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Mechanisms of immune regulation during development of atopic diseases in childhood: Analysis of T cell subpopulations considering genetic and epigenetic influences

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I hereby assure that I wrote this scientific work without the inadmissible help of others and without the use of aids other than the ones listed. Thoughts directly and indirectly taken from sources are indicated as such.

Munich, 31.07.2013

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Diana Rädler

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1. INTRODUCTION

1.1 Atopic diseases in childhood

The term atopy describes an inherited predisposition towards the development of a certain allergic hypersensitivity. Atopic subjects suffer from diseases like atopic eczema (atopic dermatitis), allergic asthma and allergic rhinitis (hay fever) (Biedermann and Rocken, 1999). The underlying pathophysiology is mediated by different immunoregulatory pathways and interaction of the innate and adaptive immune system. These two major parts of immune defence have to be efficiently regulated by itself and closely connected in their regulation in order to keep a healthy immune balance. Besides T cell mediated immune responses, the humoral and the mucosal immune system play a prominent role in the adaptive immune defence (Raedler and Schaub 2013, in press). A dysregulation of this balance is present in atopic individuals, with less robust cell-mediated innate immune responses to most antigens, but increased humoral (IgE-mediated) responses to allergens (Umetsu *et al.* 2002).

Thymus-derived T cells play a critical role in cell-mediated immunity and in atopic diseases. T cells can differentiate into several T helper cell (Th) subsets, including Th1, Th2, Th9, Th17, Th22 or follicular helper cells (Tfh), which all facilitate different types of immune responses (Figure 1; overview innate/adaptive immune system).

Allergic sensitization describes the reaction of the immune system to allergen exposure, associated with induction of Th2 cell differentiation and secretion of the respective Th2 cytokines, such as IL-4. IL-4 activates B cells which in turn produce allergen-specific immunoglobulin E (IgE) antibodies. Contact of the antibodies to other immune cells, particularly to mast cells and basophils, results in a coating of these cells with specific IgE. Further contact to the respective allergen then leads to an early/immediate allergic response, associated with release of preformed mediators such as histamine and tryptase as well as *de novo* generated leukotrienes and prostaglandin D2. A second, late phase arises after 4 to 8

hours upon allergen exposure as consequence of the mediators and cytokines released during the early phase. This phase is characterized by the recruitment of inflammatory cells and infiltration into the lamina propria of the mucosa (Dykewicz and Hamilos, 2009).

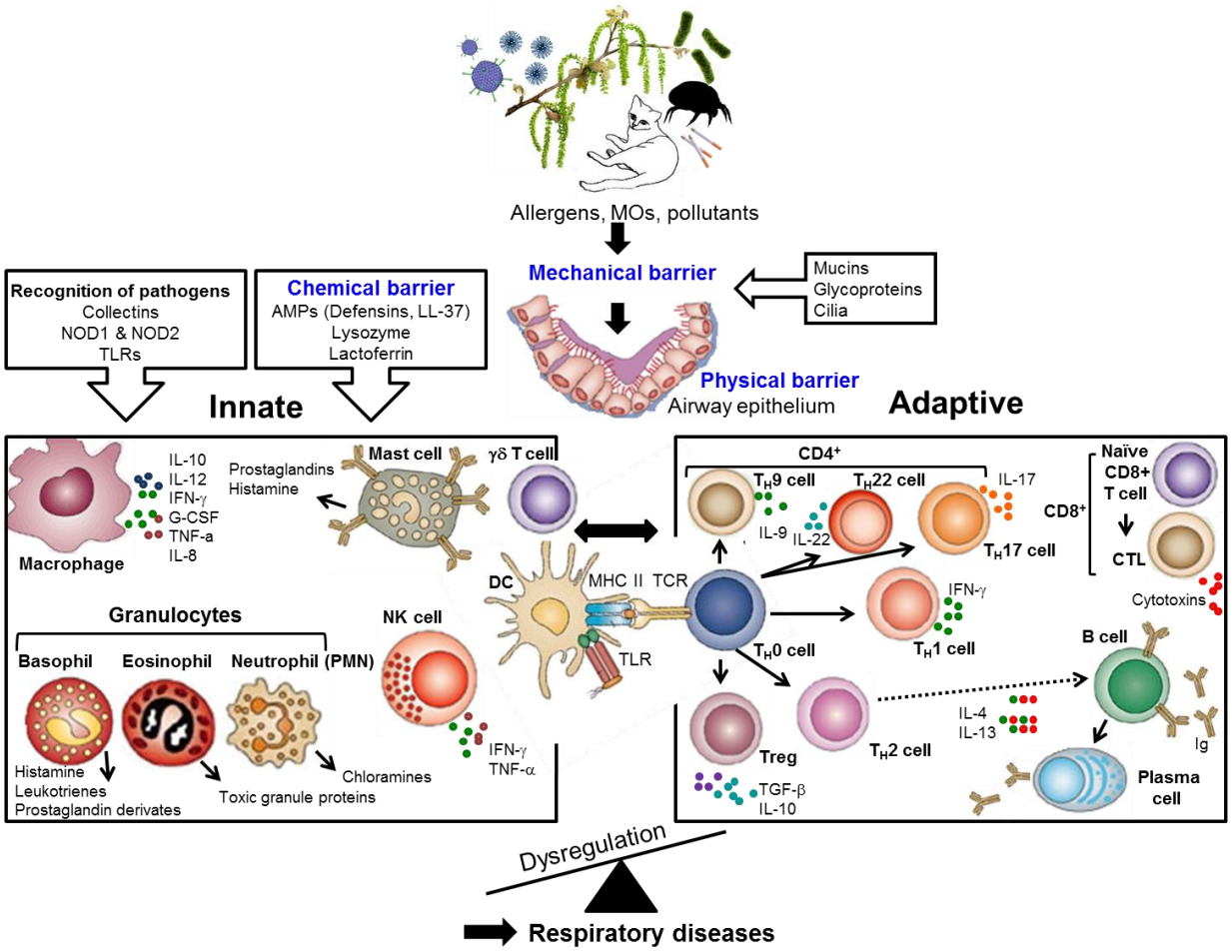

Figure 1. Overview of the initiation and interaction of the innate and adaptive immune system. CTL, cytotoxic T lymphocyte; DC, dendritic cell; G-CSF, Granulocyte-colony stimulating factor; IFN-γ, Interferon-gamma; Ig, Immunoglobulin; IL, Interleukin; LL-37, cathelicidin, antimicrobial peptide; MHC II, major histocompatibility complex; MOs, microorganisms; NK cell, natural killer cell; NOD, nucleotide-binding oligomerization domain containing; PMN, polymorphonuclear neutrophil; T_H, T helper cell; TNF-α, tumor necrosis factor-alpha; TCR, T cell receptor; TLR, Toll-like receptor (Raedler and Schaub 2013, in press).

Atopic dermatitis (AD) is a chronic inflammatory dermatosis, characterized by pruritus, xerosis and shows a close association with IgE-mediated sensitization to foods and aeroallergens. It affects small children and infants essentially. Clinically, AD can be divided in two distinct

phases, an early acute phase, which is a Th2-mediated process accompanied by high expression levels of the Th2 cytokines IL-4, IL-5 and IL-13 and a second chronic phase, in which IL-12 production by various cell types results in a switch from a Th2 to a Th1-type cytokine milieu, associated with an increase in IFN-γ expression.

More than half of the children suffering from AD also develop other allergic diseases as allergic rhinitis or asthma (paragraph 1.2); this process of sequential manifestations of allergic diseases is referred to as "atopic march" (Sabin *et al.* 2012; Rybojad M., 2012).

Allergic rhinitis is an allergic inflammation of the nasal airways which causes nasal itching, congestion, sneezing and clear rhinorrhea as a response to inhaled allergens by an already sensitized individual (Uzzaman and Story, 2012). Of note, the prevalence of allergic diseases and asthma has been increasing dramatically for the last 50 years (Devereux G., 2006).

1.2 Asthma in childhood

Asthma is a complex and heterogeneous disease with respect to its immunopathology as well as clinical phenotypes and response to therapies (Holgate S.T., 2008). Asthma is characterized by airflow obstruction, bronchial hyperresponsiveness, airway inflammation, increased mucus secretion and smooth muscle hypertrophy. It comprises wheezy breathlessness as a consequence of airway narrowing, which is partially or totally reversible (Kay A.B., 2001). Asthma is a multicellular process involving various different cell types including eosinophils, neutrophils, lymphocytes and mast cells. Asthma is not caused by a single gene or environmental factor but depends on an interplay between the aforementioned factors, entitled as gene-environment interactions (Busse and Lemanske, 2001; Holgate S.T., 2008).

Asthma in childhood is currently divided in two main phenotypes, the allergic and the non-allergic asthma (Humbert *et al.* 1999). Allergic asthma is caused by inhaltion of environmental allergens by an atopic individual, which in turn triggers an early phase response. This early

phase is associated with release of inflammatory mediators such as leukotrienes, prostaglandins, cytokines and histamines which provoke edema and bronchoconstriction. The subsequent late phase is characterized by an activation of eosinophils and infiltration of Th2 cells into the bronchial mucosa, which in turn increases the production of Th2 and pro-inflammatory cytokines (Kiley *et al.* 2007).

Asthma of non-allergic origin is caused by triggers not related to allergy such as viral infections, cold air or exercise (Romanet-Manent *et al.* 2002). It can be distinguished clinically from allergic asthma based on a negative skin prick test to common allergens (Humbert *et al.* 1999; Romanet-Manent *et al.* 2002). Childhood asthma is currently classified into allergic and non-allergic asthma based on clinical criteria and specific sensitization, whereas the detailed underlying immunological mechanisms contributing to the pathophysiology of these distinct clinical phenotypes are still unknown.

