE and Phadiatop

Total serum IgE levels (kU/l) were analysed in paper II and IV and specific IgE towards a panel of common allergens (Phadiatop) in paper IV. For both these analyses, the fluoroenzyme immunoassay UniCAP® was used according to the manufacturer's recommendations (Pharmacia Diagnostics, Uppsala, Sweden).

Cell preparation

Venous blood was drawn into heparin treated tubes and PBMC were isolated on Ficoll-Paque density gradient (Pharmacia) and then washed three times with RPMI-1640 (Life Technologies AB, Täby, Sweden) with 2% foetal calf serum (Life Technologies). To adjust the cell number, cells were counted in a Bürker chamber after staining with Türk's. Aliquots of 1 ml, containing $1 \cdot 10^6$ cells, were cultured in AIM-V serum free medium (Life Technologies AB) with 20 μ M mercaptoethanol (Sigma-Aldrich, Stockholm, Sweden) (I and III), or in RPMI-1640 with 10% foetal calf serum (FCS) (II and IV). Cells were further cultured with:

Material and methods

- 2 µg/ml of PHA (Sigma-Aldrich). Paper I and III.

- 100 pg/ml IL-12 (R&D Systems, Abingdon, UK), 5 ng/ml IL-2 (R&D Systems) or with IL-2 in combination with high and low concentrations of IL-12 (100 pg/ml and 25 pg/ml, respectively). Paper II.

- a combination of two antibodies towards CD2 (CLB-T11/1 clone 6G4 and CLB-T11.2/1 clone 4B2, 100 ng/ml, CLB, Amsterdam, the Netherlands) alone or combined with 100 ng/ml CD28 antibody (CLB-CD28/1 clone 15E8, CLB), or with PHA (2 µg/ml). Paper IV.

Medium alone was used as control culture in all studies. The cells were cultured at 37°C with 5% CO₂ (Forma CO₂-incubator model 3862, Forma Scientific Inc., Marietta, Ohio, USA) for 24 h (I and III), 72 h (II) or 70 h (IV).

In paper III, cells were cryopreserved by standard methodology in 10% dimethyl sulfoxide (Sigma-Aldrich), 50 % FCS and 40% RPMI-1640. After thawing, viable cells were resuspended to 1×10^6 cells/ml and cultured as described above. For each infant, cells from all available time-points were thawed and cultured at the same time.

Supernatants were collected after centrifugation (10-15 min, 400 g) and then stored at -70°C. For later RNA extraction, the pellets were lysed with 300 µl RLT Lysis buffer (from RNeasy™ 96 RNA extraction kit, Qiagen, Hilden, Germany) and then stored at -70°C.

The kinetics of IL-12, IL-2 and PHA were evaluated in eight individuals, four with allergy and four without. The incubation times used were 24, 48, 72 and 96 h. Optimal concentrations (25-100 pg/ml) of IL-12 were investigated in 2 non-allergic individuals. The kinetics as well as the optimal concentrations for the CD2.1, CD2.2 and CD28 antibodies were tested in samples from two healthy subjects. The different concentrations tested ranged from 12.5 to 500 ng/ml and the times used were 24, 48, 72 and 96 h.

Intracellular calcium levels

After density gradient centrifugation, cells were immediately loaded with 4 µM fura-2-acetoxymethylester (Sigma-Aldrich) in RPMI-1640 with 10 % FCS for 30 minutes in 37°C with 5% CO₂, for measurement of intracellular calcium levels. Fura-2-acetoxymethylester

methylester passes through the cell membrane and is hydrolysed into the calcium binding, fluorescent and membrane impermeable fura-2. The cells were then centrifuged (400 g, 10 min) and resuspended in HEPES-buffer (pH 7.4 at 37°C, 145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 10 mM HEPES, 10 mM glucose and 1 mM CaCl₂) to a cell-concentration of 2*10⁶ cells/ml. Aliquots of 800 µl were pre-heated at 37°C for 4 minutes.

Cells were stimulated with 500 ng/ml of antibodies to CD2, T11.2/1 followed by T11.1 after 90 s, followed by a cross-linking rabbit anti-mouse IgG Fcγ-fragment specific antibody (2.5 µg/ml, Jackson ImmunoResearch Laboratories Incorporation, West Grove, PA, USA). Ionomycin (0.1 µM), was used as positive control. Fluorescence signals were recorded on a Hitachi F-2000 fluorescence spectrofluorometer, specially designed for measurement of intracellular calcium. Fluorescence emission was measured at 510 nm during simultaneous excitation at 340 nm and 380 nm. The intracellular calcium concentration was calculated, 1 and 10 minutes after stimulation, using the general equation described by Grynkiewicz *et al*, $[Ca^{2+}]_i = K_d * (R-R_{min}) / (R_{max}-R) * (F_o/F_s)$. Maximal and minimal ratios were determined by first adding 0.1 % Triton X-100 and then 20 mM EGTA ([ethylenebis(oxyethylenenitrilo)]tetraacetic acid), respectively [221].

Initial studies were done using different concentrations (100-1000 ng/ml) of CD2 antibodies, different order of adding the antibodies as well as different cell concentrations (2-10*10⁶ cells/ml) and length of measurement after antibody addition. Control measurements with isotype matched antibodies were also done.

RNA extraction

Total ribonucleic acid (RNA) was isolated according to the RNeasy™ 96 Protocol (Qiagen), for isolation of total RNA from animal cells using vacuum/spin protocol. In brief, cells were lysed with RLT lysis buffer, mixed with ethanol, and applied to a RNeasy 96™ well plate. Contaminants were washed away by wash buffers included in the kit, RW1 1 ml followed by RPE 2 x 1 ml. The membrane was dried and the RNA was eluted in 2 x 30 µl RNase free water. The purity of the RNA was checked spectrophotometrically in some randomly chosen samples and the ratio A₂₆₀/A₂₈₀ was always >1.7. RNA was stored at -70°C.

Reverse transcription (RT)

RNA was converted to complementary DNA (cDNA) using the Reverse-it 1st strand synthesis kit (paper I-IV, Abgene Advanced Biotechnologies Ltd, Epsom, UK) or the High-capacity cDNA archive kit (paper III, Applied Biosystems, Foster City, CA, USA). The Reverse-it kit uses oligo dT-primer, random decamer primer, dNTPs, RNase inhibitor and M-MLV reverse transcriptase, and the reactions were run for 1 h at 42°C followed by 10 minutes at 75°C, according to the protocol of the supplier. The Archive kit uses random primers, dNTPs and the MultiScribe™ Reverse Transcriptase and the reactions were run for 10 min at 25°C followed by 2 h at 37°C as recommended by the supplier. The samples in paper I were run in a GeneAmp PCR System 2400 and samples for paper II-IV in GeneAmp PCR System 2700 (both from Applied Biosystems).

Real-time PCR

Quantification of IL-12R β_2 , WSX-1, T-bet, GATA-3 and Foxp3 mRNA expression was performed on the ABI Prism™ 7700 Sequence Detector System (Applied Biosystems). HPLC-purified oligonucleotide primers and probes designed for human IL-12R β_2 , GATA-3 and rRNA sequence were bought from MedProbe (Oslo, Norway) (Table 4). The TaqMan probes had the fluorophore FAM (6-carboxy-fluorescein) at the 5'-end and the quencher TAMRA (6-carboxy-tetramethylrhodamine) at the 3'-end. During the extension phase of the PCR reaction, the probe is cleaved by the 5'-nuclease activity of the Taq polymerase resulting in separation of the fluorophore and the quencher (Figure 7) [222]. The fluorescence signal increases proportionally to the PCR product. A computer algorithm compares the amount of reporter dye emission (R) with the quencher dye emission (Q) during amplification and the amount of degraded probe is given by the ΔR_n value (R/Q). A threshold is set based on the baseline of the ΔR_n during the first cycles. The algorithm calculates the cycle at which each PCR amplification reaches a significant threshold cycle (Ct), *i e* 10 times the standard deviation of the baseline. The calculated Ct is proportional to the target copies present in the sample at the start of the reaction.

Foxp3, T-bet and WSX-1 expression were analysed with Taqman® Gene Expression Assays (assay id: Hs00203958_m1, Hs00203436_m1 and Hs00175472_m1, Applied Biosystems) according to the supplier's instruction. All primer/probe pairs as well as the Taqman® Gene Expression Assays were tested and evaluated before running the samples. None of the primer-probe pairs or the assay mixes amplified genomic DNA to any significant extent.

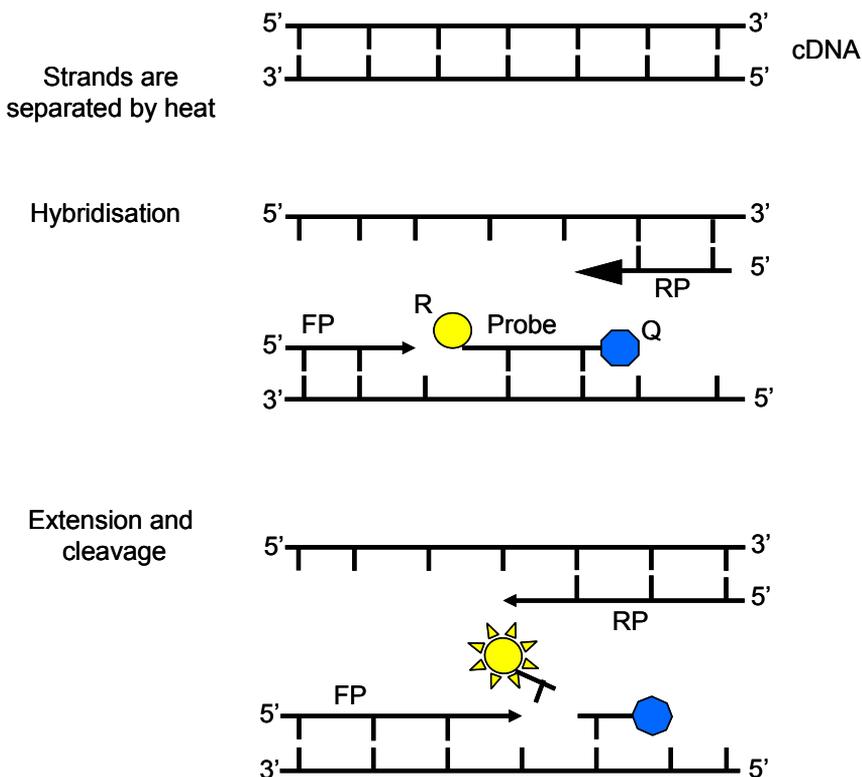


Figure 7. Principle of real-time PCR. The cDNA strands are separated by heat, forward and reverse primer (FP and RP) and the probe hybridise to cDNA. The probe has two fluorescent dyes, a reporter (R) and a quencher (Q), attached to the ends. As long as the probe is intact, the quencher absorbs the fluorescence of the reporter. During the extension phase, the polymerase cleaves the probe and once separated from the quencher, the fluorescence of the reporter is detected by the Sequence detector system. The more target gene that is present at the start of the reaction, the more fluorescence will be detected and the lower the threshold cycle (Ct) will be when product is first detected.

Preparations were done according to the instructions of the supplier. Briefly, TaqMan Universal Master mix, including AmpliTaq Gold Polymerase, Amp Erase UNG (prevents reamplification of carryover-PCR products by removing any uracil incorporated into double stranded DNA, the enzyme is then inactivated at the hot start), dNTPs with dUTP, passive reference and optimised buffer components, were mixed with primers and probe or assay mix. Then, 24 μ l of the mix was added to each well in a 96-well reaction plate (MicroAmp Optical, Applied Biosystems). Samples (1 μ l), standards or water (NTC) were added in duplicate wells, supplied with optical caps and centrifuged briefly before entering the thermocycler. The thermal cycle conditions were: 50°C for 2 minutes followed by 95°C for 10 minutes (hot start) and then 40 cycles were run with 15 seconds at 95°C and 1 minute at 60°C, as

recommended by Applied Biosystems for the SDS 7700 system.

Each target mRNA was analysed separately and the relative quantifications were done using the standard curve method as described in User Bulletin no 2 (Applied Biosystems). The results from the rRNA reactions were used as internal controls, *i.e.* the amount of target mRNA was calculated relative to the amount of rRNA present in each sample.

Table 4. Sequences and concentrations of forward (FP) and reverse (RP) primers and probes used for analyses of mRNA expression of IL-12R β_2 , GATA-3 and rRNA. Exon-exon junctions are marked in bold and italic.

Target	Primer /Probe	Sequence (5'-3')	Conc. (nM)
IL-12R β_2	FP	ACATTCTTGGACATAG GT GAGGCC	50
	Probe	TCCCTCCGTGGGACATTAGAATCAAATTTCAA	125
	RP	GTACATCTGCTCACGGAAGCC	50
GATA-3	FP	CAAATGAACGGACAGAACCG	50
	Probe	CCCCTCATTAAGCCCAAGCGAAGG	125
	RP	GCTCTCCTGGCTGCAG ACA	50
rRNA	FP	CGGCTACCACATCCAAGGAA	200
	Probe	TGCTGGCACCAGACTTGCCCTC	50
	RP	GCTGGAATTACCGCGGCT	100

Enzyme-linked immunosorbent assay (ELISA)

The cytokine production was detected in the culture supernatants using enzyme-linked immunosorbent assay (ELISA) (Figure 8). The production of IFN- γ , IL-4, IL-5, IL-10 and IL-13 was analysed in paper I and in paper III also IL-9. In paper II and IV, the production of IFN- γ , IL-5 and IL-10 was measured. Separate antibody pairs were used for most ELISA analyses, but the PeliPair reagent set (PeliPairTM reagent set, CLB) was used for measurements of IL-10 in all papers, for IFN- γ in paper IV and for IL-13 in paper III. In the PeliPair reagents sets, the concentrations of the antibodies are not given and are therefore not stated in the text below. The antibodies used in the IL-9

ELISA were derived from C57Bl/6 mice as described in [218] and were a kind gift from Dr Jacques van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium.

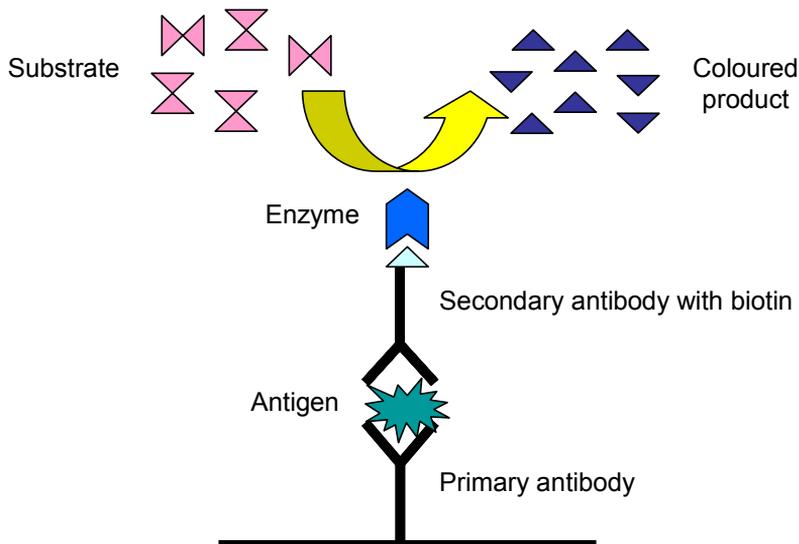


Figure 8. Principle of ELISA. Cell supernatants are transferred into a microtitre plate pre-treated with coating antibodies (primary antibody), which capture the cytokine of interest. Secondary antibodies conjugates with an enzyme, react with a substrate and form a coloured product which can be measured spectrophotometrically. The amount of substrate converted to product is proportional to the amount of the specific cytokine present in the cell supernatant.