Besides the allergic and non-allergic asthma classification, further approaches are currently discussed (Just *et al.* 2012), e.g. based on the frequency of symptoms and the age-at-onset to segment asthmatic children in early or late-onset asthmatics with transient or persistent symptoms (Bisgaard *et al.* 2001, Papadopoulos *et al.* 2012). Moreover, multiple-trigger wheeze was described for children who respond with wheezing to further triggers, like crying, laughter or tobacco. Multitrigger wheeze displays overlapping features with AA and NA, but presumably rather reflects a chronic allergic airway disease corresponding to AA (Potter P.C., 2010; Frey and von Mutius, 2009).

1.3 T cell subsets and function

Besides other immune cells, T cells are thought to be one of the key regulatory cells for the development of atopic diseases in childhood (Jutel and Akdis, 2011). Generally, T helper cells (Th cells) represent a group of leukocytes which have the expression of the surface marker

CD4 in common and play an important role in several processes of adaptive immunity. Following T cell development, thymus-derived matured naïve T cells can get activated and differentiate into several subsets of T helper cells (Figure 2). During this activation, an antigen-presenting cell (APC) presents foreign endocytosed and processed material via MHC II to the $CD4^+$ T cells, which in turn express specific T cell receptors (TCRs) against the peptide/MHC II complex. The second activation signal is among others mediated by the co-stimulatory molecules CD80 and CD86, which activate the CD28 receptor. Following this 2-signal activation, naïve Th cells can differentiate into specific Th subsets.

The first described T cell subsets were Th1 and Th2 cells (Mosmann *et al.* 1986). Th1 cells are involved in cell-mediated immunity and phagocyte-dependent inflammation, required for clearing intracellular pathogens. Th1 cells are the main producers of the cytokines IFN-γ and IL-2. Th2 cells primarily produce the cytokines IL-4, IL-5, IL-10 and IL-13 and besides evoke a strong antibody response and eosinophil accumulation. They play a major role in helminth and parasitic infections (Romagnani S., 2000). Both cell types (Th1, Th2) express characteristic transcription factors, *T-bet* and *Hlx* in Th1 and *GATA-3* and *STAT-6* in Th2 cells and their related cytokines act antagonistically (Korn *et al.* 2009). This Th1/Th2 paradigm of T helper cell differentiation, which emerged in the late 1980s, has been expanded following the discovery of further T helper cell subsets including Th9, Th17 and Th22 cells, regulatory T cells and follicular helper cells (Mosmann *et al.* 1986).

Th9 cells are related to Th2 cells as both lineages require IL-4 and the transcription factors STAT6 and GATA-3. Chang *et al.* could show that PU.1 promotes expression of IL-9 and pro-allergic chemokines and therefore suggest PU.1 as important factor for induction of Th9 lineage cells (Chang *et al.* 2010).

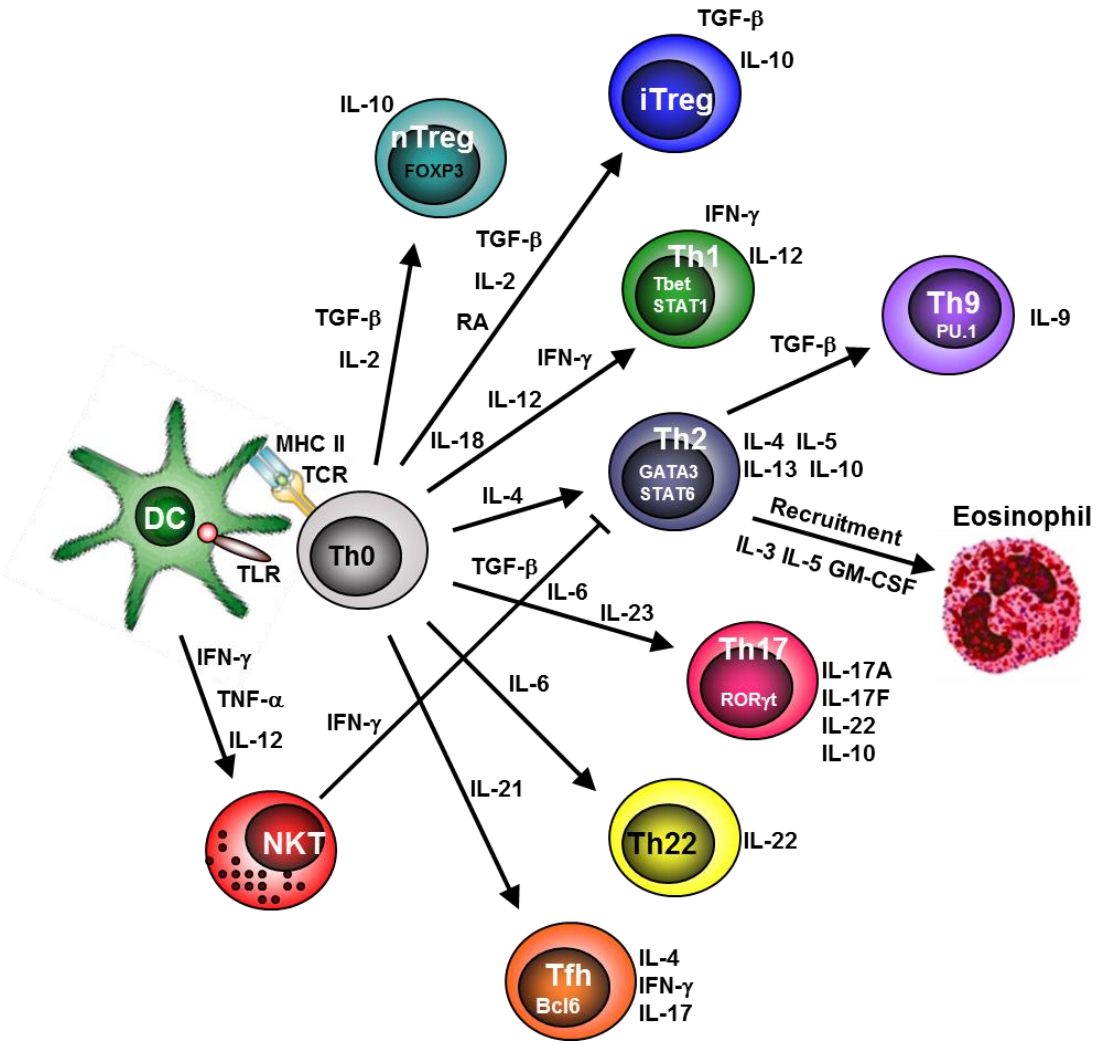

Figure 2. T cell activation and effector cell development.
A foreign protein or antigen (Ag) is taken up by an antigen presenting cell (DC, dendritic cell), processed and displayed via MHC II. Upon antigen presentation by dendritic cells, naïve $CD4^{+}$T cells differentiate into unique effector T cell subsets; this process is in part determined by the cytokine milieu. The different effector cells are classified by the dominant transcription factors in concert with the cytokines they express. Bcl6, B cell lymphoma 6; GATA-3, GATA-binding protein 3; GM-CSF, granulocyte-macrophage colony-stimulating-factor; INF-γ, interferon-gamma; IL, interleukin; MHC II, major histocompatibility complex II; PU.1 (SPI1), spleen focus forming virus (SFFV) proviral integration oncogene; RA, retinoic acid; RORγt, RAR-related orphan receptor gamma; STAT, signal-transducer and activator of transcription protein; TCR, T cell receptor; Tfh, follicular helper cell; TGF-β, transforming growth factor beta; Th, T helper cell.

Th17 cells are characterized by the cytokines IL-17A, IL-17F and IL-22, which mediate an up-regulation of pro-inflammatory cytokines, chemokines and metalloproteases. Th17 cells are involved in protection against fungi and extracellular bacteria (Akdis *et al.* 2012) and show some kind of plasticity as they can shift to Th1-like cells at inflamed sites (Annunziato *et al.* 2012).

1. INTRODUCTION

The cytokine IL-22 is produced by Th17 cells; however it was also identified as key cytokine of a distinct $CD4^+$ cell subset, the Th22 cells (Duhen *et al.* 2009, Trifari *et al.* 2009). Th22 cells express high levels of chemokine receptors *CCR4*, *CCR6*, *CCR10* and the cytokine IL-12 and secrete only low amounts of IL-17 (Duhen *et al.* 2009). Both Th17 and Th22 are involved in immune defence to extracellular bacteria and link immune responses to tissue inflammation. While IL-17 rather augments inflammation, IL-22 has a more protective function (Akdis *et al.* 2012).

Regulatory T cells (Tregs) represent a heterogeneous group of T cells, responsible for maintenance of tolerance to self-antigens, suppression of immunity to pathogens and tumors and abrogation of autoimmune diseases. Tregs are characterized by expression of the transcription factor (TF) and master regulator *FOXP3*. Two different origins of $FOXP3^+$ cells have been described. First, thymus-derived natural regulatory T cells (nTregs), expressing CD4, high amounts of the activation marker CD25 and intracellular *FOXP3*. Second, a group of induced Tregs (iTregs), in which expression of *FOXP3* is induced in the periphery (Feuerer *et al.* 2009; Sakaguchi *et al.* 2010). Moreover, two subgroups of induced regulatory T cells exist, the TGF-β secreting Th3 cells and the IL-10 secreting Tr1 cells, which have been related to adaptive regulatory functions without expressing *FOXP3* (Curotto de Lafaille and Lafaille, 2009; Shevach E.M., 2006).

Most of these different Th cell subsets have the expression or lack of characteristic and unique transcription factors and cytokines in common, which are essential for the transcriptional programs regulating their differentiation. However, this differentiation state is not completely stable and a conversion of Tregs into effector T cells can occur under certain conditions (Beyer and Schultze, 2011).

Quantification of nTregs is problematic due to the fact that activated effector T cells also express high amounts of *CD25* and can even transiently express *FOXP3* (Shevach E.M., 2006).

Additional markers can be used for classification as the Treg-associated genes Glucocorticoid-induced TNF receptor (*GITR*) and Lymphocyte activation gene-3 (*LAG3*), though expression is not completely Treg-specific (Liu and Raedler *et al.* 2011). A further possibility to distinguish between natural Tregs and activated FOXP3$^+$ conventional T cells and iTregs is to investigate epigenetic modifications at the *FOXP3* locus, particularly at a region called „Treg-specific demethylated region“ (TSDR), which was shown to be fully demethylated only in nTregs (Baron *et al.* 2007) and was shown to reliably quantify Tregs already in cord blood (Liu *et al.* 2010).

1.4 Role of regulatory T cells and Th17 cells in the development of atopic diseases

1.4.1 Regulatory T cells

Tregs are supposed to maintain a healthy immune balance and avoid excessive immune responses which might cause harmful immune pathology. Consequently, several studies suggest an important role for regulatory T cells in various atopic diseases. Tregs were already decreased in number as well as suppressive capacity in cord blood from atopic mothers and in airways of asthmatic children compared to healthy controls (Schaub *et al.* 2008; Hartl *et al.* 2007). A decrease in Treg functionality was moreover described for allergic asthmatic patients compared to healthy controls (Lin *et al.* 2008; Ling *et al.* 2004). Although, a potential role of Tregs in development of atopic diseases seems to be undisputable, the results on number and function in atopic diseases are quite contradictory. While Pumputiene *et al.* reported no differences in Treg numbers between allergic asthmatics and healthy controls (Pumputiene *et al.* 2011), Smyth *et al.* and Shi *et al.* published higher numbers of Tregs in the airways of asthmatics and in PBMCs of allergic asthmatics during acute exacerbation, respectively (Smyth *et al.* 2010; Shi *et al.* 2004). Increased numbers of Tregs were also found in PBMCs of patients with atopic dermatitis (Ou *et al.* 2004). Inconsistency in reported Treg numbers can

potentially be explained by different definitions of Treg cells, often solely based on the surface markers CD4 and CD25, but not on intracellular FOXP3. The reported cell numbers might therefore include Tregs and also activated T cells.

1.4.2 Th17 cells

Although Th17 cells were reported to play a role in protection against fungi and extracellular bacteria, they were also shown to play a key role in several autoimmune disorders and rather have pathogenic than protective function in development of autoimmune and allergic diseases (Akdis *et al.* 2012). Th17 cells are involved in the bone destruction phase of autoimmune arthritis (Sato *et al.* 2006), multiple sclerosis (Klotz *et al.* 2009), in triggering inflammation in systemic lupus erythematosus (Wong *et al.* 2000) as well as in manifestation of chronic intestinal inflammation in inflammatory bowel diseases (Yen *et al.* 2006). The key Th17 cytokine IL-17 is involved in neutrophil maturation, migration and function (Hellings *et al.* 2003; Cosmi *et al.* 2011). Since neutrophils were lately associated with important features of human asthma, including airway gland hypersecretion, bronchial hyerresponsiveness (BHR) and airway wall remodelling, IL-17 was suggested to contribute to the chronic inflammatory changes in allergic asthmatics (Molet *et al.* 2001; Hellings *et al.* 2003) as well as in the pathogenesis of severe, neutrophilic and steroid-insensitive asthma (Alcorn *et al.* 2010). In addition, Zhao *et al.* reported a relation of Th17 cell numbers with asthma severity (Zhao *et al.* 2010). In accordance, IL-17 levels were increased in peripheral blood of patients with atopic dermatitis (AD) and as well associated with disease severity in a study by Koga *et al.* (Koga *et al.* 2008). Ciprandi *et al.* suggested the use of IL-17 levels in serum as marker for severe allergy (Ciprandi *et al.* 2008).

1.5 Genetic and epigenetic changes associated with atopic diseases

Genetics seem to play a major role in atopic diseases. To reveal whether genetic changes are associated with certain diseases, several candidate-specific approaches and genome-wide association studies (GWAS) were performed in the last decades. Among others six major genomic regions were identified within different GWASs of asthma and asthma-related traits: the ORM1-like 3 and gasdermin B (*ORMDL3-GSDMB*) region, interleukin 33 (*IL-33*), the IL-1 receptor-like 1 and IL-18 receptor 1 (*IL1RL1-IL18R1*) region, the RAD50 homolog and IL-13 (*RAD50-IL13*) region, the thymic stromal lymphopoietin and WD repeat domain 36 (*TSLP-WDR36*) region and the major histocompatibility complex class II DR/DQ (*HLA-DR/DQ*) region (Moffat *et al.* 2010; Li *et al.* 2012). Several studies revealed an association of the Chr17q21 locus, including the genes *ORMDL3* and *GSDMB*, with childhood asthma (Moffatt *et al.* 2007; Moffatt *et al.* 2010; Lluis *et al.* 2011), however their role in asthma pathogenesis is not clear yet. Moreover, single nucleotide polymorphisms (SNPs) within characteristic Th1 and Th2 pathway genes were shown to influence development of atopic diseases (Cameron *et al.* 2006; Schedel *et al.* 2008; Suttner *et al.* 2009; Casaca *et al.* 2012) and SNPs in various Toll-like receptor (*TLR*) genes were shown to affect asthma and atopy risk (Eder *et al.* 2004; Lazarus *et al*. 2004; Kormann *et al.* 2009).

Expression of ten different *TLRs* was identified in humans so far, which are able to detect structural conserved pathogen-associated microbial patterns (PAMPs), including bacterial, viral and fungal products as well as damage-associated molecular patterns (DAMPs) that are released by cells undergoing necrosis (Holgate S.T., 2012). TLRs, as key molecules in recognition of microbial environment, are involved in the induction of expression of co-stimulatory molecules (Akira and Takeda, 2004).

1. INTRODUCTION

Atopic diseases like asthma were originally described to result from complex gene-environment interactions. In addition, epigenetic regulation was suggested as potential mechanism by which environmental factors could interact with genes involved in disease development (Martino and Prescott, 2010). Epigenetics in general describe a change in gene expression or the cell phenotype, not mediated by changes in the DNA sequence. Epigenetic processes include DNA methylation, histone acetylation, phosphorylation or ubiquitinylation and noncoding RNAs (Yang *et al.* 2012). Epigenetic regulation mechanisms were shown to have a major impact on T cell differentiation and regulation of the Th1 and Th2 key cytokines IFN-γ and IL-4, respectively (Wilson *et al.* 2009; Hughes *et al.* 2010). Applying a mouse model, Brand *et al.* identified epigenetic modulation as one mechanism by which prenatal, microbial exposure contributes to protection against allergy and asthma development in the offspring (Brand *et al.* 2011).

1.6 Aim of the study

The prevalence of atopic diseases in childhood increased dramatically over the past decades. However, little is known about the underlying mechanisms of immune regulation in infancy until the onset of disease. Genetic and environmental factors both contribute to development of atopic disease, most likely as interacting factors in a process entitled as gene-environment interactions.

The aim of the present work was to study the impact of SNPs in *TLRs* and *IL-10*, as key molecules of innate and adaptive immunity, on various aspects of the immune response. Both *TLR* and *IL-10* SNPs were associated with inflammatory and atopic diseases in children and adults in several studies (Eder *et al.* 2004; Bossé *et al*. 2009). We assessed immune regulation at the earliest possible time point, in cord blood and the impact of *TLR* and *IL-10* SNPs on the manifestation of atopic disease in early childhood.

In the second part, immune regulation later in life, during the manifestation of childhood asthma, was investigated. Based on clinical criteria, childhood asthma is currently classified into allergic and non-allergic asthma, whereas the underlying immunological mechanisms which contribute to the pathophysiology of the two clinical phenotypes are still unknown. Our aim was to investigate different T cell populations, with a special focus on Treg and Th17 cells, two T cell subsets which are supposed to play a major role in atopic and inflammatory diseases (Ling *et al.* 2004; Molet *et al.* 2001). In this context, several studies have demonstrated a role for epigenetic regulation in T cell differentiation and responses (Lee *et al.* 2006); however their effect on immune regulation in allergic and non-allergic asthmatic children is not clear yet and was addressed in this work.

Different studies have tried to disentangle the asthma phenotypes by Genome-wide association studies (GWAS) in which different genotypes were associated with asthma (Moffat *et al.*

2010); however their role in asthma pathogenesis is not clear yet. Aim of this study was to identify novel biomarkers and underlying immunological mechanisms of allergic and non-allergic asthma in childhood.

The following scientific questions were therefore addressed in this thesis:

1) To study whether genetic variants in Toll-like receptors or the *IL-10* gene already have an impact on neonatal immune regulation and development of atopic diseases or wheeze later in childhood.
2) To investigate whether allergic and non-allergic asthmatics and healthy children differ in number and function of Tregs and Th17 cells, characteristic T cell subset- and pro-inflammatory cytokines.
3) To study whether epigenetic changes have an impact on T cell regulation and development of childhood asthma, with a special focus on the IFN-γ promoter and the Treg-specific demethylated region (TSDR).
4) To investigate if children with allergic and non-allergic asthma can be distinguished through microarray-based identification of signalling pathways and differentially expressed genes and to identify new biomarkers important in asthma pathogenesis.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents and chemicals