Costar 3690 plates (Corning Inc., Corning, NY, USA) were coated overnight at room temperature. The following mouse anti-human coating antibodies were used; IFN- γ , IL-4, IL-13 (clone no 25718.111, 3010.211 and 32116.11 respectively, R&D Systems), IL-5 (clone no TRFK 5, PharMingen, San Diego, CA, USA) and IL-9 (clone MH9A4). The concentrations used were 2 $\mu\text{g/ml}$ for IFN- γ , 0.25 $\mu\text{g/ml}$ for IL-5, 5 $\mu\text{g/ml}$ for IL-9 and 4 $\mu\text{g/ml}$ for IL-4 and IL-13. For IL-10, as well as for IFN- γ and IL-13 when using PeliPair™ reagent set, the coating antibodies provided were used diluted 1:100. Excess free antibodies were washed off (Wellwash Ascent, Labsystems, Stockholm, Sweden) with 4 x 250 μl phosphate buffered saline (PBS)-Tween (0.05% Tween 20 in PBS, pH 7.4). The wells were then incubated for 1 h at room temperature on a plate shaker (Thermostar, Labvision, Stockholm, Sweden) with 100 $\mu\text{l/well}$ of low-fat milk to block non-specific protein binding sites.

The plates were washed as previously described and 50 μl of samples or standards; recombinant human IFN- γ , IL-4, IL-9 and IL-13 (R&D Systems), IL-5 (PharMingen) and IL-10 (CLB) were added to duplicate wells. A control using AIM-V serum free medium with 20 μM mercaptoethanol (I and III) or RPMI-1640 with 10%

FCS (II and IV) was also included. The plates were incubated and washed as before.

Biotinylated detection antibodies, goat anti-human IFN- γ , IL-4 and IL-13 (R&D Systems), rat anti-human IL-5 (clone no JES1-5A10, PharMingen), mouse anti-human IL-9 (clone MH9A3) and IL-10 (CLB), were then added (50 μ l/well). All were diluted in high performance ELISA-dilution buffer (CLB), to the final concentrations; 0.20 μ g/ml for IFN- γ , 1 μ g/ml for IL-5, 90 ng/ml for IL-9 and 0.50 μ g/ml for IL-4 and IL-13. The antibodies to IL-10 (and IFN- γ and IL-13 when using PeliPair™ reagent set) were diluted 1:100. This was followed by another incubation and wash.

Streptavidin-poly horseradish peroxidase (HRP) (50 μ l/well, 0.1 μ M, CLB) was added, and the plates were incubated as before, but for 30 minutes, and then washed. Thereafter 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich) was added (50 μ l/well), and incubated as before for 30 minutes, but in the dark. Finally, the reactions were stopped using 1.8 M H₂SO₄ (50 μ l/well). The amount of substrate converted to product was thereafter detected as optical densities (OD) at 450 nm in an Anthos ht II ELISA reader (paper I, Anthos ht II, Labdesign, Täby, Sweden) or in a VERSAmax tunable microplate reader (Göteborgs Termometer fabrik, Göteborg, Sweden). Values were expressed as pg/ml deduced from the ODs of the reference serum curve after subtracting the blanks. The sensitivity limits for quantitative determinations were as shown in Table 5. The change in detection limits for IFN- γ and IL-13 is due to the change to PeliPair reagent set. For IL-5 the detection limit changed, due to wrong calculation of the concentration of the IL-5 standard, which was discovered after paper II.

Table 5. Detection limits for cytokine analyses with ELISA.

Cytokine	IFN- γ (pg/ml)	IL-5 (pg/ml)	IL-10 (pg/ml)	IL-4 (pg/ml)	IL-9 (pg/ml)	IL-13 (pg/ml)
Paper I	12	3	2	3		16
Paper II	25	3	5			
Paper III	25	6	5	6	16	2
Paper IV	16	6	5			

Data handling and statistics

All mRNA analyses were done in duplicates (coefficient of variance always <15%) and the mean values were used. The mean mRNA levels were normalised by division of the amount of rRNA. To compare spontaneous expression, ratios were compared

directly. For comparisons of stimulated amounts, the mRNA/rRNA ratio of the control cells was withdrawn from the mRNA/rRNA ratio of the stimulated cells before comparisons were made.

Cytokines were also analysed in duplicates (coefficient of variance always <15%) and mean values, after subtracting the blanks were used. Samples below detection limit were given a value corresponding to half the cut off value to allow statistical calculations. To obtain the stimulated amounts, the mean values of the control cultures were withdrawn from the mean values of the stimulated cultures. Differences between control and stimulated cells that were smaller than half the cut off value were given half the cut off value in the statistical analyses.

Calcium responses were measured at 1 and 10 minutes after addition of stimuli. The background level, *i e* the calcium concentration before addition of stimuli was withdrawn from the calcium levels at 1 and 10 minutes before making comparisons.

Since neither the mRNA, cytokine nor calcium levels were normally distributed non-parametric tests, corrected for ties, were used. Comparisons between unpaired groups were analysed with Mann-Whitney *U*-test and comparisons between paired groups with Wilcoxon signed rank test. Correlations were analysed with Spearman's rank order correlation coefficient test. P-values <0.05 were considered significant. A statistical package, Statview 5.0 for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA), was used for paper I. Statistica™ 5.1 for Windows (Statsoft®, Tulsa, OK, USA) was used in paper II and Statview™ 5 for Windows (SAS Institute Incorporation, Cary, NC, USA) was used in paper III and IV.

Ethical considerations

All studies in this thesis were approved by the Regional Ethics Committee for Human Research at the University Hospital in Linköping, Sweden. Study III was also approved by the Ethics Review Committee on Human Research of the University of Tartu, Estonia. All parents of the participating children as well as the included adults gave their informed consent to participate in the studies.

Results and discussion

Methodological aspects

Dose-response and kinetics for the stimuli

The optimal concentration of IL-12 was found to be 100 pg/ml. We also chose to include a lower dose of IL-12 in combination with IL-2, as optimal doses sometimes overcome differences in responses that appear at suboptimal doses. The kinetics studied for IL-2 and IL-12 were not clear-cut in our eight individuals. However, we decided to use 72 h as this was optimal for most parameters, although PHA induced high responses already at 24 h. Optimal concentrations of the CD2 antibodies alone or in combination with CD28 antibodies were found to be 100 ng/ml, and the optimal time of stimulation was 72 h.

Calcium analysis

Initial studies revealed the optimal concentrations of the CD2 antibodies to be 500 ng/ml. The optimal order of antibody addition was CD2 T11.2/1 followed by CD2 T11.1 followed by the cross-linking antibody. A cell concentration of $2 \cdot 10^6$ cells/ml was considered sufficient and the calcium responses were decided to be calculated 1 and 10 minutes after addition of the cross-linking antibody.

Optimisation and evaluation of real-time PCR reactions

Before running the samples, primers and probe specific for human IL-12 receptor β_2 -chain and for GATA-3 were designed and evaluated. The forward primer for IL-12R β_2 is situated over an exon-exon junction for prevention of amplification of genomic DNA, which may be present in the samples. Despite that some amplification was found in RNA samples that were not reverse transcribed. We tested two different DNase treatments, but did not find them to be reliable since the results from the treated samples varied. The RNA samples (-RT control) had Ct-values around 35-36 indicating less than 10 target copies at the start of the reaction according to Applied Biosystems. The cDNA samples were always detected at least 5 cycles apart from the RNA. GATA-3 was not amplified at all in RNA samples. An example of a real-time PCR reaction with the standards for IL-12R β_2 mRNA expression is shown in Figure 9 and the standard curve generated from the same reaction is shown in Figure 10. The slopes of all the standard curves are shown in Table 6. At optimal reaction conditions, *i e* 100% efficiency, cDNA is duplicated at each cycle which results in a slope of -3.3.

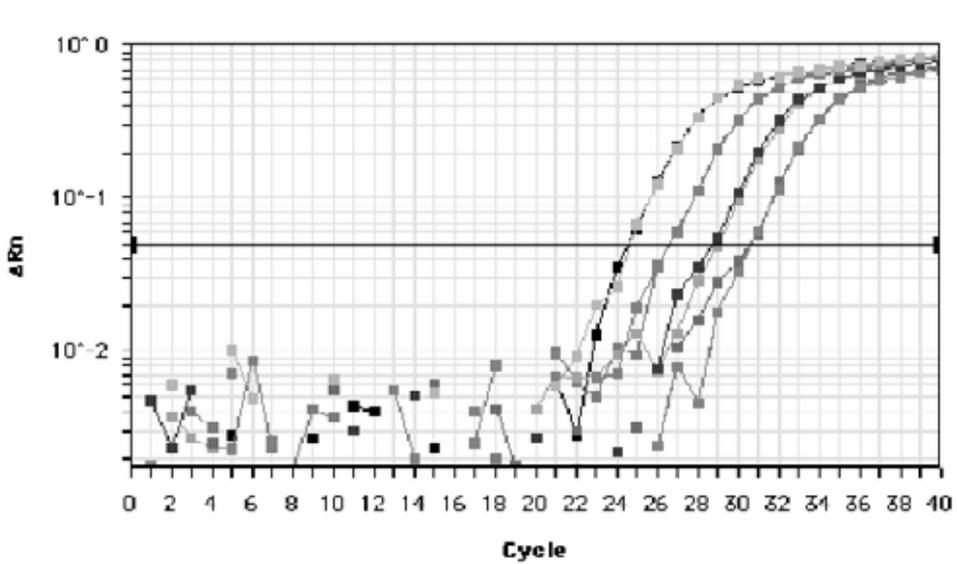
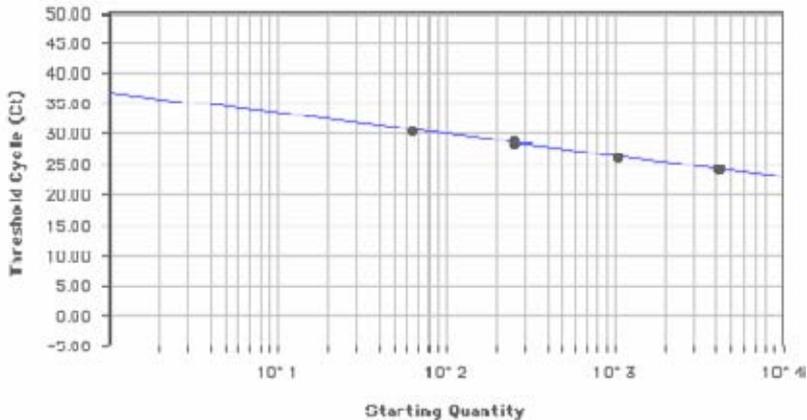


Figure 9. Real-time PCR reaction of the standard curve for IL-12R β_2 . A 1:4 dilution series of cDNA from stimulated PBMC in duplicate was used as standard and the threshold cycle thus changes with approximately 2 cycles per standard.



Slope: 3.398 Y-intercept: 36.86 Correlation coeff: 0.996

Figure 10. The Ct values obtained from the amplification plots were used to plot standard curves for quantification of unknown samples. This standard curve was generated from the amplification plots in Figure 9.

None of the used Taqman® Gene Expression Assays amplified genomic DNA and the slopes are shown in Table 6. The inter-assay variation as calculated from paper III was <15% for all markers.

Results and discussion

Three different cDNA:s were used as standards in the four studies. For analysis of rRNA and Foxp3, cDNA from PHA-stimulated cells were used. The other markers were related to CD2/CD28 stimulated cells in paper I, II and IV and also for GATA-3 in paper III. PHA in combination with IL-12 and IFN- γ stimulation was used for IL-12R β_2 , T-bet and WSX-1 in paper III.

Table 6. The slopes of the standard curves for the different markers in the four studies.

	Paper I	Paper II	Paper III	Paper IV
IL-12R β_2	-2.8 to -3.4	-3.1 to -3.8	-3.3 to -3.6	-3.4 to -4.0
GATA-3			-3.4 to -3.6	-3.4 to -4.0
T-bet			-4.3 to -5.0	-3.9 to -4.1
WSX-1			-3.4 to -3.7	-3.8 to -4.0
Foxp3			-3.9 to -4.2	-3.6 to -3.9

Paper I: PHA-induced IL-12R β_2 mRNA expression in atopic and non-atopic children

In paper I, we studied the PHA-induced mRNA expression of IL-12R β_2 -chain in relation to production of cytokines, in PBMC from 32 12-year-old children. PHA signals partly via the CD2 receptor pathway. Stimulation via the CD2 receptor increases the IFN- γ production and enhances the responsiveness of activated T cells to IL-12, possibly via up-regulation of the signal-transducing β_2 -chain of the IL-12 receptor. Previous studies have suggested an impaired CD2 function in allergic children. We hypothesised that allergic children have a reduced responsiveness to IL-12, which is associated with a defective CD2 function resulting in impaired up-regulation of the IL-12R β_2 -chain.

IL-12R β_2 mRNA expression

We analysed the mRNA expression of the IL-12R β_2 -chain after culture of PBMC in medium alone or with 2 μ g/ml of PHA for 24 h. PHA induced an up-regulation of IL-12R β_2 mRNA in all children (Figure 11).

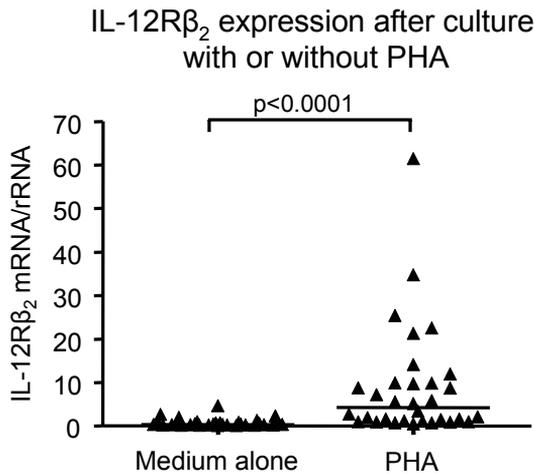


Figure 11. The amount of mRNA for IL-12R β_2 in PBMC cultured in medium alone or with PHA for 24 h. PHA-stimulated cells expressed significantly higher IL-12R β_2 mRNA levels than the control cells. Values are given as ratios of IL-12R β_2 and rRNA, which we used as internal control in the real-time PCR reaction. Median levels are indicated and the groups were compared with Wilcoxon signed rank test, n=32.

The spontaneous expression was similar in SPT positive and SPT negative children (Figure 12a). After withdrawal of the spontaneous expression, the PHA-

induced responses were compared. We found that SPT negative children induced significantly more mRNA for IL-12R β_2 than the SPT positive children did in response to PHA (Figure 12b). As PHA signals partly via the CD2 receptor [103], these results support our hypothesis that an impaired CD2 pathway may be present in allergic children.

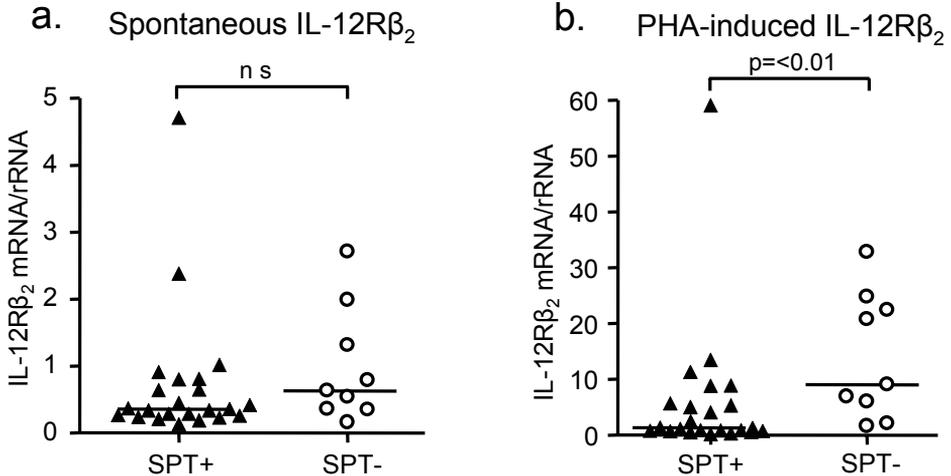


Figure 12. Spontaneous (a) and PHA-induced (b) IL-12R β_2 mRNA expression in PBMC cultured in medium alone or with PHA for 24 h. The values are given as ratios of IL-12R β_2 and rRNA. Median levels are indicated and the groups were compared with Mann-Whitney *U*-test, n=23 in the skin prick test positive (SPT+) group and n=9 in the SPT negative group.