100bp DNA ladder (500µg/ml)	New England BioLabs, Ipswich, USA
ACK Lysis Buffer	Cambrex, East Rutherford, USA
Anti-human CD4-APC H7 antibody	BD Biosciences, Heidelberg, Germany
Anti-human CD25-APC antibody	BD Biosciences, Heidelberg, Germany
Anti-human CD49d-FICT antibody	Beckmann Coulter, Fullerton, USA
Anti-human FOXP3-PE antibody	eBioscience, San Diego, USA
Anti-human IL-17-Alexa488 antibody	eBioscience, San Diego, USA
Anti-human IL-22-efluor660 antibody	eBioscience, San Diego, USA
Anti-human CD3-PE antibody	BD Biosciences, Heidelberg, Germany
Anti-human CD4-APC antibody	BD Biosciences, Heidelberg, Germany
Anti-human CD8-Alexa488 antibody	BD Biosciences, Heidelberg, Germany
Anti-human CD4-FITC antibody	Beckmann Coulter, Fullerton, USA
Anti-human IgG1-APC antibody	BD Biosciences, Heidelberg, Germany
Anti-human IgG2a-PE antibody	eBioscience, San Diego, USA
Anti-human IgG1-Alexa488 antibody	eBioscience, San Diego, USA
Anti-human IgG1-efluor660 antibody	eBioscience, San Diego, USA
Boric acid	Sigma-Aldrich, Steinheim, Germany
Bromphenol blue	Roth, Karlsruhe, Germany
D. pteronyssinus allergen 1 (Derp1)	Biotechnologies, Charlottesville, USA
EDTA	Sigma-Aldrich, Steinheim, Germany
Ethanol 100%	Merck, Darmstadt, Germany
Ethidiumbromide (10mg/ml)	Biorad, Hercules, USA
FACS Clean Solution	BD Biosciences, Heidelberg, Germany
FACS Flow™ Sheath Fluid	BD Biosciences, Heidelberg, Germany
FACS Rinse Solution	BD Biosciences, Heidelberg, Germany
Ficoll-Paque™ PLUS	GE Healthcare, Piscataway, USA
Fixation/Permeabilization Concentrate	BD Biosciences, Heidelberg, Germany
Fixation/Permeabilization Diluent	BD Biosciences, Heidelberg, Germany
Foetal Bovine Serum Gold (FBS)	PAA Laboratories GmbH, Pasching, Austria
Glycerol	Sigma-Aldrich, Steinheim, Germany
H_2O bidest.	H. Kerndl GmbH, Weißenfeld, Germany
Human serum	Sigma-Aldrich, Steinheim, Germany
Hydrogen chloride (HCl)	Sigma-Aldrich, Steinheim, Germany
Ionomycin	Sigma-Aldrich, Steinheim, Germany
Isolation buffer	Miltenyi Biotec, Bergisch Gladbach, Germany
Isopropanol 100%	Merck, Darmstadt, Germany
LiChrosolv H_2O (HPLC)	Merck, Darmstadt, Germany
Lipid A	Sigma-Aldrich, Steinheim, Germany
Paraformaldehyde	Sigma-Aldrich, Steinheim, Germany
Penicillin/Streptomycin	Gibco, Carlsbad, USA
Phosphate-Buffered Saline (PBS)	Gibco, Carlsbad, USA
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich, Steinheim, Germany
Phytohemagglutinin	Sigma-Aldrich, Steinheim, Germany
Primers	Life technologies, Invitrogen, Carlsbad, USA
Rat serum	eBioscience, San Diego, USA
RPMI 1640 + GlutaMax	Gibco, Carlsbad, USA

Trizma Base	Sigma-Aldrich, Steinheim, Germany
Trypan Blue	Sigma-Aldrich, Steinheim, Germany
TRIzol Reagent	Invitrogen, Carlsbad, USA
Tween 20	Merck, Darmstadt, Germany
Water DEPC (0.1 %)	Serva Electrophoresis GmbH, Heidelberg, Germany
X-Vivo 15	Lonza, Cologne, Germany
Xylene cyanol	Merck, Darmstadt, Germany

2.1.2 Solutions and buffers

1% PFA	10g paraformaldehyde Ad 900ml ddH_2O 800µl 1N NaOH 30 min at 65°C 100ml PBS 10X until pH 7.4
5X TBE buffer	54g trizma base 27.5g boric acid 20ml 0.5M EDTA (pH 8.0) Ad 1l H_2O bidest.
DNA ladder	10µl 100bp DNA ladder 80µl 0.5x TBE-Buffer 10µl loading dye diluent
Ethidiumbromide [500µg/ml]	100µl ethidiumbromide 1.9 ml H_20
FACS buffer	25ml 10X PBS 12.5ml FBS (5%) 1.25ml Tween 20 (0.5%) Ad 250ml LiChrosolv H_2O
Loading dye stock solution	0.25g bromphenol blue 0.25g xylene cyanol 30% glycerol 70ml dH_2O
Loading dye diluted solution	5ml loading dye stock solution 13.5ml glycerol 31.5ml dH_2O
Medium with 10% human serum	440ml RPMI 1640 + GlutaMAX 10ml Penicillin/Streptomycin 50ml inactivated human serum

2.1.3 Reagent systems (Kits)

CellTrace™ CFSE Cell Proliferation Kit	Invitrogen, Carlsbad, USA
CD3 MicroBeads	Miltenyi Biotec, Bergisch Gladbach, Germany
CD4+CD25+ Regulatory T Cell Isolation Kit	Miltenyi Biotec, Bergisch Gladbach, Germany
FlexiGene DNA Kit	Qiagen, Hilden, Germany
Human Cytokine Multiplex Assay Kit	Biorad, Hercules, USA
QIAamp DNA Mini Kit	Qiagen, Hilden, Germany
QuantiTect Reverse Transcription Kit	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
SYBR Green PCR Master Mix	Applied Biosystems, Foster City, USA

2.1.4 Consumables

96-Well White Shell PCR Plates	BD Biosciences, Heidelberg, Germany
Biosphere® filter tips 10µl M 40mm type D	Sarstedt, Nümbrecht, Germany
Biosphere® filter tips 100µl	Sarstedt, Nümbrecht, Germany
Multiwell™ 6 well plates	BD Biosciences, Heidelberg, Germany
Multiwell™ 12 well plates	BD Biosciences, Heidelberg, Germany
Multiwell™ 24 well plates	BD Biosciences, Heidelberg, Germany
Multiwell™ 96 well plates	Greiner bio-one, Frickenhausen, Germany
SafeGuard Filter tips 100-1000µl	Peqlab, Erlangen, Germany

2.1.5 Laboratory equipment

Centrifuge 5810 R / 5417 R / 5415 R	Eppendorf, Hamburg, Germany
Centrifuge Rotanta 460R / S	Hettich, Tuttlingen, Germany
Electrophoresis power supply	VWR International, Radnor, USA
Gel iX Imager	Intas Science Images Instruments, Göttingen, Germany
CFX96 Touch™ Real-time PCR Detection System	Biorad, Hercules, USA
Incubator Hera Cell 240	Heraeus, Hanau, Germany
Incubator Heraeus 6000	Heraeus, Hanau, Germany
LUMINEX 100 IS System	Luminex Corp., Austin, USA
MACS® MultiStand	Miltenyi Biotec, Bergisch Gladbach, Germany
Micro Centrifuge II	NeoLab, Heidelberg, Germany
Microscope Axiovert 40C	Zeiss, Göttingen, Germany
Neubauer chamber	Karl Hecht KG Assistent, Sondheim, Germany
Thermocycler Eppendorf Mastercycler	Eppendorf, Hamburg, Germany

2.1.6 Software

Bio-plex Manager Software 4.1.1	Biorad, Hercules, USA
Biorad CFX Manager 2.1	Biorad, Hercules, USA
CHIP Bioinformatics Tools	http://snpper.chip.org/bio/
EndNote X3	ISI ResearchSoft, Berkeley, USA
Ensembl Genome Browser	http://www.ensembl.org/

Haploview	http://www.broad.mit.edu/mpg/haploview/
National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
R	http://www.R-project.org/
SAS software package version 9.2	SAS Institute, Cary, USA
SNPper	CHIP Bioinformatics Tools
SPSS version 20	SPSS IBM Inc., Armong, USA
Vector NTI 10 Advance 11.5	Invitrogen, Carlsbad, USA

2.2 Methods

2.2.1 Study population

Two different study populations were investigated in this work. The PAULINA (Paediatric Alliance for Unselected Longitudinal Investigation of Neonates for Allergies)/PAULCHEN (Prospective Cord Blood Study in Rural Southern Germany) study population, including 200 neonates with cord blood samples from the Munich outer area (Schaub *et al.* 2008; Schaub *et al.* 2009) and the CLARA (Clinical Asthma Research Association) childhood asthma population, with children aged 4 to 15 years, the latter recruited at the University Children´s Hospital, Munich, Germany.

2.2.1.1 PAULINA/PAULCHEN

Umbilical cord blood (n = 200; 72 samples from atopic mothers, 128 from non-atopic mothers) was obtained from healthy neonates born in the Munich metropolitan area, Germany. Subjects were recruited from July 2005 to September 2007 during the last trimester of pregnancy. Inclusion criteria comprised healthy neonates and mothers with uncomplicated pregnancies. Exclusion criteria included preterm deliveries, perinatal infections, maternal use of antibiotics in the last trimester and chronic maternal diseases. Detailed information about parental health and socioeconomic status was assessed by questionnaires. Maternal atopy was defined as doctor's diagnosis of asthma and/or eczema and/or hay fever. Maternal total and specific IgE (RAST) were measured. A positive specific IgE was defined as ≥0.35 IU/mL to one or more common allergens from a panel of 20 allergens (Mediwiss Analytic, Moers, Germany; d*ermatophagoides pteronyssinus* [D1], *dermatophagoides farina* [D2], birch pollen [T3], gras pollen [GX], egg white [F1], cow´s milk [F2], soybean [F14], hazelnut [F17], latex [K82], hazel [T4], cat epithelium [E1], horse epithelium [E3], dog epithelium [E5], *Alternaria*

alternate [M6], peanut [F13], carrot [F31], wheat [F4], mugwort [W6], timothy [G6], plantain [W9]).

The laboratory investigators were blinded to clinical information; samples were analysed based on sample availability to perform the laboratory studies. Informed consent was obtained from the mothers for their participation in the study, including cord blood collection. Approval was obtained from the human ethics committee of the Bavarian Ethical Board, LMU Munich, Germany. A total number of 200 children was recruited, the number for the single analyses varied due to sample availability or non-participation in the follow up.

At the age of 3 years, a follow-up of the study population was performed. All data were collected in detailed questionnaires answered by the parents. The outcomes included atopic dermatitis (AD), food allergy, wheeze and asthma (clinical symptoms and doctor´s diagnosis). A positive allergen test was defined by at least one positive RAST to one of the 20 common inhaled or food allergens. Wheeze was defined by wheezy symptoms in the last three years. Atopy was defined by doctor´s diagnosis of atopy (obstructive bronchitis, asthma, atopic eczema or food allergy) or atopic symptoms. AD was defined as doctor´s diagnosis of inflammatory, relapsing, non-contagious and pruritic skin disorder and asthma as doctor´s diagnosis of chronic inflammatory airway disease characterized by variable and recurring symptoms, bronchospasm and reversible airflow obstruction (according to the guidelines of ATS/ERS Society Statement, 2005: Beydon *et al.* 2007; Nielsen and Bisgaard, 2005; Crapo *et al.* 2000).

2.2.1.2 CLARA

Peripheral blood (n= 230) was sampled from children age 4 to 15 years in the CLARA study, Munich, Germany. Enrollment occurred from January 2009 and is still ongoing. Parents were approached for consent and completed a detailed questionnaire that assessed infant data.

Upon enrollment, all asthmatic children underwent full clinical examination, pulmonary function test, chest radiograph and blood count. In addition, total and specific IgE (RAST) were measured. A positive specific IgE was defined as one or more positive reactions [≥0.35 IU/ml] to a panel of 20 common allergens (Mediwiss Analytic, Moers, Germany; for allergen details see 2.2.1.1). Reversible airway obstruction was defined as significant difference of the forced expiratory volume in one second before and after inhalation of the short-acting β_2-adrenergic receptor agonist salbutamol (ΔFEV1), analogous to ATS/ERS criteria (Beydon *et al.* 2007; Nielsen and Bisgaard, 2005; Crapo *et al.* 2000).

Inclusion criteria for asthmatic children comprised at least 3 attacks of obstructive bronchitis and/or a doctor´s diagnogis of recurrent episodes of obstructive bronchitis and/or a history of asthma medication and a typical lung function showing reversible pulmonary obstruction. Children were defined as asthmatics if they met the inclusion criteria for asthmatics and had a ΔFEV1 of higher than 10%. Allergic asthmatics (AA) were defined based on the criteria above and a positive (>0.35 IU/ml) specific allergic sensitization assessed by RAST test. Non-allergic asthmatics (NA) were based on the criteria above and a negative specific allergic sensitization assessed by RAST test. The definition for healthy children (HC) was based on having no allergies and any chronic diseases. Children with other pulmonary, chronic or autoimmune diseases were excluded, similarly children with immunodeficiency and subjects taking steroids, antibiotics, probiotics or suffering from an infection within 14 days before blood withdrawal.

2.2.2 Isolation and culture of CBMCs and PBMCs

Cord blood mononuclear cells (CBMCs) cells and peripheral blood mononuclear cells (PBMCs) were isolated within maximum 24 hours after blood withdrawal by density-gradient centrifugation with Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden) after dilution in

phosphate buffer saline (PBS, Gibco, Karlsruhe, Germany). Cells were washed with RPMI 1640.

CBMCs were diluted to a concentration of $5x10^6$ cells/ml in 10% human serum (Sigma Aldrich, Steinheim, Germany). CBMCs were cultured unstimulated or stimulated with lipid A (LpA, 0.1µg/ml), peptidoglycan (Ppg, 10µg/ml), phytohemagglutinin (PHA, 5µg/ml), *Dermatophagoides pteronyssinus* (Derp1, 30µg/ml) or a combination of LpA and Derp1 for 3 days.

PBMCs were diluted to a concentration of $5x10^6$ cells/ml in X-Vivo (Lonza, Cologne, Germany). For RNA isolation and cytokine measurement, $2x10^6$ cells were cultured (24-well plate; BD) in X-Vivo for 48 hours either without stimulation or stimulated with platebound anti-CD3 (3µg/ml) plus soluble anti-CD28 (1µg/ml), lipid A (LpA, 0.1µg/ml) or peptidoglycan (Ppg, 1mg/ml) at 37°C. Cell pellets were used to isolate RNA for quantitative RT-PCR and supernatants were used to determine cytokine levels. The peptidoglycan was extracted from bacterial culture by the group of Prof. Dr. Otto Holst (Institute of Structural Biochemistry, Research Center Borstel, Germany). For flow cytometry staining, $2.5x10^6$ cells were cultured (12-well plate, BD) in X-Vivo for 48 hours either without stimulation or stimulated with platebound anti-CD3 (3µg/ml) plus soluble anti-CD28 (1µg/ml) and lipid A (LpA, 0.1µg/ml).

2.2.3 Quantitative analysis of regulatory T cells and Th17 cells

PBMCs were analysed using three-colour flow cytometry (FACS canto II, BD, Heidelberg, Germany). For surface staining, PBMCs were incubated in aliquots of $2.5x10^5$ cells in FACS buffer with saturating concentrations of fluorochrome-labeled antibodies for 20 minutes at 4°C. Cells were stained with 1µl CD4 APC-H7, 1µl CD25 APC, 1µl CD49d FICT and corresponding isotype controls were used. To distinguish alive and dead cells, cells were stained with 7-Aminoactinomycin (7-AAD) for 10 minutes. The samples were washed with

1ml RPMI buffer and resuspended in 200µl RBMI buffer. For intracellular staining $5x10^5$ cells were incubated with surface antibodies and 7AAD and once washed with RPMI buffer. Thereafter cells were resuspended in 250µl freshly prepared fixation/permeabilization solution and incubated at 4°C for 30 minutes in the dark. After centrifugation, cells were washed twice with 1x CytoPerm/Wash. After blocking with 2% rat serum at 4°C for 15 minutes, intracellular antibodies (5µl Foxp3 PE, 5µl IL-17 Alexa488, 1.25µl IL-22 efluor660 and corresponding isotype controls) were added and cells incubated at 4°C for 30 minutes in the dark. After two further washing steps with 1ml of 1x Perm/Wash, cells were resuspended in 200µl buffer and fixed in 200µl 1% PFA. Flow cytometry data were analysed using the FACS DIVA software.

2.2.4 Qualitative analysis of regulatory T cells

$CD3^-$ cells were isolated with the human CD3 isolation kit (Miltenyi Biotec) and irradiated at 30 Gy for 10 minutes. $CD4^+CD25^{high}$ and $CD4^+CD25^-$ cells were isolated in a two-step procedure by depleting the non-$CD4^+$ cells, followed by a positive selection of the $CD25^{high}$ cells (human $CD4^+CD25^{high}$ isolation kit, Miltenyi Biotec). The purity of all isolated cell types was determined by flow cytometry analysis and was above 90%. Cells were stained by mixing equal volumes of cell suspension ($5x10^6$ cells/ml) with 5µM CFSE in PBS with 10% FBS for 5 minutes at room temperature in the dark. CFSE treated $CD4^+CD25^-$ cells ($2x10^4$ cells/well) were incubated with irradiated $CD3^-$ cells ($4x10^4$ cells/well) in coculture with or without $CD4^+CD25^{high}$ T cells at indicated cell:cell ratios ($2.5x10^3$ to $6x10^4$ cells/well).

Results were compared before and after stimulation with 0.8 ng/µl PHA. After 72 hours of culture, division of $CD4^+CD25^-$ T cells was analysed with FACS Diva Software. The suppressive capacity (SC) was calculated with the following formula:

$$\textbf{SC [\%]} = \frac{\text{(cell division of effector T cells alone)} - \text{(cell division of effector T cells with Tregs)}}{\text{(cell division of effector T cells alone)}} \times 100$$

Cytokine concentrations of IL-1β, IL-2, IL-5, IL-9, IL-10, IL-13 and IFN-γ were measured in the Treg suppression assay supernatants of two conditions (irradiated $CD3^-$ cells, PHA-stimulated effector cells without and with Tregs [Teffs:Tregs = 1:0.5]) with the Human Cytokine-Multiplex-Assay-Kit (Biorad, Hercules, USA).

2.2.5 Cytokine secretion

Cytokine concentrations of Interleukin (IL)-2, IL-4, IL-5, IL-10, IL-13, IL-17, Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α) and Granulocyte macrophage colony-stimulating factor (GM-CSF) were measured in cell supernatants by using the Human Cytokine-Multiplex-Assay-Kit according to the manufacturer's instructions (Bio-Rad, Munich, Germany) by LUMINEX technology. The lower detection limits of the assay (pg/ml) were 0.15 (IL-2), 0.05 (IL-4), 0.12 (IL-5), 0.09 (IL-13), 0.08 (IL-10), 1.04 (IL-17), 0.58 (IFN-γ), 0.25 (TNF-α) and 1.0 (GM-CSF).

2.2.6 DNA and RNA extraction

DNA from frozen EDTA blood was isolated using the FlexiGene DNA Kit (Qiagen, Hilden, Germany). DNA of frozen $CD4^+$ cells was isolated using the QIAamp DNA Mini Kit (Qiagen) and RNA of PBMCs and $CD4^+$ cells was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

2.2.7 Microarrays

For microarray experiments, PBMCs from a subgroup of 14 AA, 8 NA and 14 HC ("microarray population") and isolated $CD4^+$ cells from 5 AA and 3 HC were analysed in cooperation with the Institute for Medical Microbiology, Immunology and Hygiene, TUM, Munich. Total RNA was labeled and hybridized to Affymetrix GeneChip® Human Gene 1.0

ST arrays according to the manufacturer's instructions. The quality of the scanned arrays was checked by MvA plots, density plots and RNA degradation plots. All steps were performed using *R* and *Bioconductor* (Gentleman *et al.* 2004). RMA, „robust multichip average" was used for background correction, normalization and to control for technical variation between arrays within the study (Irizarry *et al.* 2003). As the samples were labeled and hybridized at different timepoints, batch effects had to be included as a covariate into the linear model.