IL-12R β_2 mRNA expression in relation to cytokine production

In addition to analysis of the IL-12R β_2 mRNA expression, we wanted to relate that expression to the production of different cytokines, namely IFN- γ , IL-4, IL-5, IL-10 and IL-13. These cytokines were analysed in the cell supernatants using ELISA. For measurement of IL-4, separate cultures were performed with 2 μ g/ml monoclonal antibodies to the IL-4 receptor (R&D Systems) to prevent consumption of the produced IL-4. The production of IFN- γ was lower in the SPT positive group compared to the SPT negative group (Figure 13), and there was a strong correlation between the mRNA expression of the IL-12R β_2 -chain and the production of IFN- γ (Figure 14). The other cytokines were secreted in similar amounts among SPT positive and negative children (data not shown). The production of IL-5, IL-10 and IL-13 showed weaker correlations with IL-12R β_2 expression ($\rho=0.5$, $p<0.05$) and IL-4 did not correlate at all. However, the ratio of IFN- γ /IL-4 correlated well with IL-12R β_2 expression, indicating that up-regulation of the β_2 -chain is preferably a Th1 phenomenon. This is supported by the findings that Th1, but not Th2 development

results in IL-12R β_2 up-regulation [131].

We divided the children as SPT positive or negative but the differences were apparent, although not statistically significant, when the children were divided according to allergic symptoms as well. All but one of the SPT positive children also had symptoms and, with one exception, none of the SPT negative children had any symptoms.

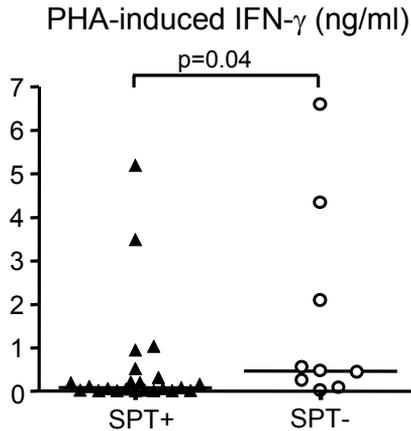


Figure 13. PHA-induced IFN- γ production by PBMC from SPT positive (n=23) and SPT negative (n=9) 12 year-old children. IFN- γ secretion was detected in cell supernatants with ELISA. Median levels are indicated and the groups were compared using Mann-Whitney U-test.

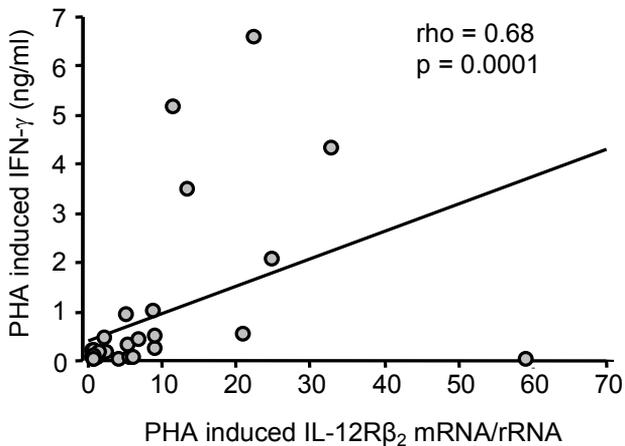


Figure 14. Correlation between IL-12R β_2 mRNA expression and production of IFN- γ from PBMC stimulated with PHA for 24 h. IL-12R β_2 mRNA was analysed with real-time PCR and production of IFN- γ with ELISA. The correlation was analysed with Spearman's rank order correlation coefficient test.

Discussion

We found that stimulation of PBMC with PHA, which acts partly via the CD2 pathway, induces an up-regulation of the mRNA expression of the IL-12R β_2 -chain. Together with the IL-12R β_1 -chain, the signal-transducing β_2 -chain forms a high affinity receptor for IL-12 [127]. CD2 signalling has previously been shown to interact with the response to IL-12, *e g* blocking the CD2 receptor inhibits IL-12-induced proliferation and IFN- γ production [84]. The interaction between CD58 on monocytes and CD2 on T cells is important in monocyte stimulated IFN- γ production by T cells as well as for proliferation of the T cells [85, 99]. Our results indicate that the increased IL-12 responsiveness seen in CD2 signalling may be caused by an up-regulation of the β_2 -chain of the IL-12 receptor. A direct effect on the IL-12 receptor expression by interaction of CD2-CD58 has to our knowledge not been shown previously. A reduced function of the CD2 pathway could have important consequences for the development of allergy, since a decreased IL-12 responsiveness would result in difficulties to induce Th1 type immune responses [105, 106].

We also found lower PHA-induced expression of the IL-12R β_2 -chain in the SPT positive, compared with SPT negative, children. This is in agreement with the lower IL-12R β_2 expression Wright *et al* found in nasal biopsies from patients with allergic rhinitis compared with non-allergic individuals [148]. Another study did not find such a difference in nasal biopsies from patients with allergic rhinitis and patients with non-allergic rhinitis (chronic sinusitis) [223]. Lower IL-12R β_2 expression has also been found in patients with allergic asthma, compared with non-asthmatic individuals, after culture of PBMC with IL-12. In contrast to our results, which showed similar baseline mRNA expression of IL-12R β_2 , they reported lower spontaneous mRNA expression in patients with asthma compared to non-asthmatics [144]. At the protein level, IL-12R β_2 was detected in non-asthmatic individuals but not at all or weakly in asthmatic individuals [144]. In addition, the IL-12 induced IFN- γ production is low in PBMC from patients with atopic dermatitis [224] and allergic rhinitis [225]. This production is negatively correlated with serum IgE levels [226].

The lower IL-12R β_2 mRNA expression we found in the allergic children supports our theory that they fail to up-regulate the β_2 receptor component. Since the responsiveness to IL-12 is dependent on this up-regulation [127], allergic children have a reduced ability to respond to IL-12. As IL-12 is important in promoting Th1 like responses [105] impaired expression of its receptor may be important in the development of allergic diseases. Our results also supports previous findings of reduced CD2 function in atopic children, *i e* lower numbers and proportions of CD2

expressing cells as well as lower responsiveness to PHA [80, 81, 83, 102].

The strong correlation we found between IL-12R β_2 mRNA expression and IFN- γ production supports previously reported results on the enhancing effect IL-12 has on IFN- γ production [105, 227, 228]. This is accompanied by increased expression of high affinity IL-12 receptors [227]. IL-12 as well as IFN- γ has been demonstrated to enhance up-regulation of the IL-12R β_2 subunit creating a positive feed-back loop between IL-12, IL-12R β_2 and IFN- γ [96, 133]. As we analysed the IL-12R β_2 -chain at the mRNA level, the good correlation with IFN- γ production also supports that the enhanced mRNA expression actually results in higher protein expression of IL-12R β_2 .

In summary, we found lower PHA-induced up-regulation of IL-12R β_2 expression in allergic compared to non-allergic children. This may be caused by impaired CD2 signalling. An impaired responsiveness to IL-12 may be one contributing factor in the development of allergic diseases since IL-12 is important in mounting Th1 like responses.

Paper II: Reduced IL-2-induced IL-12 responsiveness in atopic children

In paper II, we studied the effect of IL-2 and IL-12 on the expression of the IL-12R β_2 -chain in 38, 7-year-old allergic or non-allergic children. The expression was related to IFN- γ , IL-5 and IL-10 production. Lower IL-12-induced IFN- γ production has been reported in allergy and we hypothesised that it may be caused by a deficient up-regulation of the IL-12R β_2 -chain, which is crucial for IL-12 responsiveness. Thus, we here approached another signalling pathway, besides the CD2-pathway studied in paper I, for IL-12 responsiveness.

IL-12R β_2 mRNA expression and cytokine production in response to IL-2 and IL-12 stimulation

We used IL-2 and IL-12 alone or IL-2 combined with two different concentrations of IL-12. All these stimuli resulted in significant up-regulation of the IL-12R β_2 -chain when compared to control cells cultured in medium alone (Figure 15). IL-2 was more potent than IL-12 and the combinations were more potent than IL-2.

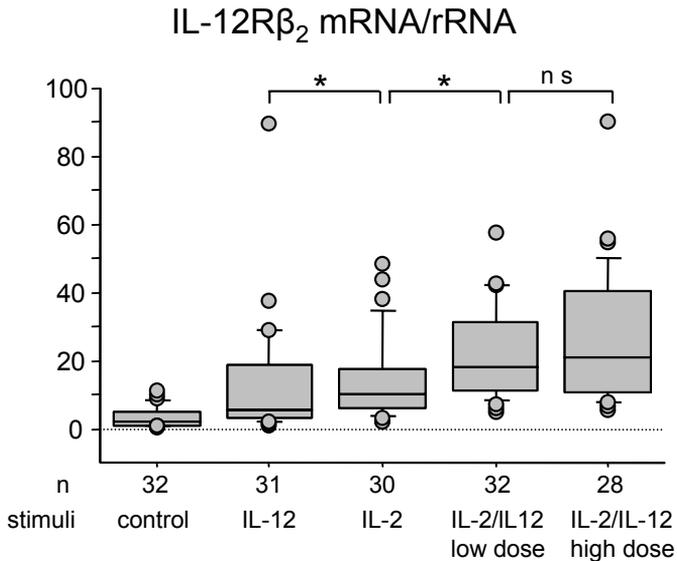


Figure 15. PBMC from 7-year-old children stimulated with IL-12 (100 pg/ml), IL-2 (5 ng/ml), IL-2 combined with low (25 pg/ml) or high (100 pg/ml) IL-12 for 72 h. IL-12R β_2 mRNA expression was analysed with real-time PCR and the different stimuli were compared with Wilcoxon signed rank test. All stimuli resulted in significant up-regulation of IL-12R β_2 compared with control cells ($p < 0.0001$). IL-2 induced more IL-12R β_2 than IL-12 and the combinations were even more efficient. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers. * $p < 0.05$.

The IFN- γ and IL-10 production followed the same pattern as the IL-12R β_2 mRNA expression in response to IL-12 and IL-2 stimulation, *i e* all stimulations resulted in enhanced production of these cytokines (Figure 16a-b). IL-2 induced more IFN- γ and IL-10 than IL-12, the combination of IL-2 and low IL-12 induced more than IL-2 alone and the production was further enhanced with the higher dose of IL-12.

Increased IL-5 secretion was found after IL-2 ($p < 0.0001$), but not IL-12, stimulation. The combination of IL-2 and high dose IL-12 inhibited the IL-2-induced IL-5 secretion ($p < 0.01$) (data not shown).

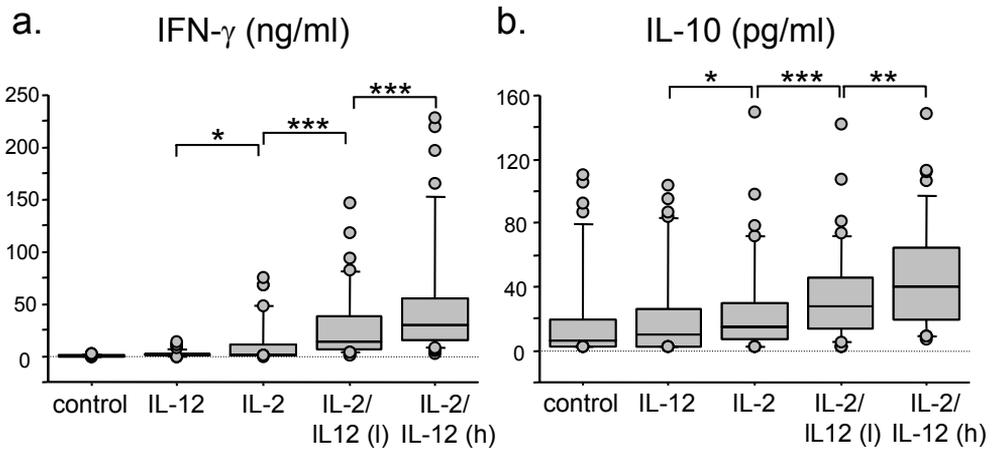


Figure 16. IFN- γ (a) and IL-10 (b) production by PBMC cultured for 72 h with IL-12 (100 pg/ml), IL-2 (5 ng/ml), IL-2 combined with low (25 pg/ml) or high (100 pg/ml) IL-12. All stimulations induced IFN- γ ($p < 0.0001$) and IL-10 ($p < 0.05 - p < 0.0001$). IL-2 was a stronger stimulus than IL-12, the low dose combination was stronger than IL-2 and the high dose combination was stronger than the low dose combination. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, $n = 38$.

IL-12R β_2 mRNA expression in relation to cytokine production

The spontaneous IL-12R β_2 mRNA expression correlated well with both spontaneous and stimulated IFN- γ production ($\rho = 0.5-0.7$, $p < 0.01$). One of the strongest correlations was found between spontaneous IL-12R β_2 mRNA and IL-12-induced IFN- γ (Figure 17). Thus, high IL-12 receptor expression resulted in higher IL-12-induced levels of IFN- γ . As IL-12 signalling is dependent on the expression of the IL-12R β_2 -chain, this agrees with previous studies reporting enhanced IFN- γ production by IL-12 [105, 227, 228]. IL-12-induced production of IL-10 or IL-5 did not correlate with spontaneous IL-12R β_2 mRNA.

IL-2-induced IL-12R β_2 expression correlated with IFN- γ secretion induced by IL-2 and the low dose of IL-12 (Figure 18), with a similar trend for IL-2 alone (rho=0.33, p=0.08). No correlations were found between IL-2-induced IL-12R β_2 and IL-10 or IL-5 secretion.

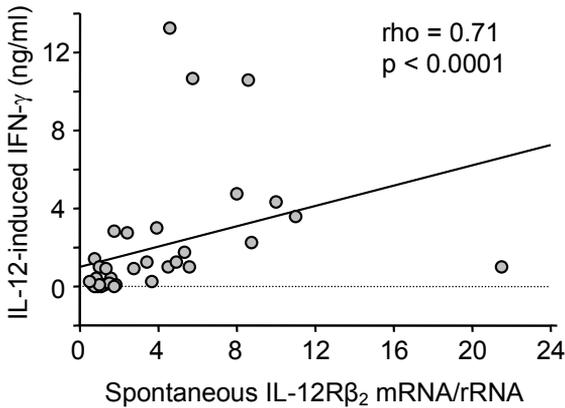


Figure 17. The correlation between the spontaneous expression of IL-12R β_2 mRNA and the IL-12 induced IFN- γ secretion in PBMC from 7-year-old children. The PBMC were cultured for 72 h and the correlation was analysed with Spearman's rank order correlation coefficient test, n=35.

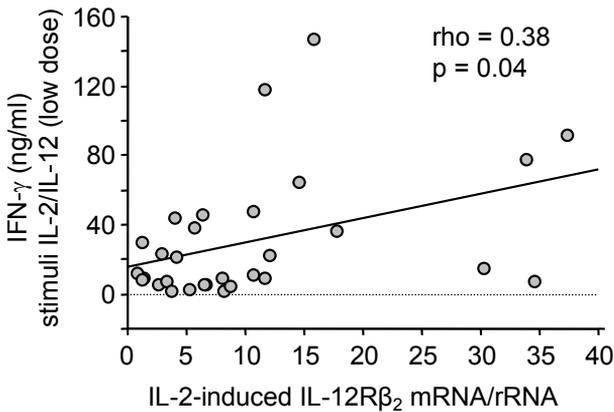


Figure 18. The correlation between the IL-2-induced expression of IL-12R β_2 and the IL-2/IL-12 (low dose) induced IFN- γ secretion in PBMC from 7-year-old children. PBMC were cultured for 72 h. The correlation was analysed with Spearman's rank order correlation coefficient test, n=30.