2.2.8 Quantitative RT-PCR

Total RNA (1µg) of CBMCs and PBMCs isolated with TRI reagent and RNeasy mini Kit, respectively, was processed with reverse transcriptase (Qiagen, Hilden, Germany). For qRT-PCR we used 0.24µl cDNA [12ng] of CBMCs or 0.12µl cDNA [6ng] of PBMCs per well. Gene-specific PCR products were measured continuously by using the iCycler iQ-multicolor Real-Time PCR Detection-System for CBMCs and the CFX96 TouchTM Real-time PCR Detection System (Bio-Rad) for PBMCs for 40 cycles. Direct detection of the PCR product was monitored by measuring the increase in fluorescence caused by binding of SYBR Green to dsDNA. The cyle threshold (CT) describes the number of PCR cycles required for the fluorescence signal to exceed the detection threshold. The difference in CT values relative to the housekeeping gene *18S* was used to calculate the delta CT (ΔCT). A higher ΔCT corresponds to a lower gene expression. The difference in CT values relative to *18S* rRNA was used to calculate the fold difference with the formula $2^{-\Delta\Delta CT}$. The relative quantitative results were used to determine changes of stimulated/unstimulated samples. All experiments were run in duplicates with the same thermal-cycling parameters. Non-template controls and dissociation curves were used to detect primer-dimers and nonspecific amplification. PCR products were separated on a 3% agarose gel to control for specificity and the expected size of

the PCR fragment. mRNA-specific oligonucleotide primers (forward/reverse) were designed with *Vector NTI Advance 11.5* (Invitrogen, Carlsbad, USA).

2.2.9 Genetics: Polymorphism selection and genotyping

For the selection of polymorphisms in different *TLR* genes, haplotype tagging SNPs were selected for genotyping from all *TLR* polymorphisms with a minor allele frequency >0.10 (Haplotype software). Based on previous knowledge, 12 polymorphisms within the human *TLR* genes *TLR1, TLR2, TLR4, TLR6* and *TLR10* were selected due to their association with asthma and their putative functional implications (Liu, Raedler *et al.* 2011; Eder *et al.* 2004; Kormann *et al.* 2008).

Tagging SNPs within the *IL-10* gene were selected based on the American (CEU) and European (TSI) population samples, genotyped in the HapMap project (Raedler *et al.* 2012). All SNPs located from the proximal promoter (-4000bp) to the 3´UTR and with a minor allele frequency >0.1 in both populations were included in the analyses. Polymorphisms with $R^2 \geq 0.8$ were defined as linkage disequilibrium (LD) block (Figure 6).

Using Haploview (Version 3.32), four tagging SNPs (rs1800890, rs1800871, rs1878672, rs3024498) were selected from all 13 frequent polymorphisms located in the *IL-10* region (Figure 6). Two further polymorphisms, rs1800893 and rs3024496, which are in close LD with rs1878672 and two putative functional variations rs79309463 (deletion) and rs10494879, located in the promoter (-7616 and -6424), were genotyped. (Kube *et al.* 2003; Rieth *et al.* 2004; Wilk *et al.* 2007). In total, seven *IL-10* polymorphisms and one deletion were investigated. The LD-plot of the eight *IL-10* polymorphisms within our study population is shown in Figure 7.

Genotyping was performed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom Inc., San Diego, CA, USA; Ding and Cantor, 2003) at the Institute of

Epidemiology (Helmholtz Centre Munich, Neuherberg, Germany). Polymerase chain reaction assays and associated extension reactions were designed with the SpectroDESIGNER software (Sequenom Inc.). All amplification and extension reaction conditions have been previously described (Kormann *et al.* 2005). Deviations from Hardy-Weinberg Equilibrium (HWE) were assessed for quality control of genotyping procedures. Allele frequencies were compared to the HapMap CEPH (Centre d'Etude du Polymorphisme Humain) population.

2.2.10 Epigenetic regulation of IFN-γ and FOXP3

2.2.10.1 *IFN-γ* promoter and *FOXP3* TSDR methylation

For assessment of the *IFN-γ* promoter methylation, $CD4^+$ cells were isolated with the autoMACS Pro Seperator, depleting the non-$CD4^+$ cells, as recommended by the manufacturer (human $CD4^+CD25^{high}$ isolation kit, Miltenyi Biotec). The purity was determined by flow cytometry analysis and was above 90%. Bisulfite conversion of genomic DNA and template preparation was performed by the group of Prof. Dr. Harald Renz (Institute for Laboratory Medicine and Pathobiochemistry, Philipps University of Marburg, Germany) as described elsewhere (Waterland *et al.* 2006; Tost and Gut, 2007). Pyrosequencing was performed with the PyroMark ID System (Biotage AB, Uppsala, Sweden).

For assessment of the TSDR methylation status, $1.5x10^6$ PBMCs were cultured in 24-well plates in X-Vivo without stimulation or following stimulation with platebound anti-CD3 (3µg/ml) and soluble anti-CD28 (1µg/ml) for 48 hours at 37°C. DNA of PBMCs was isolated with QIAamp DNA mini kit (Qiagen). The methylation status of the TSDR region was determined with an immune monitoring assay from Epiontis GmbH (Berlin, Germany).

Bisulfite conversion of genomic DNA, bisulfite sequencing of *FOXP3* and *GAPDH*, and quantitative PCR have been previously described (Sehouli *et al.* 2011). Real-time PCRs were performed using the Roche LightCyclerH 480 (Roche, Mannheim, Germany) chemistry or

Epitect-MSP (Qiagen) in 20µl, containing 30pmol of methylation- or non-methylation-specific forward and reverse primers for TSDR, 5pmol hydrolysis probe, 50ng lambda-DNA (New England Biolabs, Frankfurt, Germany) and 60 ng bisulfite-treated genomic DNA template and or the respective amount of plasmid standard.

2.2.10.2 *IFN-γ* and *FOXP3* acetylation

For *IFN-γ* and *FOXP3* acetylation analysis, $CD4^+$ cells were isolated by negative selection (autoMACS Pro Seperator, Miltenyi Biotec) and $CD4^+$ DNA was isolated with QIAamp DNA Mini Kit (Qiagen). Chromatin immunoprecipitation (ChIP) was performed by the group of our cooperation partner Prof. Dr. Harald Renz in Marburg. Antibodies against acetylated H4 and H3 (Upstate Biotechnology, Charlottesville, USA) were used. Purification of the immunoprecipated and eluted DNA was performed with QIAquick columns (Qiagen) and amplified by means of quantitative PCR. All amplifications were performed in duplicates, using 2 µL of DNA per reaction. The duplicate mean values were displayed as the percentage of immunoprecipitated DNA compared with total DNA (100% input) (Brand *et al.* 2011).

2.2.11 Statistical analyses

Epidemiologic parameters were analysed for all study populations and screened for possible confounders. Descriptive statistics were calculated depending on phenotype or genotype. Significant differences depending on the phenotype or genotype were analysed by Kruskal-Wallis-test for ordinal/continuous data or by Chi-square test/Fisher´s exact test for categorical data. Data analysis was performed with SAS9.2 (SAS Institute, Cary, NC, USA) and with *R* (Developmental core team, 2005).

For the SNP analyses, all SNPs were tested for deviation from HWE by using the Chi-square test. The LD between the *IL-10* SNPs was calculated by using R^2 and the threshold was set to

0.80 to determine tagging SNPs, each representing 1 LD block. The frequency of SNPs and genetic analyses were analysed with Chi-square test (two groups) or Mantel-Haenszel test for trend (three groups). Not normally distributed data of cytokine concentrations and gene expression were analysed with Mann-Whitney rank test (two groups) or Kruskal-Wallis rank test (three groups) and results were reported as median (±25-75% interquartile range).

Additionally, rank transformed gene expression data were analysed by linear regression models, including the allele count as linear variable. Data were stratified for maternal atopy. For the *IL-10* SNPs, diplotype frequencies were estimated by using a combination of homozygous carriers of the selected variations. As this was an explorative study and the immunologic data were highly correlated, no adjustment for multiple testing was applied. Statistical significance was defined as $p \leq 0.05$.

For the microarray analyses, the single gene ranking was performed with the moderated Bayesian t-statistics as implemented in LIMMA (Smyth G.K., 2004). Genes which still showed a small variability across the samples following normalization were removed with an intensity filter (the intensity had to be above 100 in at least 25% of the samples). A further variance filter was applied where the interquartile range of log2 intensities was set to at least 0. Multiple testing was taken into account by controlling the FDR according to Benjami and Hochbergs method (Benjamini and Hochberg, 1995). Acceptance or rejection of each hypothesis test was decided upon a series of related t-statistics and classified as up-regulated, down-regulated, or not significantly changed (threshold cutoffs: p-value=0.05 and log2 fold-change=0). The GlobalAncova analysis was applied to investigate the role of functional gene groups and pathways within AA, NA and HC. Differentially expressed genes were analysed using *Ingenuity Pathway Analysis* (IPA) software (Ingenuity® Systems, Redwood City, USA).

As PCR data were skewed and partly censored, they were analysed by Tobit regression on rank transformed data (Ballenberger *et al.* 2012). Accordingly, the gene expression values as delta

CT (ΔCT) were rank transformed and entered in the model as dependent variable, the phenotype as independent variable and the stimulation conditions were treated as covariates for the gene comparison analysis. The ranks were retransfomed into the original ΔCT unit and estimates of each phenotype were presented as medians.

For analysis of flow cytometry data, ANOVA on log-transformed data was calculated; data were normally distributed following log-transformation. The phenotype was used as independent variable. Data were presented as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots represent outliers, black line represents the geometric mean).

Treg suppression assays were analysed by a linear mixed model on log-transformed data. "Phenotype" and "addition of Tregs" were treated as independent variables. An interaction term was entered into the model to assess whether the effect of phenotype on "suppressive capacity" depends on "addition of Tregs".

Cytokine data were log-transformed in order to meet the assumption of normality. As the data contained left censored observations (below detection threshold), a Tobit regression was applied with "phenotype" as independent variable and data were presented as boxplots.

IFN-γ methylation data were analysed by ANOVA, with "phenotype" as independent variable.

TSDR acetylation and TSDR demethylation data were analysed by Mann-Whitney test.

3. RESULTS

3.1 Immune regulation early in life (Cord blood)

In order to study immune regulation at the earliest possible timepoint, cord blood mononuclear cells (CBMCs) were investigated. These cells were used to study the role of genetic variants in several candidate genes, which were previously associated with immune-mediated diseases such as asthma. For this work, I have focused on single nucleotide polymorphisms in Toll-like receptor (*TLR*) genes, as key modulators of the adaptive and especially innate immune system, and in the *IL-10* gene, coding for a crucial cytokine with pleiotrophic effects, secreted by several cells of the adaptive immune system.

3.1.1 Role of TLR SNPs in innate and adaptive immunity in cord blood

TLRs represent an evolutionarily conserved family of innate pattern recognition receptors, capable of activating innate and adaptive immunity (Akira *et al.* 2001).

Besides its function in mediating a cellular response to microbial exposure, TLR signalling is involved in the generation of Th cell subsets and production of their effector cytokines, expression of chemokines and antimicrobial peptides (AMPs) and production of reactive oxygen species (Akira *et al.* 2001, Akira and Takeda, 2004). Moreover, TLRs were proposed to directly and indirectly modulate Tregs (Liu and Zhao, 2007), a cell population required to keep a healthy immune balance. Thus we studied the impact of *TLR* polymorphisms on Treg-associated genes in cord blood.

3.1.1.1 *TLR* SNP characteristics

Twelve polymorphisms in *TLR1, TLR2, TLR4, TLR6* and *TLR10* were genotyped within the PAULINA and PAULCHEN study population based on their putative functional implications in allergy or asthma development (Eder *et al.* 2004; Kormann *et al.* 2008) (Table 1). The

genotyping success rate was at least 97% and none of the polymorphisms significantly deviated from Hardy-Weinberg equilibrium (data not shown).

Table 1: Biological characteristics of the analysed *TLR* SNPs

Gene	rs no.	Position rel. to ATG*	Allele	Location	Δ AA	Δ TF	ESE/ ESS	MAF ISAAC Munich	MAF PAULINA/ PAULCHEN
TLR1	rs4833095	743	A/G	CDS	X		X	0.26	0.29
TLR1	rs5743595	-2192	T/C	Intron		X		0.20	0.21
TLR2	rs1898830	-15607	A/G	Promoter		X		0.33	0.33
TLR2	rs3804100	1350	T/C	CDS			X	0.08	0.07
TLR2	rs3804099	597	T/C	CDS			X	0.45	0.43
TLR2	rs4696480	-16934	T/A	Promoter				0.51	0.54
TLR4	rs4986790	4435	A/G	CDS	X	X	X	0.04◊	0.04
TLR4	rs4986791	4735	C/T	CDS	X		X	0.06	0.06
TLR4	rs6478317	-6687	A/G	Promoter		X		0.29	0.32
TLR4	rs10759932	-5724	T/C	Promoter		X		0.12	0.14
TLR6	rs5743789	-2079	T/A	Promoter				0.22	0.22
TLR10	rs4129009	2323	A/G	CDS	X			0.19	0.21

*, based on March 2006 human reference (NCBI build 36.1). ISAAC Munich, Munich study population of the international study of asthma and allergies in childhood, case-control study. ◊, not genotyped in Munich, HapMap data; rs no., reference SNP ID number; CDS, coding sequence; MAF, minor allele frequency; ΔAA, SNP leads to an amino acid change; ΔTF, SNP changes transcription factor binding based on FastSNP analysis; ESE/ESS, SNP changes exonic splicing enhancer or exonic splicing silencer based on FastSNP analysis (Liu and Raedler *et al.* 2011).

3.1.1.2 Impact of *TLR* SNPs on gene expression of Treg-associated genes and Th1, Th2 and TNF-α cytokine secretion

To study the impact of *TLR* SNPs on Tregs, we investigated mRNA expression of the Treg-associated genes *FOXP3, GITR* and *LAG3.* Importantly, *FOXP3* as Treg transcription factor and master regulator is currently representing the most specific Treg marker (Feuerer *et al.* 2009). *FOXP3* mRNA expression was decreased in homozygous carriers of the *TLR1* SNP rs4833095 and *TLR10* SNP rs4129009 following innate stimulation with LpA or Ppg and in homozygous carriers of *TLR2* rs3804099 and *TLR4* rs10759932 SNP after PHA-stimulation (Table 2). Neither the other three *TLR4* SNPs nor the *TLR6* SNP rs5743789 significantly influenced Treg marker gene expression.

3. RESULTS

As maternal atopy in general was identified as important confounder (Schaub *et al.* 2006; Schaub *et al.* 2008) and differences in allele frequency were detected for the *TLR2* SNPs rs4696480 and rs1898830 depending on maternal atopy, expression of Treg-associated genes was stratified for maternal atopy (Table 2; Figure 3, 4).

Table 2: Influence of the 12 assessed SNPs on gene expression of Treg-associated genes and Th1, Th2, and TNF-α cytokine secretion

Gene	rs no. SNP		Treg-associated genes						Th1		Th2		TNF-α	
			FOXP3		GITR		LAG3							
TLR1	rs4833095 GG	all	↓	LpA* Ppg*		n.d.		n.d.		n.d.		n.d.	↑	Derp1*
TLR1	rs5743595 CC	all		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.
TLR2	rs1898830 GG	CBMCs [A]	↓	U* Derp1*	↓	U* Derp1	↓	U* PHA* Ppg Derp1		n.d.	↑	**IL-13:** U* PHA LpA	↑	PHA LpA* Derp1*
		CBMCs [NA]	↑	U* LpA*	↑	U* LpA* Ppg* Derp1	↑	U* LpA* Ppg*		n.d.		n.d.		n..d.
TLR2	rs4696480 AA	CBMCs [A]	↑	U* Derp1*	↑	U Ppg* Derp1*	↑	U* PHA Derp1		n.d.	↑	**IL-5**: PHA* LpA	↑	PHA LpA Derp1
											↑	**IL-13**: PHA* LpA* Derp1*		
		CBMCs [NA]	↓	U* PHA* LpA* Ppg	↓	U* PHA Ppg* Derp1*	↓	U* PHA LpA Ppg* Derp1		n.d.		n.d.		n.d.
TLR2	rs3804100 CC	all		n.d.		n.d.		Derp1*		n.d.		n.d.	↑	U*
TLR2	rs3804099 CC	all	↓	PHA*		n.d.		n.d.		n.d.		n.d.	↑	U*
TLR4	rs4986790 GG	all		n.d.		n.d.		n.d.		n.d.	↑	**IL-13:** Derp1*		n.d.
TLR4	rs4986791 TT	all		n.d.		n.d.		n.d.		n.d.	↑	**IL-13:** Derp1*		n.d.
TLR4	rs6478317 GG	all		n.d.		n.d.		n.d.	↑	**IL-12:** LpA*		n.d.		n.d.
TLR4	rs10759932 CC	all	↓	PHA*		n.d.		n.d.		n.d.		n.d.		n.d.
TLR6	rs5743789 AA	all		n.d		n.d.		n.d.		n.d.		n.d.		n.d.
TLR10	rs4129009 GG	all	↓	LpA Ppg		n.d.		n.d.		n.d.		n.d.		n.d.

↑: expression up-regulated; ↓: expression down-regulated; Th1, secretion of IFN-γ and IL-12; Th2, secretion of IL-5 and IL-13; TNF-α, secretion of TNF-α; U, unstimulated; PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan; Derp1, *Dermatophagoides pteronyssinus.* CBMCs [A], CBMCs of atopic mothers; CBMCs [NA], CBMCs of mothers without atopy; all, no differentiation according to maternal atopy. rs no., reference SNP ID number; n.d., no difference; *, p≤0.05 (Liu and Raedler *et al.* 2011).

The *TLR2* SNPs rs4696480 and rs1898830 did not influence Treg marker expression if all samples were analysed together. In CMBCs of atopic mothers, homozygous carriers of the *TLR2* rs4696480 AA genotype showed higher *FOXP3*, *GITR*, and *LAG3* expression in

unstimulated cells (Figure 3 A/C/E, p=0.02/0.08/0.009) and after Derp1-stimulation (p=0.01/0.004/0.1) and non-significantly higher expression after PHA-, LpA- and Ppg-stimulation, compared to carriers of AT and TT. As opposed to this, *FOXP3*, *GITR* and *LAG3* expression was lower in the AA genotype compared to AT and TT following all stimulation conditions in CBMCs of non-atopic mothers (Figure 3 B/D/F).