IL-12R β_2 mRNA expression and cytokine expression in relation to allergy

The children were grouped as non-allergic if they never had had allergic symptoms or positive SPT. These children were compared with children having a cumulative history of allergy (symptoms 0-7 years) or with children having any current symptoms (within the last year), or with children having current airway symptoms (AB and/or ARC). The results are summarised in (Table 7). In addition, to make the groups even more distinct, non-allergic children with low IgE levels were compared with allergic

children with high IgE levels. The median value of total IgE in all children was 72 kU/l. In the comparisons, values below and above the median value are referred to as low and high, respectively.

We found similar spontaneous expression of IL-12R β_2 mRNA in allergic and non-allergic children (Table 7). Children with current allergic airway symptoms and high levels of IgE had significantly lower IL-2-induced IL-12R β_2 mRNA expression than non-allergic children with low levels of IgE (Figure 19). A negative correlation was found between total IgE levels and the IL-12R β_2 expression induced by IL-2 in combination with the high dose IL-12 ($\rho=-0.48$, $p=0.01$, $n=32$). A similar trend was found when IL-2 was used alone ($\rho=-0.32$, $p=0.09$, $n=30$).

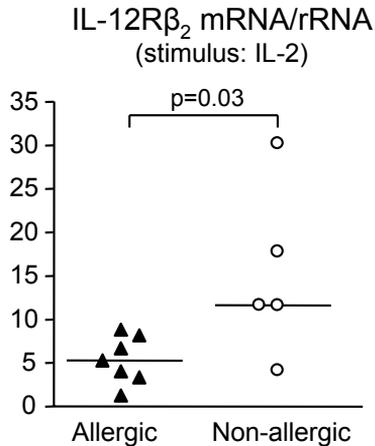


Figure 19. IL-2-induced IL-12R β_2 mRNA expression in PBMC from children with current allergic airway symptoms and total IgE levels above median (allergic) and non-allergic children with IgE levels below median (non-allergic). The groups were compared with Mann-Whitney *U*-test and the medians are shown.

The IFN- γ production was lower in children with current airway symptoms and positive SPT than in children without allergic symptoms and negative SPT when PBMC were stimulated with IL-2 in combination with the lower dose of IL-12 used (Figure 20). The same was found among children with a cumulative history of allergy and high IgE levels compared to children without a history of allergy and low IgE levels (Figure 21), as well as in children with current airway symptoms and high IgE ($p=0.04$) or current skin symptoms and high IgE ($p=0.02$).

Total levels of IgE correlated negatively with the IFN- γ production induced by IL-2 in combination with the lower dose IL-12 ($\rho=-0.40$, $p=0.01$, $n=38$).

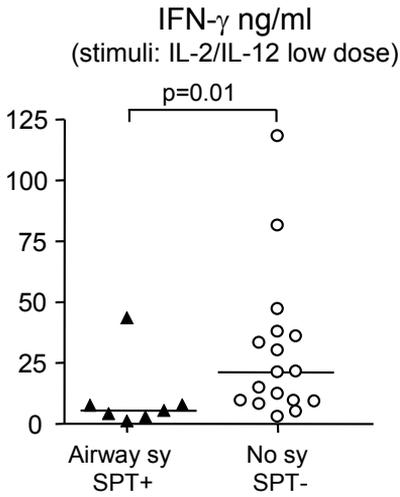


Figure 20. IFN- γ levels after addition of IL-2 and IL-12 (25 μ g/ml), produced by PBMC from children with current airway symptoms and positive SPT and non-allergic children with negative SPT. The groups were compared with Mann-Whitney *U*-test and the medians are shown. Sy=symptoms.

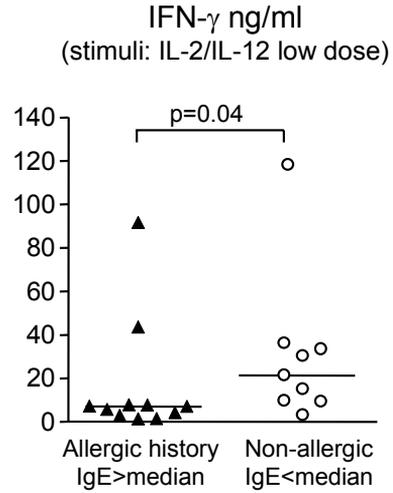


Figure 21. IFN- γ levels after addition of IL-2 and IL-12 (25 μ g/ml), produced by PBMC from children with a cumulative history of allergic disease and high total IgE and non-allergic children with low IgE. The groups were compared with Mann-Whitney *U*-test and the medians are shown.

Table 7. Data of spontaneous and cytokine-induced IL-12R β_2 mRNA expression and production of IFN- γ , IL-10 and IL-5. PBMC from 7-year-old children were cultured for 72 h and mRNA expression was analysed with real-time PCR and the cytokine productions with ELISA. The median values are shown for IL-12R β_2 mRNA/rRNA ratios and for concentrations of the cytokines. Comparisons between non-allergic children and children with a cumulative history of allergic disease, current (within the last year) allergic disease and current allergic airway symptoms were performed. Significant p-values (<0.05) and tendencies (<0.15) are shown. No=number of samples analysed for mRNA and number of positive samples (above cut off level) for cytokines, h=high dose IL-12, l=low dose IL-12.

Stimuli	No.	Non-allergic	No.	Cumulative history of allergic disease	No.	Current allergic disease	No.	Current allergic airway symptoms
IL-12Rβ_2 mRNA/RNA ratio								
Spontaneous	14/17	1.8	21/21	2.8	16/16	1.7	10/10	1.7
IL-2	10/17	9.4	20/21	6.5	16/16	5.9	10/10	6.5
IL-12	11/17	4.2	20/21	4.5	16/16	7.5	10/10	7.5
IL-2/IL-12 (h)	13/17	15.9	19/21	18.1	15/16	12.2	9/10	11.9
IL-2/IL-12 (l)	10/17	18.0	18/21	14.0	14/16	13.7	8/10	6.4 p=0.12
IFN-γ secretion (pg/ml)								
Spontaneous	8/17	192	11/21	402	7/16	402	4/10	559
IL-2	17/17	527	20/21	2996	16/16	2996	10/10	395
IL-12	15/17	1219	19/21	976	14/16	939	9/10	912
IL-2/IL-12 (h)	17/17	34165	21/21	28500	16/16	24576	10/10	16230
IL-2/IL-12 (l)	17/17	21243	21/21	9003	16/16	7736	10/10	7736
IL-10 secretion (pg/ml)								
Spontaneous	9/17	8.0	14/21	13.4	9/16	13.3	6/10	13.3
IL-2	9/17	9.1	18/21	11.6 p=0.001	14/16	10.7 p=0.02	8/10	12.6 p=0.06
IL-12	7/17	7.1	12/21	5.9	10/16	5.9	5/10	5.6
IL-2/IL-12 (h)	15/17	16.6	21/21	23.6	16/16	26.0	10/10	21.5
IL-2/IL-12 (l)	12/17	22.6	19/21	19.9	15/16	20.2	9/10	20.2
IL-5 secretion (pg/ml)								
Spontaneous	5/17	4.7	7/21	4.0	7/16	4.0	5/10	4.3
IL-2	17/17	38.5	20/21	31.8	15/16	31.2	10/10	34.2
IL-12	3/17	5.1	1/21	4.4	1/16	4.4	1/10	4.4
IL-2/IL-12 (h)	15/17	34.2	19/21	24.8	14/16	23.4	9/10	24.8
IL-2/IL-12 (l)	15/17	42.8	20/21	23.4	15/16	22.4	10/10	24.4

Discussion

Children with allergic airway symptoms and high IgE levels had lower IL-2-induced IL-12R β_2 mRNA expression than non-allergic children with low IgE levels. This was accompanied with lower IFN- γ production in response to IL-2 combined with IL-12. Lower IL-12 responsiveness in the form of IFN- γ production, has previously been demonstrated in allergic subjects although the underlying mechanisms are not fully

understood [224-226, 229, 230]. Our findings support our theory that an impaired up-regulation of the IL-12R β_2 unit is one contributing factor to the lower IL-12 responsiveness. Lower IL-12R β_2 expression has previously been reported in nasal biopsies from patients with allergic rhinitis compared to controls [148]. Further, our own findings in paper I, *i e* lower PHA-induced IL-12R β_2 expression in SPT positive, as compared to negative, children also supports this hypothesis. In addition, mutations in the IL-12R β_2 gene have been associated with atopy [149].

IL-2 is important in T cell proliferation and long-term survival (reviewed in [231]). A reduced up-regulation of the IL-12R β_2 -chain in response to IL-2 may therefore contribute to the Th2-skewed responses in allergy.

We found a negative correlation between total levels of IgE and IL-2 and IL-12 induced IL-12R β_2 , with a similar trend for IL-2-induced IL-12R β_2 . As the IgE levels were higher among the children with airway symptoms (n=10, median 443 kU/l) than in children with skin symptoms (n=12, median 150 kU/l), the differences in IL-12R β_2 mRNA expression found in children with airway symptoms in this study may not be associated with the type of allergic disease but the presence of high levels of IgE. Total IgE was also negatively correlated to IFN- γ production following addition of IL-2 together with IL-12. The negative correlation found between total IgE and IL-12R β_2 and IFN- γ production, is in agreement with the fact that IL-12 promotes Th1 like responses and inhibits Th2 like responses, including the effects of IL-4 on IgE synthesis [122]. In addition, IL-12 has been shown to inhibit IgE synthesis in human helminth infections, partly by regulating IFN- γ and IL-4 [232]. An inverse relationship between serum IgE levels and IFN- γ production has previously been reported after culture of PBMC with IL-12 [226], although another study did not find such a relationship [229].

We found higher IL-2-induced production of IL-10 in allergic, compared to non-allergic children. Although the properties of IL-10 include stimulation of humoral immunity [233], most of the IL-10 mediated effects are related to anti-inflammatory functions and regulatory T cells [234, 235]. The role of IL-10 in human allergy is however, somewhat contradictory. Similar to our results, increased spontaneous IL-10 mRNA expression has been reported in skin lesions from patients with atopic dermatitis [236]. Further, peripheral monocytes from the atopic dermatitis patients secreted higher levels of IL-10 than monocytes from control subjects [236]. In asthma, an increased number of cells expressing IL-10 in BAL fluid has been reported, and allergen challenge further increased the IL-10 signalling in T cells [237]. Considering

the anti-inflammatory properties of IL-10, elevated levels may be induced to suppress the ongoing inflammatory process. In contrast, lower IL-10 secretion has been demonstrated in BAL fluid and by PBMC from asthma patients compared to controls [238]. Further, lipopolysaccharide (LPS) stimulation of PBMC induced lower IL-10 production in asthmatics [238]. In addition, grass pollen immunotherapy has been associated with increased numbers of IL-10 expressing cells in the nasal mucosa and with elevated IL-10 production in response to grass pollen [239].

In summary, a reduced IL-2-induced IL-12R β_2 expression was found in children with allergic airway symptoms and high IgE levels compared to non-allergic children. This was accompanied by lower IFN- γ production in response to IL-2 in combination with IL-12, possibly due to impaired up-regulation of the IL-12R β_2 -chain in allergic children. As IL-2 is involved in initiation and sustaining of immune responses, this may contribute to the Th2-skewed immune responses in allergic children.

Paper III: Development of Th1, Th2 and Treg associated immunity during the first two years of life in relation to allergy

In paper III, we followed 35 Swedish and 26 Estonian children prospectively from birth to two years of age. During this period we studied the spontaneous and PHA-induced expression of Th1, Th2 and Treg associated factors in relation to development of allergy. The cytokine receptors IL-12R β_2 and WSX-1, and the transcription factors T-bet, GATA-3 and Foxp3 were analysed at the mRNA level. The production of IFN- γ , IL-4, IL-5, IL-9, IL-10 and IL-13 was analysed at the protein level. We hypothesised that the Th1 associated factors IL-12R β_2 , WSX-1, T-bet and IFN- γ as well as the Treg associated Foxp3 and IL-10 would increase with age. We also hypothesised that the Estonian children, coming from an area with low incidence of allergy would show an earlier induction of immune regulation.

Due to low numbers of Estonian children with available samples from several time-points, comparisons over age are only reported for the Swedish children. As only three Estonian children had allergic symptoms, comparisons between allergic and non-allergic Estonian children could not be done either. Thus, all results reported only refer to the Swedish children, except for the direct comparisons between Sweden and Estonia.

mRNA expression of the cytokine receptors IL-12R β_2 and WSX-1 in PBMC from Swedish children

The spontaneous WSX-1 expression remained similar from birth to 24 months whereas the IL-12R β_2 increased from 6 to 24 months (Table 8). When comparing allergic and non-allergic children separately, the increase of IL-12R β_2 was only found in the allergic children, significantly so between 3 and 12 months (Figure 23a). Spontaneous IL-12R β_2 and WSX-1 expressions were similar at birth and at 24 months however (Figure 23a and c). It was also similar between allergic and non-allergic children at all ages, except for higher IL-12R β_2 expression in the allergic children at 24 months of age (Figure 23a and c).

Culture with PHA resulted in significant up-regulation of both IL-12R β_2 and WSX-1 mRNA expression at all time-points. In the whole group, the PHA-induced expression of WSX-1 did not change over age, but the IL-12R β_2 expression decreased from 3 months. When dividing the children according to allergic symptoms, the decrease of IL-12R β_2 expression was only observed among the non-allergic children (Figure 23b). The PHA-induced WSX-1 expression on the other hand, increased from birth to 24 months among the non-allergic children (Figure 23d). The PHA-induced

expression was similar in allergic and non-allergic children at all ages, except for higher WSX-1 expression in allergic children at birth (Figure 23d).

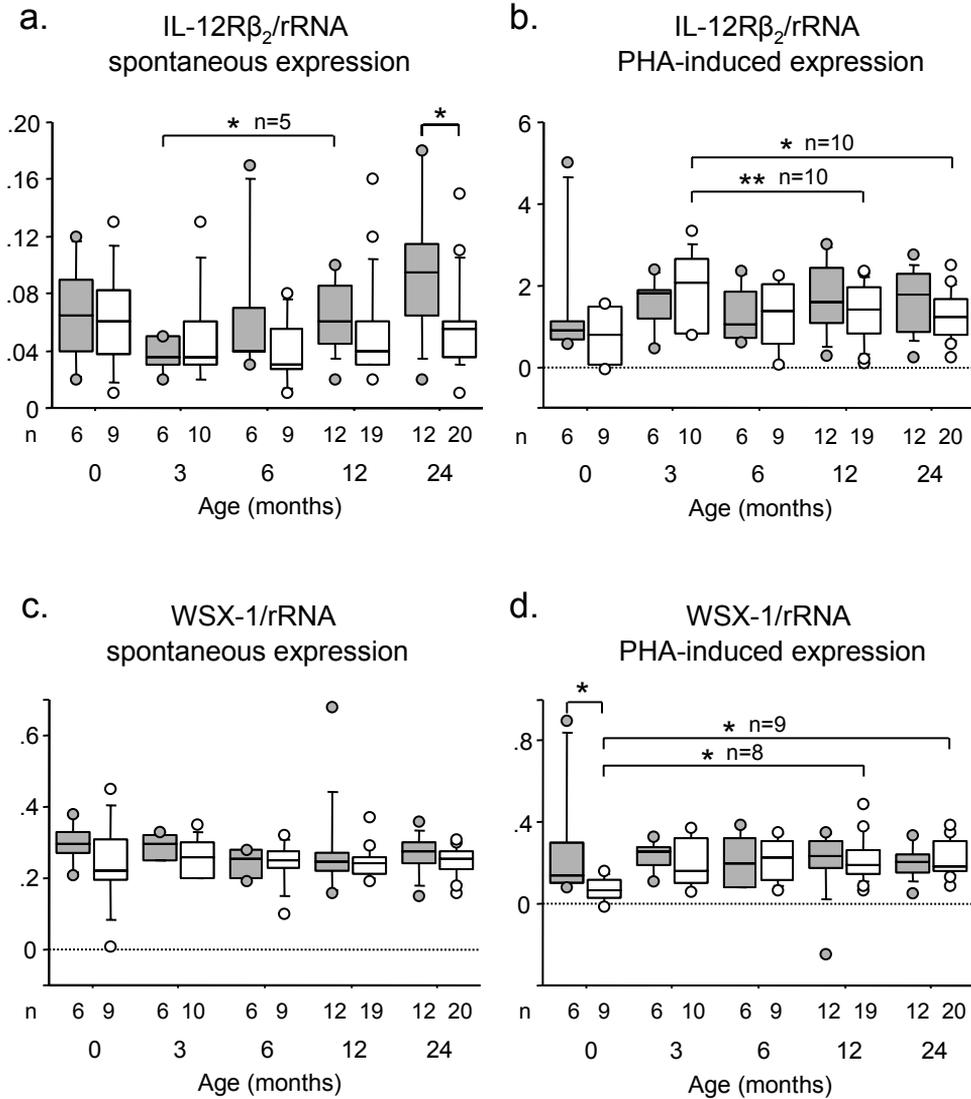
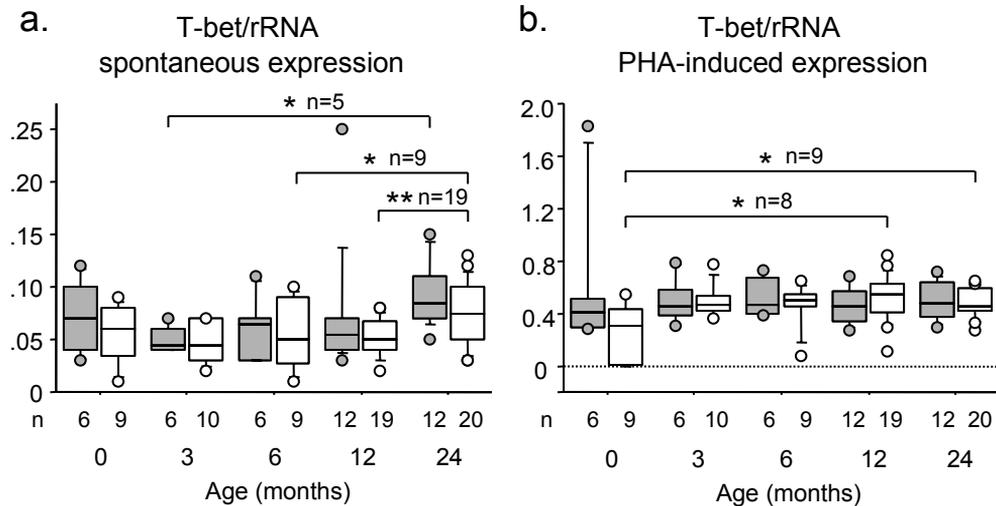


Figure 23. Spontaneous and PHA-induced mRNA expression of IL-12R β_2 (a and b) and WSX-1 (c and d) in allergic (grey boxes) and non-allergic (white boxes) Swedish children followed from birth to 24 months. PBMC were cultured with or without 2 μ g/ml PHA for 24 h. mRNA expressions were analysed with real-time PCR. Paired comparisons were analysed with Wilcoxon signed rank test and unpaired comparisons with Mann-Whitney *U*-test, * $p < 0.05$ and ** $p < 0.01$. The n given next to significant differences corresponds to the number of children in the paired comparison. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers.

mRNA expression of the transcription factors T-bet, GATA-3 and Foxp3 in PBMC from Swedish children

The spontaneous expression of GATA-3 and Foxp3 among all the Swedish children was similar over age, but the T-bet levels increased from 3 to 24 months. The increase of T-bet expression was found in both allergic and non-allergic children, significantly so from 3 to 24 months in allergic and from 6 to 24 months in non-allergic children (Figure 24a). The allergic children also increased their spontaneous expression of GATA-3 from 6 to 24 months of age (Figure 24c). The spontaneous levels of T-bet, GATA-3 and Foxp3 at birth and at 24 months did not differ, and there were no significant differences between allergic and non-allergic children at any specific age (Figure 24a, c and e).

In the whole group, PHA induced significant up-regulation of T-bet and Foxp3 at all ages. GATA-3 was up-regulated at birth and 24 months, but no up-regulation was observed at 3, 6 and 12 months. When analysing the allergic and non-allergic children separately, GATA-3 up-regulation was found among the allergic children at 6 and 12 months of age (Figure 25a-b). PHA-induced expression of T-bet increased from birth to 24 months in the whole group. When dividing the children as allergic or not, this increase was only apparent in the non-allergic group of children (Figure 24b). No changes over age were observed concerning PHA-induced GATA-3 or Foxp3 expressions among all children, or in allergic and non-allergic children analysed separately (Figure 24d and f).



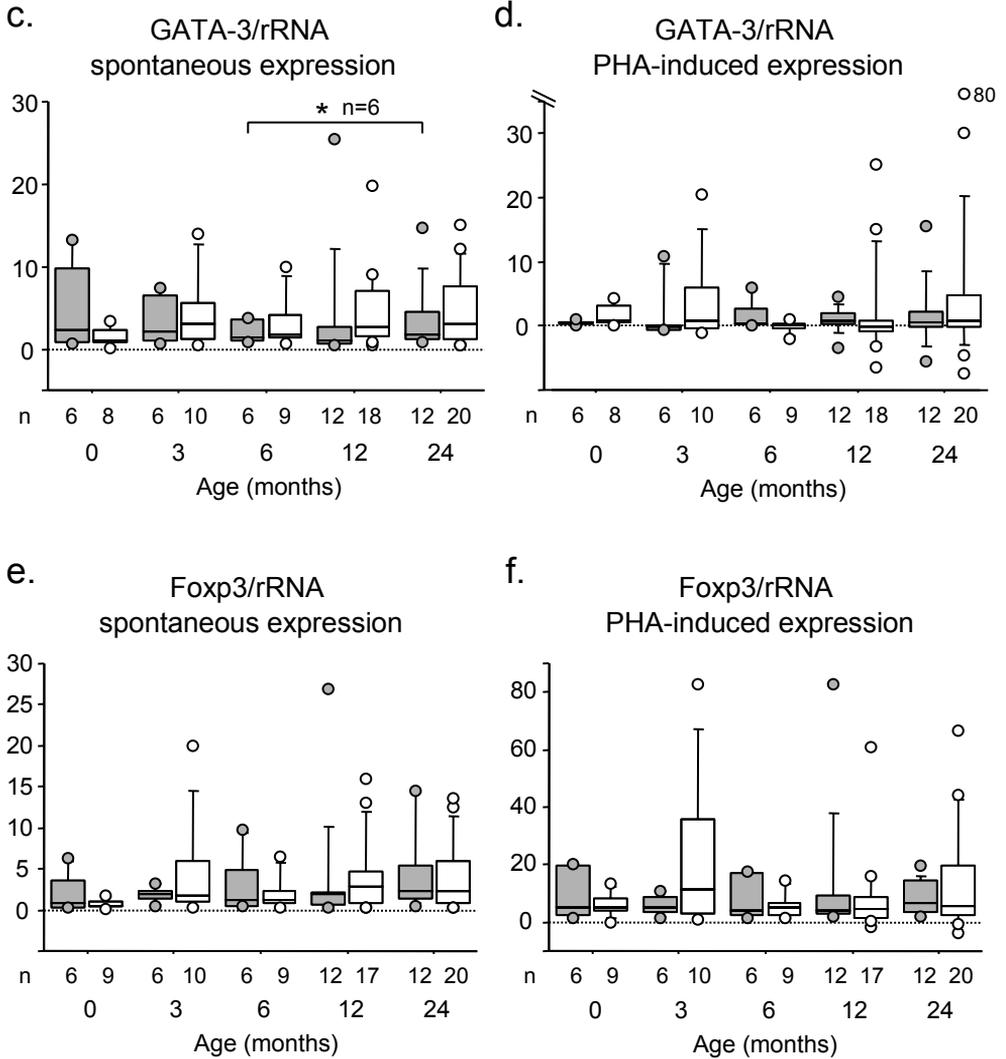


Figure 24. Spontaneous and PHA-induced mRNA expression of T-bet (a and b), GATA-3 (c and d) and Foxp3 (e and f) in allergic (grey boxes) and non-allergic (white boxes) Swedish children followed from birth to 24 months. PBMC were cultured with or without 2 µg/ml PHA for 24 h. mRNA expressions were analysed with real-time PCR. Paired comparisons were analysed with Wilcoxon signed rank test and unpaired comparisons with Mann-Whitney *U*-test, * $p < 0.05$ and ** $p < 0.01$. The n given next to significant differences corresponds to the number of children in the paired comparison. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers.

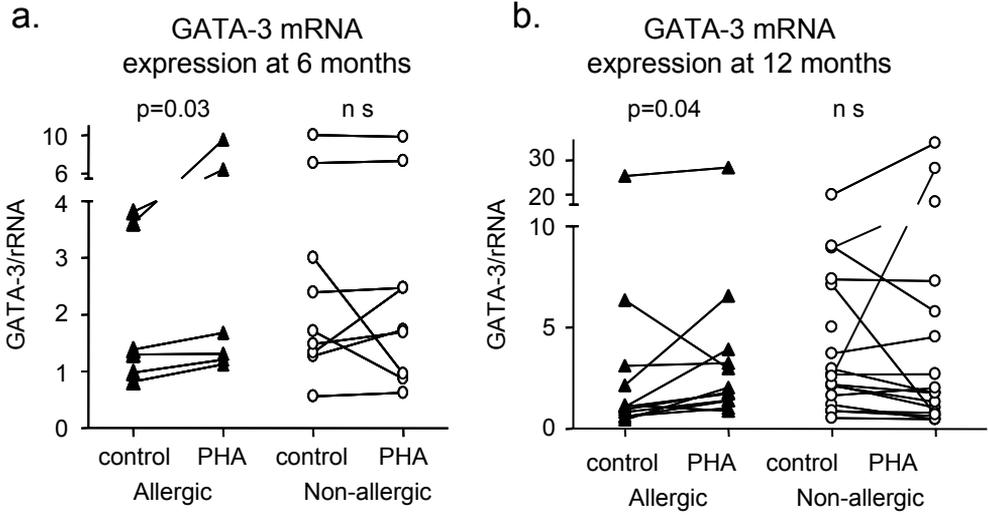


Figure 25. GATA-3 mRNA expression at 6 (a) and 12 months (b) in allergic (n=6 at 6 months and n=9 at 12 months) and non-allergic (n=9 at 6 months and n=18 at 12 months) Swedish children. PBMC were cultured in medium alone (control) or with 2 $\mu\text{g/ml}$ PHA for 24 h. mRNA expressions were analysed with real-time PCR. P-values are given for statistical significant differences between unstimulated and stimulated cell cultures, analysed with Wilcoxon signed rank test.

Table 8. mRNA ratios of spontaneous and PHA-induced WSX-1, IL-12R β_2 , T-bet, GATA-3 and Foxp3 at different ages in the Swedish children. PBMC were cultured with or without 2 μ g/ml PHA for 24 h. Median values as well as minimum and maximum values are given. Significant differences ($p < 0.05$) are indicated with a letter and explained below the table. Spont=spontaneous expression relative rRNA, PHA=PHA-induced expression relative rRNA, spontaneous expression withdrawn.

mRNA/rRNA	0 months median (min-max)	3 months median (min-max)	6 months median (min-max)	12 months median (min-max)	24 months median (min-max)	
WSX-1	spont	0.28 (0.01-0.45)	0.29 (0.20-0.35)	0.25 (0.10-0.32)	0.24 (0.16-0.49)	0.26 (0.15-0.36)
	PHA	0.11 (-0.01-0.90)	0.24 (0.06-0.37)	0.24 (0.07-0.39)	0.22 (-0.25-0.49)	0.20 (0.05-0.40)
IL-12Rβ_2	spont	0.06 (0.01-0.13)	0.04 ^a (0.02-0.13)	0.04 ^b (0.01-0.17)	0.05 ^c (0.02-0.16)	0.06 (0.01-0.18)
	PHA	0.90 (-0.04-5.03)	1.88 ^g (0.45-3.34)	1.28 (0.07-2.34)	1.57 (0.10-3.02)	1.32 (0.24-2.77)
T-bet	spont	0.06 (0.01-0.12)	0.05 ^d (0.02-0.07)	0.06 ^e (0.01-0.11)	0.05 ^f (0.02-0.25)	0.08 (0.03-0.15)
	PHA	0.36 (0.00-1.83)	0.46 (0.30-0.79)	0.52 (0.08-0.73)	0.53 (0.11-0.93)	0.48 ^h (0.27-0.72)
GATA-3	spont	1.48 (0.18-19.33)	2.54 (0.51-13.95)	1.60 (0.56-14.21)	2.18 (0.44-25.36)	2.56 (0.39-40.96)
	PHA	0.59 (-7.21-4.18)	0.01 (-5.25-20.50)	0.22 (-6.20-5.93)	0.26 (-6.43-25.04)	0.67 (-7.33-80.40)
Foxp3	spont	0.50 (0.09-14.13)	2.02 (0.26-20.01)	1.32 (0.37-16.04)	2.05 (0.29-26.90)	2.56 (0.22-17.47)
	PHA	5.30 (0.09-33.37)	6.53 (0.79-82.75)	4.68 (1.25-23.31)	4.46 (-1.50-82.56)	6.45 (-3.60-120.93)

^a \uparrow 3-12 mo, n=17

^b \downarrow 0-6 mo, n=7 and \uparrow 6-24 mo, n=16

^c \uparrow 12-24 mo n=32

^d \uparrow 3-24 mo, n=16

^e \uparrow 6-24 mo, n=16

^f \uparrow 12-24 mo, n=32

^g \downarrow 3-12 mo, n=17

^h \uparrow 0-24 mo, n=15

mRNA expression Sweden – Estonia

The spontaneous and PHA-induced expression of WSX-1, GATA-3, Foxp3 and T-bet was similar in Swedish and Estonian children at all ages except for higher spontaneous expression of all these markers at 3 months in Swedish children (Table 9). In addition, PHA-induced WSX-1 at birth was lower in Swedish as compared to Estonian children. Spontaneous as well as PHA-induced IL-12R β_2 expression was similar at all ages in Swedish and Estonian children (Table 9).

When comparing non-allergic Swedish and Estonian children, lower PHA-induced WSX-1 at birth was still observed among the Swedish children. Comparisons of non-allergic children only, also revealed higher spontaneous GATA-3 expression at 12 months, lower PHA-induced IL-12R β_2 at birth and higher PHA-induced T-bet at 3 months among the Swedish children.

Table 9. mRNA ratios of spontaneous and PHA-induced WSX-1, IL-12R β_2 , T-bet, GATA-3 and Foxp3 at different ages in Swedish and Estonian children. PBMC were cultured with or without 2 μ g/ml PHA for 24 h. Median values are given and significant differences ($p < 0.05$) between Estonian and Swedish children are shown in bold and with arrows. Spont=spontaneous expression relative rRNA, PHA=PHA-induced expression relative rRNA, spontaneous expression withdrawn.

mRNA/rRNA		0 months median		3 months median		6 months median		12 months median	
		Swe	Est	Swe	Est	Swe	Est	Swe	Est
WSX-1	spont	0.28	0.24	0.29 ↔ 0.17		0.25	0.22	0.24	0.23
	PHA	0.11 ↔ 0.19		0.24	0.12	0.24	0.20	0.22	0.24
IL-12R β_2	spont	0.06	0.06	0.04	0.04	0.04	0.06	0.05	0.05
	PHA	0.90	1.67	1.88	1.42	1.28	1.22	1.57	1.26
T-bet	spont	0.06	0.04	0.05 ↔ 0.03		0.06	0.05	0.05	0.05
	PHA	0.36	0.27	0.46	0.40	0.52	0.52	0.53	0.61
GATA-3	spont	1.48	0.88	2.54 ↔ 0.70		1.60	0.99	2.18	0.90
	PHA	0.59	0.61	0.01	0.37	0.22	0.16	0.26	0.00
Foxp3	spont	0.50	0.84	2.02 ↔ 0.68		1.32	0.85	2.05	1.03
	PHA	5.30	3.70	6.53	2.13	4.68	4.61	4.46	3.14

Cytokine secretion by PBMC from Swedish children

The production of IFN- γ , IL-4, IL-5, IL-9 and IL-13 increased with age, although the IL-13 production decreased between birth and 3 months (Figure 26). Both allergic and non-allergic children increased their cytokine production with age, significantly so from birth to 24 months for IL-4 and IL-5, from 3 months for IL-13 and from 6 months for IFN- γ . The IL-9 production increased between 3 and 24 months in non-allergic and between 12 and 24 months in allergic children (Figure 27a).

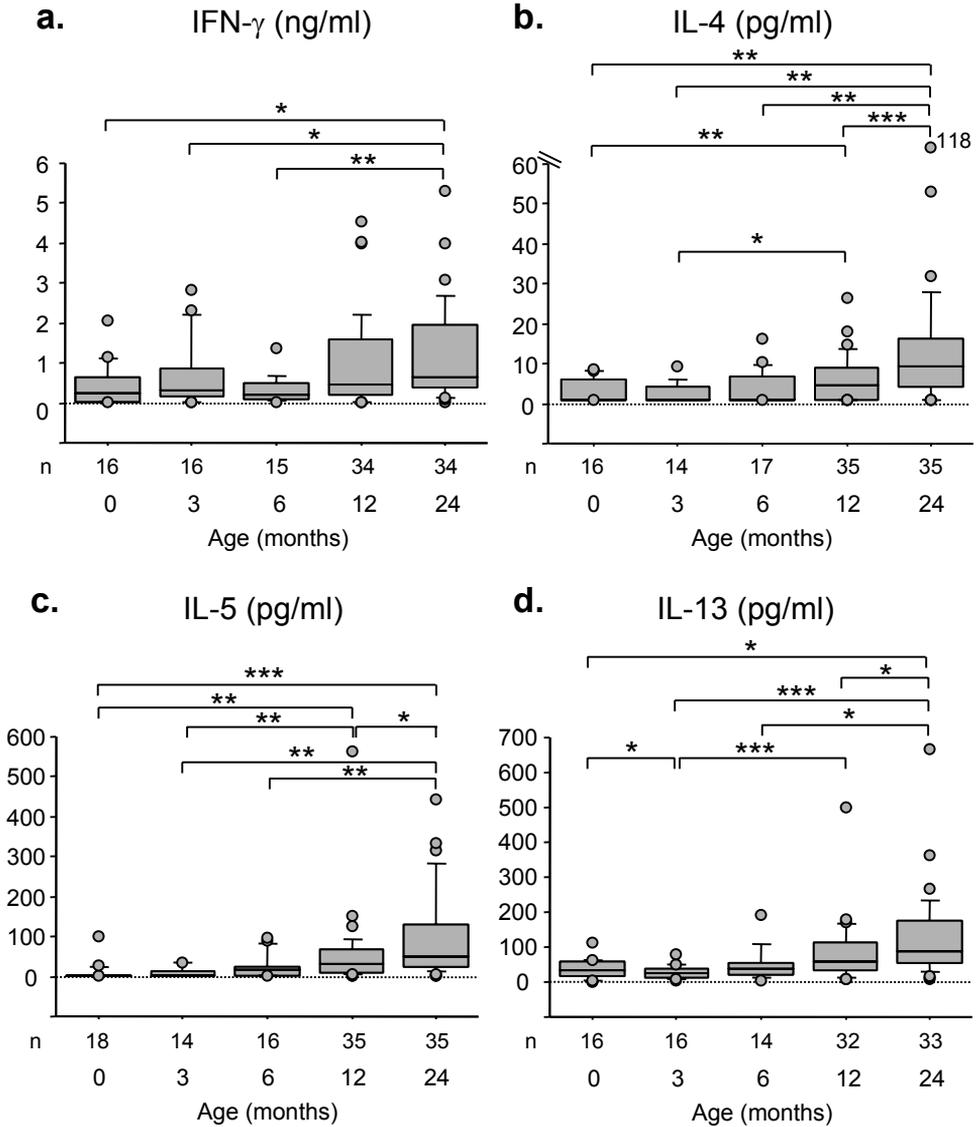


Figure 26. PHA-induced production of IFN- γ (a), IL-4 (b), IL-5 (c) and IL-13 (d) by PBMC from Swedish children followed from birth to two years of age. The cytokines were analysed with ELISA after culture of PBMC with 2 μ g/ml PHA for 24 h. Comparisons were made using Wilcoxon signed rank test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers.

The levels of IL-10 were similar at birth and 24 months, although there was an increase between 6 and 24 months. When analysing the allergic and non-allergic children separately, the IL-10 secretion increased with age in the allergic children whereas no difference was found in the non-allergic group (Figure 27b).

At a given age, the allergic and non-allergic children produced similar levels of all cytokines except for IL-9, which was produced in higher amounts in allergic children at 24 months (Figure 26 and Figure 27).

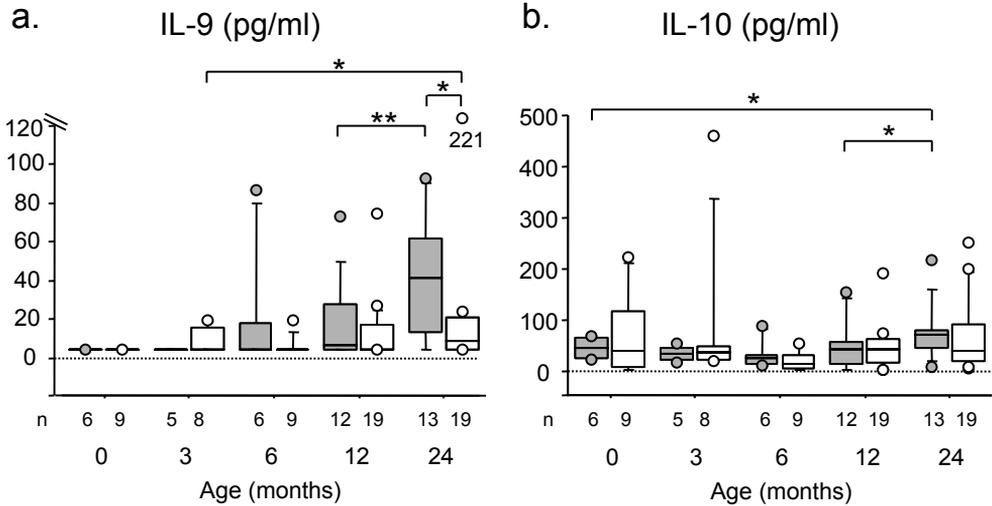


Figure 27. PHA-induced production of IL-9 (a) and IL-10 (b) by PBMC from allergic (grey boxes) and non-allergic (white boxes) Swedish children followed from birth to two years of age. The cytokines were analysed with ELISA after culture of PBMC with 2 µg/ml PHA for 24 h. Paired comparisons were made using Wilcoxon signed rank test and unpaired test using Mann-Whitney *U*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers.

Cytokine secretion Sweden – Estonia

The Swedish children produced significantly more IL-5 and IL-13 at 6 months and significantly more IL-4, IL-5 and IL-10 at 24 months compared to the Estonian children. When comparing non-allergic Swedish children with non-allergic Estonian children, IL-5, IL-10 and IL-13 secretion were still higher among the Swedish children at 24 months. No other differences were found regarding cytokine secretion in the Swedish and Estonian group.

Correlations

Among the Swedish children, the transcription factors GATA-3 and Foxp3 showed the strongest correlation. The spontaneous expression showed rho-values around 0.9 at all ages ($p < 0.0001$) whereas the strongest correlations after PHA stimulation was found at older ages (12 months, $\rho = 0.5$ and 24 months, $\rho = 0.7$ $p < 0.01$). From 6 months the PHA-induced Foxp3 also correlated well with PHA-induced T-bet ($\rho = 0.7-0.8$,

$p < 0.01$). The same was also found for PHA-induced T-bet and WSX-1 in the Swedish children ($\rho = 0.6-0.9$, $p = 0.01$).

PHA-induced IFN- γ correlated well with the PHA-induced expression of IL-12R β_2 at all ages in the Swedish children ($\rho = 0.5-0.9$, $p \leq 0.01$). PHA-induced IFN- γ also correlated with spontaneous expression of IL-12R β_2 , but only at older ages (12 months $\rho = 0.45$, $p = 0.01$, 24 months $\rho = 0.57$, $p < 0.01$). The PHA-induced IL-13 production also correlated with IL-12R β_2 expression, the correlation being weaker with age ($\rho = 0.4-0.9$, $p < 0.05$). The other cytokines and mRNA expressions did not show a clear correlation pattern.

Despite the lower numbers of Estonian children a clear correlation pattern was found between PHA-induced IFN- γ and IL-12R β_2 mRNA expression, but it was only significant at birth and 3 months ($\rho = 0.7-1.0$, $p < 0.05$).

Discussion

In this study we followed the expression of Th1, Th2 and Treg associated factors during the first two years of life. In general, we found more fluctuations in the Th1 associated markers IL-12R β_2 , WSX-1 and T-bet at different ages, while the Th2 associated GATA-3 and the Treg associated Foxp3 were similarly expressed during this time period of life.

The fluctuations found in the Th1 associated factors may relate to the Th2 deviated environment in the uterus and may suggest that the Th1 like responses are more affected by the development of the immune system [68]. However, the levels at birth and at two years of age were similar, with the exception of WSX-1. This may indicate that the maturation of the immune system, regarding the factors studied here, do not come that far during the first two years of life, but may need further time. On the other hand, it is reasonable to believe that the birth process influences and activates the immune system in several ways. Therefore, the use of cord blood may not reflect the “true” status of the immune system in the newborn child. A blood sample taken from the newborn 1 or 2 weeks post partum may have resulted in different, possibly lower levels than those found in cord blood.

None of the factors studied here has to our knowledge been studied during childhood before. However, there are some reports where the expression of IL-12R β_2 , T-bet, GATA-3 and Foxp3 has been compared in cord blood and in cells from adults [75-78]. Lower spontaneous T-bet [77], but similar Foxp3 expression [76] were

reported in CD4 positive cells from cord and adult blood, respectively. Stimulation with anti-CD3 and anti-CD28 induced similar levels of IL-12R β_2 in naïve T cells (CD45RA⁺), although the expression was undetectable in memory cells (CD45RO⁺) from cord, compared to adult blood [75]. Depending on stimuli, different patterns of GATA-3 and T-bet induction have been reported in cord and adult peripheral blood [78].

In response to PHA, the expression of the Th1 associated WSX-1 and T-bet followed the same pattern, *i e* increased with age in non-allergic children and displayed steady high levels in the allergic children. Spontaneous expression of T-bet also increased with age, in both allergic and non-allergic children, whereas the spontaneous WSX-1 expression was similar in both groups from birth to two years of age. The increase of T-bet with age fits well with the data on the Th2 deviation of immune responses present in newborns, but can not explain the earlier induction of T-bet in children who later develops allergy. T-bet is required for Th1 differentiation and is expressed in Th1, but not Th2, committed cells [174]. Asthma has previously been associated with lower T-bet expression [176], but similar levels have been reported in patients with atopic eczema and non-atopic controls [180]. All but one of the allergic children in our study had eczema. Most studies on WSX-1 and its ligand IL-27 have been done in mice, and to our knowledge there are no reports on WSX-1 expression in children or cord blood. WSX-1 was first discovered to have important effects in mounting strong Th1 responses early in infection [154, 158]. Lately the importance of WSX-1 in down-regulation of immune responses, protecting the host from lethal immune mediated damages, has been emphasised [161]. In mice, WSX-1 has been shown to have inhibitory properties on allergic asthma responses [167]. Considering its involvement in mounting Th1 responses it could be speculated that this might also be the case in humans. Our results, showing similar levels or even higher levels of WSX-1 in allergic children at birth, do not indicate such a function in humans, however.

The spontaneous expression of the IL-12R β_2 -chain was the same at birth and at 24 months, with lower levels in between. This pattern was most evident in the allergic children and they displayed higher IL-12R β_2 levels than the non-allergic children at 24 months. Different spontaneous expression of IL-12R β_2 between allergic and non-allergic individuals was not found in our other study groups, comprising children (paper I-II) or adults (paper IV). Another group reported lower spontaneous IL-12R β_2 in asthma patients compared to non-asthmatics [144]. The high levels in cord blood may indicate that the birth process itself may induce up-regulation of IL-12R β_2

mRNA expression, which then is down-regulated after birth. Like the spontaneous expression, PHA-induced IL-12R β_2 was similar at birth and at 24 months. Similar levels of IL-12R β_2 have also been reported after anti-CD3/anti-CD28 stimulation of naïve T cells from cord blood and from peripheral blood from adults [75]. The non-allergic children showed a decrease in PHA-induced expression from 3 months, however. At a specific age, PHA stimulation induced similar levels of IL-12R β_2 in allergic and non-allergic children, in contrast to the findings in paper I, but similar to the findings in paper IV. The age of the children, and related to that, the predominant allergic symptoms in the two study groups, *i e* eczema in this group of infants and airway symptoms in the group of 12-year-olds in paper I, are probably contributing factors to the different findings in this study and in paper I.

Elevated levels of the Th2 associated transcription factor GATA-3 have been reported in adults with asthma [201-203] and atopic dermatitis [180]. We did not find any different expression of GATA-3 in our allergic and non-allergic children. However, at some ages only the allergic children up-regulated GATA-3 expression in response to PHA. In addition, the allergic children increased their spontaneous GATA-3 expression from 6 to 24 months.

Spontaneous and PHA-induced Foxp3 was equally expressed in allergic and non-allergic children from birth up to two years. The proportion of CD4⁺CD25⁺ positive T cells, as well as their expression of Foxp3, have recently been demonstrated to be similar in neonates and adults [76]. Foxp3 in relation to allergy is an unknown field, although one recent study demonstrated low levels of Foxp3 mRNA in atopic dermatitis skin, despite high amounts of CD25⁺ cells [215]. The same study showed a significant inhibition by CD4⁺CD25⁺ T cells on *Der p1*-specific proliferation of PBMC from house dust mite allergic individuals. Foxp3 was not analysed in that experiment, however. Similar to our results, spontaneous Foxp3 expression has been reported to be similar in CD4 positive T cells from patients with asthma and control subjects [216].

In response to PHA stimulation, the secretion of all analysed cytokines except IL-10, *i e* IFN- γ , IL-4, IL-5, IL-9 and IL-13 increased from birth to 24 months of age. The IL-10 secretion increased with age in the allergic group but not in the non-allergic group. Similar to our results, previous studies have also demonstrated an increased PHA-induced production of both Th1 and Th2 cytokines with age [61, 62, 64].

Like in paper I and II, a strong correlation was found between the IL-12R β_2 expression and the secretion of IFN- γ , again supporting the connection between IL-12

responsiveness and IFN- γ production [96, 105, 133, 227, 228]. IL-12R β_2 expression also correlated with the IL-13 production, but this was most pronounced at young ages and had disappeared by the age of two years.

Among the transcription factors there was a strong correlation between Foxp3 and GATA-3, especially at baseline, perhaps indicating that the regulatory type of responses are more related to the Th2 type of responses than to the, in general more aggressive, Th1 like responses. However, after PHA stimulation Foxp3 also correlated strongly with T-bet, suggesting that the regulatory responses are induced both during Th2 and Th1 activation.

The mRNA expression of the studied factors did not differ in Swedish and Estonian children at a specific age, except for at three months. At that age, several factors were expressed at higher levels in the Swedish children. Considering the higher incidence of allergy in Sweden, different maturation patterns may occur in the two countries, although our results did not indicate that. The expression of these factors will also be analysed following allergen stimulation, which may be more accurate concerning allergy development. Unfortunately, we were not able to perform paired statistical analyses in the Estonian group of children, due to the low number of Estonian children with samples from several time-points.

In summary, the expression of the receptors IL-12R β_2 and WSX-1 and the transcription factors T-bet, GATA-3 and Foxp3 fluctuated in part during the first two years of life. However, similar levels were found at birth and two years except for increasing PHA-induced WSX-1 and T-bet in non-allergic children. The production of the cytokines analysed increased with age in response to PHA. Different developmental patterns of Th1 and Th2 associated factors may have implications for the development of allergic diseases in childhood.

Paper IV: CD2 controlled expression of regulatory T cell associated Foxp3 and IL-10 in humans

In paper IV, we studied the CD2 signalling pathway more closely by the use of stimulatory antibodies towards the CD2 receptor. PBMC from 10 allergic and 10 non-allergic adults were cultured with CD2 antibodies alone or in combination with antibodies to CD28, or with PHA. The expression of the cytokine receptors IL-12R β ₂ and WSX-1, the transcription factors T-bet, GATA-3 and Foxp3 as well as the production of IFN- γ , IL-5 and IL-10 was analysed in response to CD2, CD2/CD28 or PHA stimulation. We hypothesised that the allergic subjects would have a reduced CD2 function and thereby have diminished responses to the CD2 antibodies concerning the Th1 and Treg associated markers, compared with the non-allergic subjects.

mRNA expression and production of cytokines

The use of anti-CD2 antibodies alone increased the mRNA expression of the Treg associated Foxp3 and the cytokine production of IL-10, indicating a role for CD2 in the action of regulatory T cells (Figure 28). Interestingly, addition of anti-CD28 together with anti-CD2 resulted in decreased amounts of Foxp3 compared to control cells. PHA stimulation induced an up-regulation of both Foxp3 mRNA and IL-10 secretion, which was higher than the CD2-induced levels. CD2 and PHA-induced Foxp3 mRNA correlated ($\rho=0.54$, $p=0.02$), and a similar trend was found for IL-10 secretion.

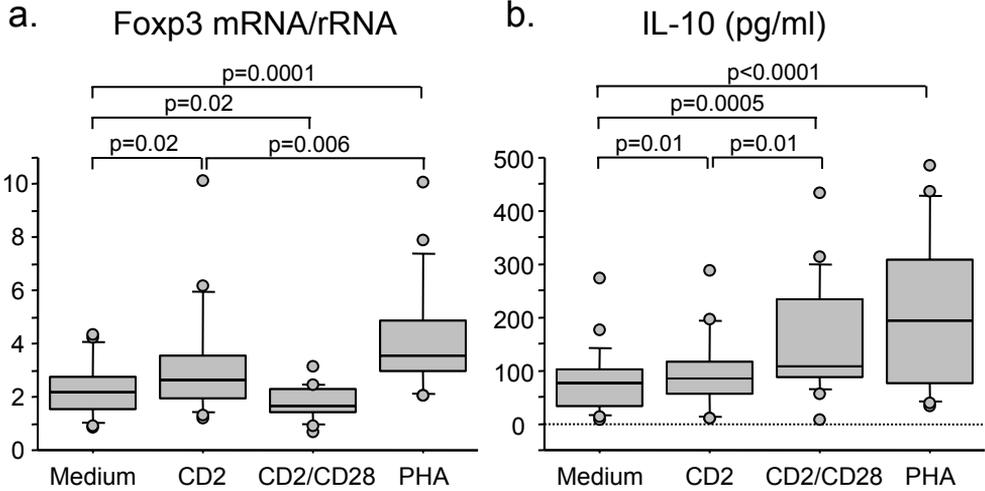


Figure 28. mRNA expression of Foxp3 (a) and secretion of IL-10 (b) in PBMC from adults. The cells were cultured in medium alone, with 100 ng/ml of a pair of anti-CD2 antibodies, with 100 ng/ml anti-CD2 and 100 ng/ml anti-CD28 antibodies or with 2 μ g/ml PHA for 70 h. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers. P-values below 0.05 after Wilcoxon signed rank test are given, n=20 except for PHA-stimulated Foxp3 where n=19.

CD2 stimulation alone did not change the expression of IL-12R β ₂, WSX-1, T-bet or GATA-3 compared to the unstimulated cells (Figure 29a-d). The combination of the CD2 and CD28 antibodies as well as PHA resulted in higher expression of these factors, however. The CD2/CD28 and the PHA-induced mRNA expression did not correlate for any of the factors, which indicates different signalling pathways. PHA was more potent in stimulating the IL-12R β ₂-chain and WSX-1 up-regulation than the CD2/CD28 combination.

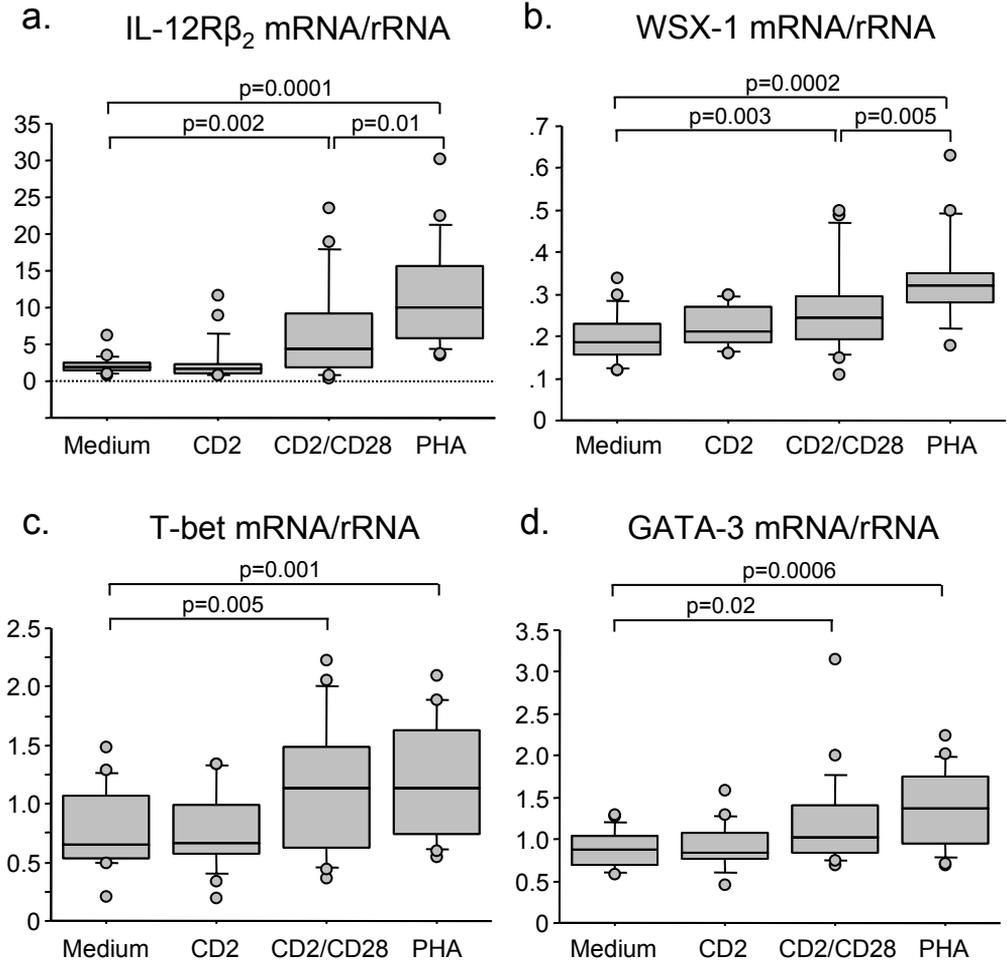


Figure 29. mRNA expression of IL-12R β_2 (a), WSX-1 (b), T-bet (c) and GATA-3 (d) in PBMC from adults. The cells were cultured in medium alone, with 100 ng/ml of a pair of anti-CD2 antibodies, with 100 ng/ml anti-CD2 and 100 ng/ml anti-CD28 antibodies or with 2 μ g/ml PHA for 70 h. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers. P-values below 0.05 after Wilcoxon signed rank test are given, n=20 for all stimulations except for PHA where n=19.

As mentioned previously, stimulation via CD2 alone induced production of IL-10. In contrast, the IFN- γ secretion was lower in CD2 stimulated as compared to the control cells, whereas the IL-5 production was similar in CD2 and control cultures (Figure 30 and Figure 31). CD2/CD28 and PHA induced increased levels of all three cytokines (Figure 28b, Figure 30 and Figure 31). The levels were similar after CD2/CD28 and PHA but they were not correlated to each other.

Allergic and non-allergic subjects displayed similar responses to CD2,

CD2/CD28 and PHA stimulation, concerning all factors studied.

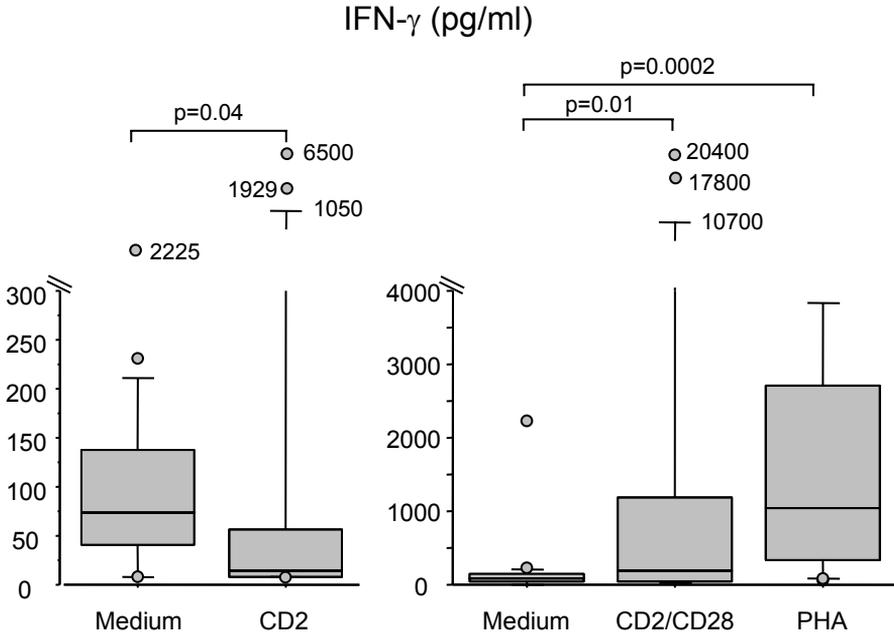


Figure 30. Secretion of IFN- γ after culture of PBMC with 100 ng/ml of a pair of anti-CD2 antibodies, 100 ng/ml anti-CD2 and 100 ng/ml anti-CD28 antibodies or with 2 μ g/ml PHA. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers. The stimulations were compared using Wilcoxon signed rank test, $p < 0.05$ are shown, $n = 20$.

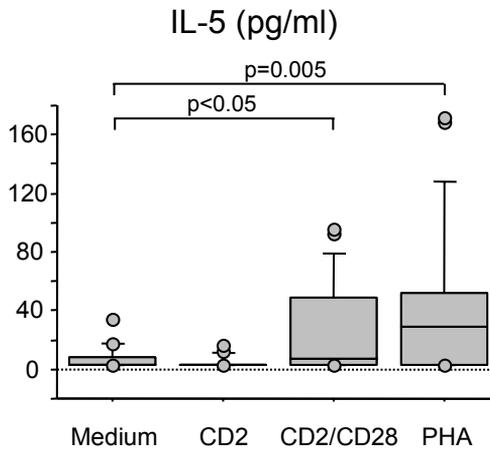


Figure 31. Secretion of IL-5 after culture of PBMC with 100 ng/ml of a pair of anti-CD2 antibodies, 100 ng/ml anti-CD2 and 100 ng/ml anti-CD28 antibodies or with 2 μ g/ml PHA. The stimulations were compared using Wilcoxon signed rank test, $p < 0.05$ are shown, $n = 20$. The 10th, 25th, 50th, 75th and 90th percentiles are indicated as well as outliers.

Correlations

The parameters up-regulated by CD2/CD28 activation, *i e* all but Foxp3, correlated to each other after CD2/CD28 stimulation ($\rho=0.53-0.96$, $p<0.0001-0.02$), except for WSX-1 and IL-5. The strongest correlations were found between CD2/CD28 up-regulated IL-12R β_2 mRNA and T-bet mRNA ($\rho=0.96$, $p<0.0001$), IL-12R β_2 mRNA and IFN- γ production ($\rho=0.94$, $p<0.0001$) and T-bet mRNA and IFN- γ production ($\rho=0.89$, $p=0.0001$). The strongest correlations after PHA stimulation were found between IL-12R β_2 and T-bet mRNA ($\rho=0.85$, $p=0.0003$) and between IL-12R β_2 mRNA and IFN- γ production ($\rho=0.74$, $p=0.002$).

Intracellular calcium

CD2 stimulation resulted in a slow and small increase of the intracellular calcium concentration, median increase after 1 minute 7 nM (min-max 2-28 nM) and after 10 minutes 38 nM (min-max 21-97 nM). Our positive control ionomycin gave higher calcium levels in all samples (median; 1 min 110 nM, 10 min 375 nM), indicating that the cells were not responding with maximum calcium mobilisation after addition of the CD2 antibodies. The calcium responses were similar in allergic and non-allergic individuals.

Discussion

In this study we found that CD2 stimulation up-regulated the Treg associated Foxp3 as well as induced the production of the anti-inflammatory and immuno-suppressive cytokine IL-10 [240]. A role of CD2 in T cell energy has previously been implicated in a couple of studies [107, 108]. Wakkach *et al* found that co-stimulation via CD2 alone induced differentiation of regulatory T cells secreting high levels of IL-10 [108]. These cells were able to suppress proliferation of bystander resting T cells in response to anti-CD3 and anti-CD28 stimulation. Further, the same study showed that simultaneous co-stimulation via CD2 and CD28 did not induce regulatory cells, but stimulated high levels of IL-2 and IFN- γ . IL-10 has previously been shown to down-regulate the co-stimulatory molecules CD80 and CD86 and the adhesion molecule CD54 (ICAM-1) on monocytes, but the CD58/LFA-3, *i e* the CD2 ligand was unaffected [241]. This also supports a role for CD2-CD58 signalling in regulatory actions, although conflicting results, *i e* down-regulation of CD58 and not of CD80 and CD54, have been reported [242].

Several properties of CD2 in T cell activation have been reported, *i e* adhesion [93], direct promoter of T cell activation [92], co-stimulatory effects [87, 91, 94], or induction of energy [107, 108]. CD2 signalling also promotes Th1 like responses as it

enhances the IL-12 responsiveness of activated T cells [84], which according to paper I may be caused by an up-regulation of the IL-12R β_2 chain. Co-stimulation via CD28 on the other hand, drives activation and expansion of naïve cells and prevents induction of anergy [111].

Addition of anti-CD2 in combination with anti-CD28 antibodies induced an up-regulation of the IL-12R β_2 -chain although the CD2 antibodies alone did not. As reported in the previous papers in this thesis, PHA also increased the IL-12R β_2 -chain expression. The IL-12R β_2 expression correlated with the Th1 associated IFN- γ secretion and T-bet mRNA expression. This was not observed after CD2 stimulation alone, however. On the contrary, CD2 signalling alone seemed to hamper Th1 like immunity as mirrored by a reduced IFN- γ secretion. Addition of CD2 and CD28, but not CD2 alone, also up-regulated the other Th1 and Th2 associated factors studied, *i.e.* WSX-1, GATA-3 and IL-5. In contrast to the initiation of Th1 and Th2 like responses, and to some extent also the increased IL-10 secretion, by CD2 and CD28 activation, the regulatory associated Foxp3 was down-regulated. Similar to our results, CD28^{+/+} T cells from mice down-regulated Foxp3 expression in response to antigen, whereas CD28^{-/-} T cells induced up-regulation of the Foxp3 expression [118]. This makes sense as activation of Th1 and Th2 like responses naturally should lead to down-regulation of regulatory functions, at least temporarily. Thus, CD2 signalling may induce T regulatory cells and functions when working without other co-stimulatory signals, whereas it can enhance responses and break T cell anergy in co-operation with other co-stimulatory signals. To our knowledge this is the first study reporting CD2 and CD28 controlled expression of the cytokine receptors IL-12R β_2 and WSX-1 and the transcription factors T-bet, GATA-3 and Foxp3.

We did not find any differences between allergic and non-allergic individuals in the CD2/CD28 or PHA-induced mRNA expression of IL-12R β_2 , WSX-1, T-bet, GATA-3 or Foxp3 nor in the production of IFN- γ , IL-5 or IL-10. Lower T-bet expression in the airways has been associated with human asthma and T-bet deficient mice develop airway changes consistent with human asthma [176]. On the contrary, patients with atopic dermatitis have similar spontaneous T-bet mRNA expression in PBMC as control subjects [180]. High expression of GATA-3 has been reported to be associated with both atopic dermatitis [180] and asthma [201, 203, 204]. The spontaneous expression of GATA-3 in PBMC from house dust mite sensitised individuals is lower than in non-atopic individuals [243]. Stimulation with house dust mite allergen or with anti-CD3/IL-2 resulted in increased levels of GATA-3 in the sensitised individuals, whereas the levels in non-atopics decreased after allergen

stimulation and was unaffected after CD3/IL-2 stimulation. Similar to our results, PHA stimulation did not result in any different GATA-3 expression in atopic and non-atopic individuals, however [243].

Foxp3 in relation to allergy is mostly an unknown field. Low levels of Foxp3 mRNA and protein have been reported in human atopic dermatitis skin [215]. Another study investigating the effect of glucocorticoid treatment on the expression of regulatory T cell associated markers, reported similar Foxp3 expression in non-stimulated CD4 T cells in patients with moderate asthma and non-asthmatic controls [216]. The expression of WSX-1, has to our knowledge not been studied in relation to allergy in humans, but WSX-1 deficient mice show enhanced airway hyperresponsiveness, increased bronchoalveolar infiltration of lymphocytes and eosinophils and enhanced production of antigen-specific IgE, IL-4, IL-13 and IFN- γ [167].

Lower spontaneous mRNA expression of IL-12R β_2 has been observed in patients with asthma compared to non-asthmatic controls [144]. This is in contrast to the results in this paper but also to our previous findings in the children in paper I and II. In contrast to our findings of lower PHA-induced IL-12R β_2 in allergic children, we did not observe any difference in IL-12R β_2 expression in allergic and non-allergic adults after addition of PHA. The different results in this paper and in paper I may be due to a greater signal redundancy in the allergic adult compared to the allergic child, different time of stimulation, *i e* 70 h here and 24 h in paper I, or to the rather small material.

Although these results in adults do not indicate impaired CD2 signalling in allergic individuals, allergic disease has previously been associated with reduced CD2 functions, *e g* lower proportion of CD2 positive lymphocytes [81, 102] and reduced CD2-induced proliferative responses (reviewed in [79, 80]). Most previous studies on CD2 function in relation to allergy have been performed in children, mainly infants [81, 102, 244]. The lack of association between CD2 function and allergy in this study, which was performed in adults, might mirror a delayed immune maturation in allergic individuals. Several studies have shown that immunological differences found between allergic and non-allergic individuals that appear early in life disappear with age [245-247]. It is possible that the reduced expression and function of CD2 early in life affects primary immune responses and the clinical outcome, but that the differences regarding CD2 function disappear with age while the allergic phenotype remains.

The increased calcium levels found after engagement of the CD2 receptor agrees with previous reports showing increased intracellular calcium levels after CD2 engagement [89, 90]. Further, blocking of the calcium-calmodulin dependent phosphatase calcineurin inhibits CD2 co-stimulation induced cytokine secretion [91]. The use of two CD2 antibodies directed towards two different epitopes (T11.1 and T11.2) of the CD2 molecule, as well as pre-incubation with the T11.2 antibody has previously been described as efficient in inducing intracellular calcium responses [248]. We did not observe any differences in intracellular calcium levels in allergic and non-allergic adults, again indicating that there are no CD2 related abnormalities in allergic adults.

In conclusion, we found that CD2 stimulation, in the absence of other co-stimulatory molecules, induces mRNA expression of the T regulatory associated marker Foxp3 as well as production of the anti-inflammatory IL-10. CD2 stimulation also hampered the Th1 response in the form of IFN- γ . Both Th1 and Th2 associated factors are dependent on CD2/CD28 activation for induction, whereas the regulatory marker Foxp3 was down-regulated. This may have implications for future therapeutic goals aiming at *in vitro* differentiate antigen specific regulatory T cells for treatment of *e g* autoimmune or inflammatory diseases. Neither the responses induced by CD2 and CD28, nor by PHA were related to allergic disease in adults in this study.

General discussion

Allergic diseases are associated with an overweight of Th2 like responses to allergens and possibly also to reduced regulatory functions. With that in mind, the general aim of this thesis was to study the mechanisms behind the reduced Th1 and possibly Treg responses in allergic children. The CD2 signalling pathway has been suggested to enhance Th1 like responses and to be impaired in allergic children. CD2 signalling enhances the responsiveness to the strong Th1 promoter IL-12. The IL-12 responsiveness in turn, is dependent on the expression of the signal-transducing β_2 subunit of the IL-12 receptor, which we have studied in all papers. Other signalling pathways, *i e* IL-2, IL-12 and CD28, have also been investigated with regard to IL-12R β_2 expression, as well as other Th1 and Th2 associated factors.

We hypothesised that one way for CD2 signalling to enhance the response of activated T cells to IL-12 may be via induction of the highly regulated IL-12R β_2 -chain. Impaired CD2 function, demonstrated by lower proportions and responsiveness to CD2, has been observed in allergic children. Provided that CD2 signalling up-regulates the IL-12R β_2 -chain, the impaired CD2 function could be associated with the Th2-skewed immune responses observed among allergic children. In accordance with that, one of the main aims in this thesis was to investigate the effect of CD2 stimulation on the expression of the IL-12R β_2 -chain. In order to do so, we used the mitogen PHA or a pair of stimulatory antibodies directed to the CD2 receptor.

As expected, PHA stimulation resulted in an up-regulation of the IL-12R β_2 -chain in all study groups (paper I, III and IV). On the contrary, the CD2 antibodies alone did not induce a significant up-regulation of the IL-12R β_2 expression according to the results in paper IV. PHA-signalling is CD2 dependent [103], but PHA also signals via the T cell receptor [104]. It could be argued that the PHA-induced up-regulation is mainly due to T cell receptor stimulation. However, we consider that unlikely, as the use of anti-CD3 stimulation alone in human cells did not induce any detectable IL-12R β_2 mRNA after 24 h, although small amounts were found after 48 h and it was readily detected after 96 h [96].

Although CD2 stimulation alone did not induce significant up-regulation of the IL-12R β_2 -chain, simultaneous stimulation via CD28 did. Stimulation via CD28 in the absence of other stimulatory factors is generally not considered to induce signals of activation, and in control assays we could hardly detect any IFN- γ , IL-5 or IL-10 production in response to the anti-CD28 antibody alone. In addition, considering the evidence concerning a role for CD2 in IL-12 responsiveness, we do not believe that

this up-regulation is a result of the CD28 antibodies alone, but rather that distinct and stronger stimulatory signals than those induced by CD2 alone are needed. This is provided by CD28 in paper IV and by PHA in paper I and III.

The IL-12R β_2 -chain was studied in all papers and the expression pattern did not fully agree; lower PHA-induced expression was found in allergic children in paper I but not in paper III and IV. One explanation could be the age difference, which may be associated with type of allergic disease, referred to as the allergic march. Whereas the predominant disease in the infants in paper III was eczema, the older children in paper I more often suffered from airway symptoms. The difference in expression in paper II was only found in children with airway symptoms and high IgE levels. Those children may be most comparable with the children in paper I, where rhinoconjunctivitis was the dominating allergic disease. Concerning the adults, they may have evolved more redundancy in their IL-12 signalling pathways and may compensate for impaired signalling function.

Another aspect, besides different allergic diseases, is the IgE levels. As suggested by the negative correlation between the IL-2 and IL-12 induced IL-12R β_2 expression and the total IgE levels in paper II, the IL-12R β_2 expression may be related to IgE levels besides the type of allergic disease. This was not investigated in paper I and III however, leaving a possible connection within these groups unknown. Among the adults, the IgE levels did not correlate significantly with the IL-12R β_2 expression, but the PHA-induced IFN- γ secretion correlated negatively with IgE ($\rho=0.5$, $p=0.03$). The same was also observed in paper II, following stimulation with IL-2 together with IL-12. These negative correlations between total IgE and IL-12R β_2 and IFN- γ production is consistent with the Th1 promoting properties of IL-12 as well as its inhibitory function on Th2 responses, including the effects of IL-4 on IgE synthesis [122].

In the studies presented within this thesis, mRNA expression of the cytokine receptors and the transcription factors have been analysed. The apparent disadvantage with that is that the mRNA levels do not always correlate to the amount of synthesised protein. Therefore it would have been interesting to also analyse the protein levels of these factors, for example with flow cytometry. The correlation between the IL-12R β_2 mRNA expression and the IFN- γ secretion, which was found in all papers, strongly suggests that at least for IL-12R β_2 the mRNA levels correspond to protein expression.

The results in paper I and IV support a role for CD2 signalling in promoting

Th1 responses when strong stimulatory signals are present and induction of regulatory functions in the absence of other co-stimulatory signals. Both these properties have previously been suggested by other groups, although IL-12R β_2 or Foxp3 have not been analysed directly [84, 85, 99, 108].

A reduced CD2 function in allergic children was found in the group of 12-year-old children, supporting previous findings of reduced CD2 function in allergic children [80, 81]. Reduced CD2 function has been suggested even prior to the first allergic symptoms [82]. Our results on the infants followed from birth, which up-regulated similar PHA-induced IL-12R β_2 regardless of allergy or not, did not support that, however. It may be that the lower IL-12R β_2 expression found in paper I is a consequence of the allergic disease and not the cause, as we do not know what comes first. A continued follow up of the infants would be interesting, especially to see if the IL-12R β_2 expression will differ when airway symptoms get more common.

The use of peripheral blood mononuclear cells does not allow us to discriminate which cells are responsible for the expression and production of the different factors studied. Concerning Foxp3, the expression seems to be restricted to the CD4⁺CD25⁺ regulatory T cell population, indicating that the up- and down-regulations found actually occur in these cells. However, it would be valuable to investigate this, for example by the use of flow cytometry. The IFN- γ produced may also originate from NK cells, besides the T cells. However, NK cells do not produce IFN- γ after CD2 stimulation alone [249], and they do not express CD28 [250].

Among the transcription factors, there was a strong correlation between Foxp3 and GATA-3, perhaps indicating that the regulatory type of responses are more related to the Th2 type of responses than to the, in general more aggressive, Th1 like responses. In paper III, it could be argued that this was simply due to methodological reasons as Foxp3 and GATA-3 were analysed with one reverse transcription kit and the other factors with another. However, similar results were observed after PHA-stimulation in paper IV, where all factors were analysed with the kit used for GATA-3 and Foxp3 in paper III, indicating that this is not the case.

Indeed, allergic diseases are complex and in the individuals developing allergy there are probably a vast range of factors contributing to the clinical outcome. Studies of CD2 signalling and the expression of IL-12R β_2 , as well as the expression of the other factors studied within this thesis, may contribute with a couple of pieces to the puzzle, although further studies are needed.

Summary and concluding remarks

In this thesis, Th1, Th2 and Treg associated factors have been studied in four different study populations aiming to investigate mechanisms behind the reduced Th1 and/or Treg function in allergic diseases.

In paper I, the PHA-induced mRNA expression of the IL-12R β_2 -chain was analysed and related to the production of several cytokines in 12-year-old children, with or without positive skin prick tests. The PHA-induced expression was lower in the skin prick test positive children, although both SPT positive and negative children had similar spontaneous IL-12R β_2 expression. The PHA-induced expression of the IL-12R β_2 correlated strongly to the production of IFN- γ . As PHA stimulates the T cells partly via the CD2 pathway, we concluded that the allergic children have a reduced IL-12R β_2 up-regulation, which may be associated with an impaired function of the CD2 pathway. This in turn may be one contributing factor to the Th2-skewed immune responses seen in allergy.

In paper II, the effect of IL-2 and IL-12 on the IL-12R β_2 mRNA expression was analysed in allergic and non-allergic 7-year-old children. The expression was related to the production of IFN- γ , IL-5 and IL-10. We found an impaired up-regulation of the IL-12R β_2 -chain after IL-2 stimulation in children with current allergic airway symptoms and high IgE levels compared with non-allergic children with low levels of IgE. This was accompanied by a low IL-2 and IL-12 induced IFN- γ production, which may reflect a reduced capacity of allergic children to up-regulate the IL-12R β_2 -chain. As IL-2 is important for induction and to sustain immune responses and as IL-12 is a strong promoter of Th1 like responses, this may also contribute to the Th2-skewed pattern in allergy.

In paper III, the spontaneous and PHA-induced development of Th1, Th2 and Treg associated factors were followed during the first two years of life, in relation to development of allergic disease in Swedish and Estonian children. More precisely, the Th1 associated cytokine receptors IL-12R β_2 and WSX-1, the transcription factors T-bet (Th1), GATA-3 (Th2) and Foxp3 (Treg) and the production of the cytokines IFN- γ , IL-4, IL-5, IL-9, IL-10 and IL-13 were studied over age. The spontaneous expression was similar at birth and at two years of age, although T-bet increased from 3 months and IL-12R β_2 from 6 months to 24 months of age. GATA-3 increased from 6 months in allergic children.

Stimulation with PHA resulted in up-regulation of all factors at all ages with

exception for GATA-3, which at 6 and 12 months was up-regulated in allergic children only. PHA-induced expression of WSX-1 and T-bet increased with age in non-allergic children, whereas IL-12R β_2 , GATA-3 and Foxp3 expression were similar at birth and 2 years. Besides higher spontaneous expression of IL-12R β_2 in allergic children at 2 years and higher PHA-induced WSX-1 at birth also in allergic children, the levels were similar in allergic and non-allergic children at a specific age. No clear differences were observed among the Swedish and Estonian children. All cytokines increased with age. Swedish children produced higher levels of IL-5 and IL-13 at 6 months and more IL-4, IL-5 and IL-10 at 2 years compared to the Estonian children. We concluded that differences in the development of Th1 and Th2 markers, *i e* WSX-1, T-bet and GATA-3 may influence the development of allergy in childhood.

In paper IV, the effect of signalling via CD2 alone, or in combination with CD28 signalling on the mRNA expression of IL-12R β_2 , WSX-1, T-bet, GATA-3 and Foxp3 were studied in allergic and non-allergic adults. The cytokine secretion of IFN- γ , IL-5 and IL-10 was also analysed. We found that stimulation via CD2 alone induced up-regulation of the Treg associated factors Foxp3 and IL-10. The induction of the Th1 associated IL-12R β_2 , WSX-1, T-bet and IFN- γ as well as the Th2 associated GATA-3 and IL-5 needed further stimulation with CD28 to be up-regulated or secreted. No differences were observed between allergic and non-allergic subjects. These results indicate a role for CD2 in the induction of regulatory functions when other co-stimulatory signals are lacking, whereas in conjunction with other stimuli it enhances stimulation of immune responses.

In the future, it would be interesting to analyse the protein expression of the IL-12R β_2 , WSX-1, T-bet, GATA-3 and Foxp3 in allergic and non-allergic individuals to see if the mRNA levels correspond to the presence of the corresponding protein, using the stimulations we have done. By the use of flow cytometry, it would also be possible to investigate which cells express and up-regulate the different factors. It could also be valuable to include other factors, *e g* the Th2 associated transcription factor c-maf and/or factors involved in intracellular signalling via IL-12 and IL-4, *e g* STAT4 and STAT6. Besides Foxp3, expression of surface markers associated with Treg would be interesting to study and follow during childhood, *e g* CD25, CTLA-4 and GITR. Functional studies of regulatory T cells, induced after CD2 stimulation would also be interesting and also to follow the regulatory function in relation to CD2 expression during childhood.

A continued observation of the development of the different factors studied would be

interesting, and is now possible as the children in paper III, have been clinically examined and blood samples collected at the age of 5 years. There are also samples available where cells have been stimulated with allergens. The expression of the different factors in response to allergens may be more accurate concerning the development of allergic disease.

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