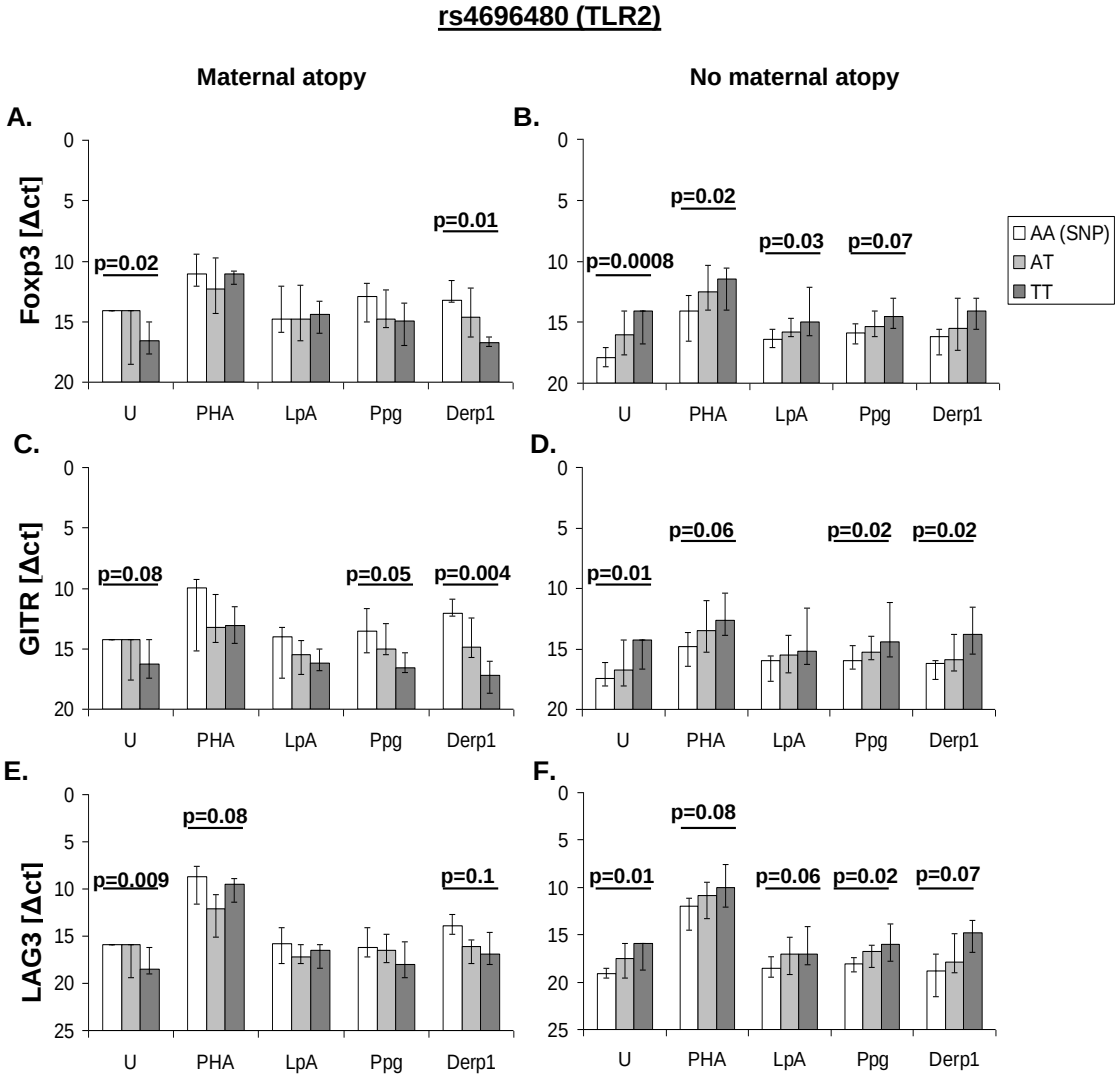

Figure 3. Association of rs4696480 with *FOXP3*, *GITR* and *LAG3* expression, unstimulated (U) and following stimulation (phytohemagglutinin, lipid A, peptidoglycan, *dermatophagoides pteronyssinus*). Values presented as median Δct (±interquartile range); higher values reflect lower expression (axis vice versa). Kruskal-Wallis test was performed to compare AA (single nucleotide polymorphism), AT, and TT. n (maternal atopy) = 15(AA), 16(AT), 8(TT); n (no maternal atopy) = 16(AA), 33(AT), 18(TT). Gene expression in CBMC of atopic (A, C, E) and nonatopic mothers (B, D, F) for *FOXP3*, *GITR*, *LAG3* (Liu and Raedler *et al.* 2011).

Homozygous carriers of the *TLR2* SNP rs1898830 (GG) showed lower *FOXP3, GITR* and *LAG3* expression in unstimulated CBMCs in neonates with atopic mothers (Figure 4 A/C/E, p≤0.04) and after Derp1-stimulation (p=0.05/0.08/0.06), compared to the AG and AA genotype. Opposite effects were observed in unstimulated CBMCs of non-atopic mothers (Figure 4 B/D/F, all p≤0.02) or following stimulation with LpA, Ppg (p≤0.04, except for *FOXP3*_Ppg), or Derp1-stimulation for *GITR* (p=0.09). None of the other *TLR* SNPs was significantly influenced by maternal atopy.

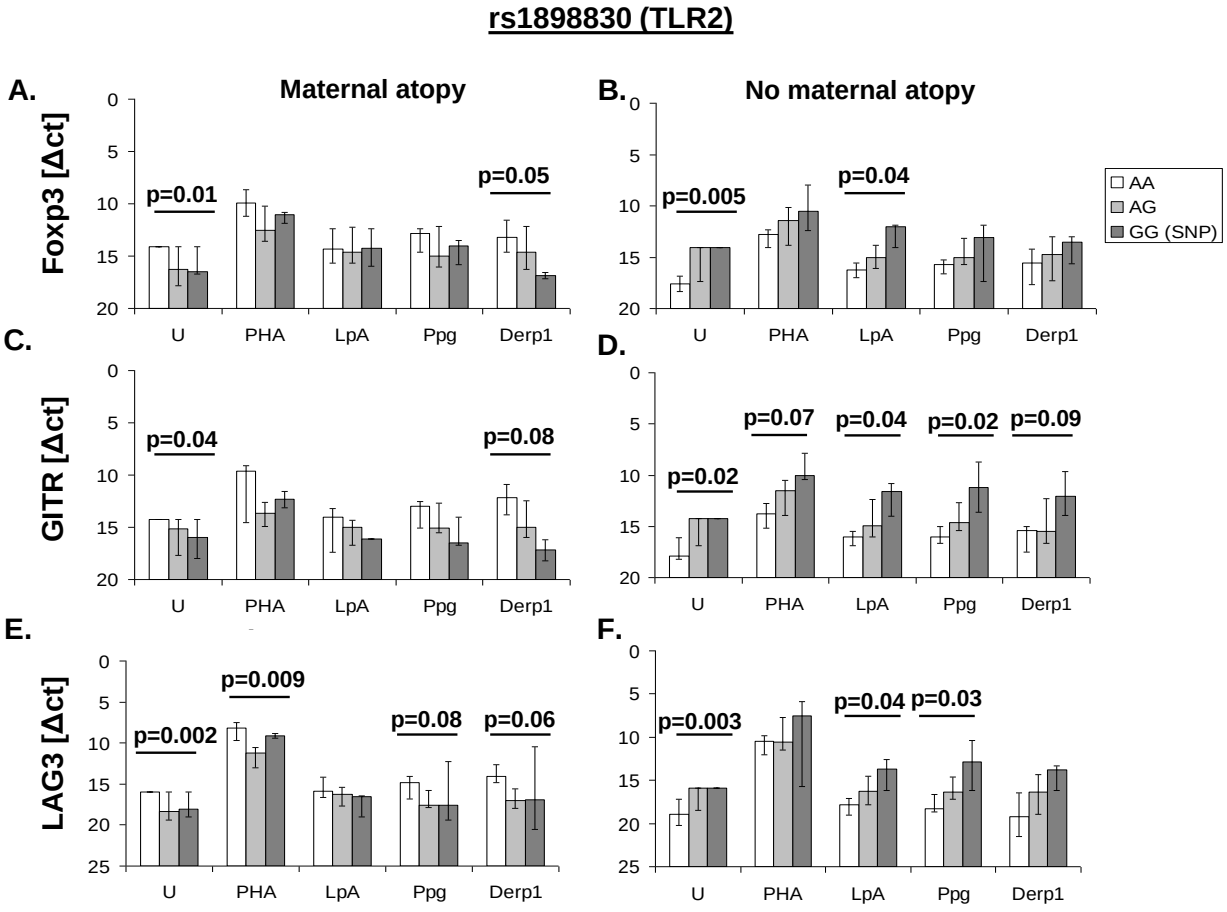

Figure 4. Influence of the rs1898830 genotype on *FOXP3, GITR* and *LAG3* expression, unstimulated (U), and following stimulation (phytohemagglutinin, lipid A, peptidoglycan, *dermatophagoides pteronyssinus*). Values presented as median Δct (±interquartile range); higher values reflect lower expression (axis vice versa). Kruskal-Wallis test was used to compare GG (single nucleotide polymorphism), AG, and AA. n (maternal atopy) = 15(AA), 11(AG), 3(GG); n (no maternal atopy) = 11(AA), 28(AG), 5(GG). Gene expression in CBMCs of atopic (A, C, E) and nonatopic mothers (B, D, F) for *FOXP3, GITR, LAG3* (Liu and Raedler *et al.* 2011).

Besides effects on Treg marker expression, the *TLR2* SNPs rs4696480 and rs1898830 also altered Th2 and TNF-α secretion. In CBMCs of atopic mothers with *TLR2* rs4696480 AA a significantly or boarderline higher secretion of IL-5, IL-13 and TNF-α was detected, while no differences were oberserved if mothers were not atopic (Table 2). No differences were oberserved for the Th1 cytokine IFN-γ. In CBMCs of atopic mothers with *TLR2* rs1898830 GG, IL-13 secretion was lower before and after PHA- and LpA-stimulation and TNF-α was decreased following LpA-, Ppg- and Derp1-stimulation (Table 2). Again, no effects on cytokine secretion were observed depending on the *TLR2* rs1898830 genotype in CMBCs of mothers without atopy.

3.1.1.3 Influence of *TLR2* rs4696480 and rs1898830 on *TLR2* mRNA expression

We hypothesized that the effect of *TLR2* SNPs might be mediated through changes in *TLR2* mRNA expression. There were no differences in *TLR2* levels depending on the genotype if *TLR2* expression was analysed in all cord blood samples independent of maternal atopy (Figure 5).

In CBMCs of homozygous carriers of the *TLR2* SNP rs4696480 (AA) with atopic mothers, *TLR2* mRNA was decreased compared to AT and TT carriers following LpA-stimulation (Figure 5A, p=0.04). CBMCs of non-atopic mothers with the rs4696480 AA genotype showed significantly increased *TLR2* expression unstimulated and following PHA-stimulation (Figure 5C, p=0.05/0.03). In CBMCs of homozygous carriers of the SNP rs1898830, gene expression did not differ depending on the genotype.

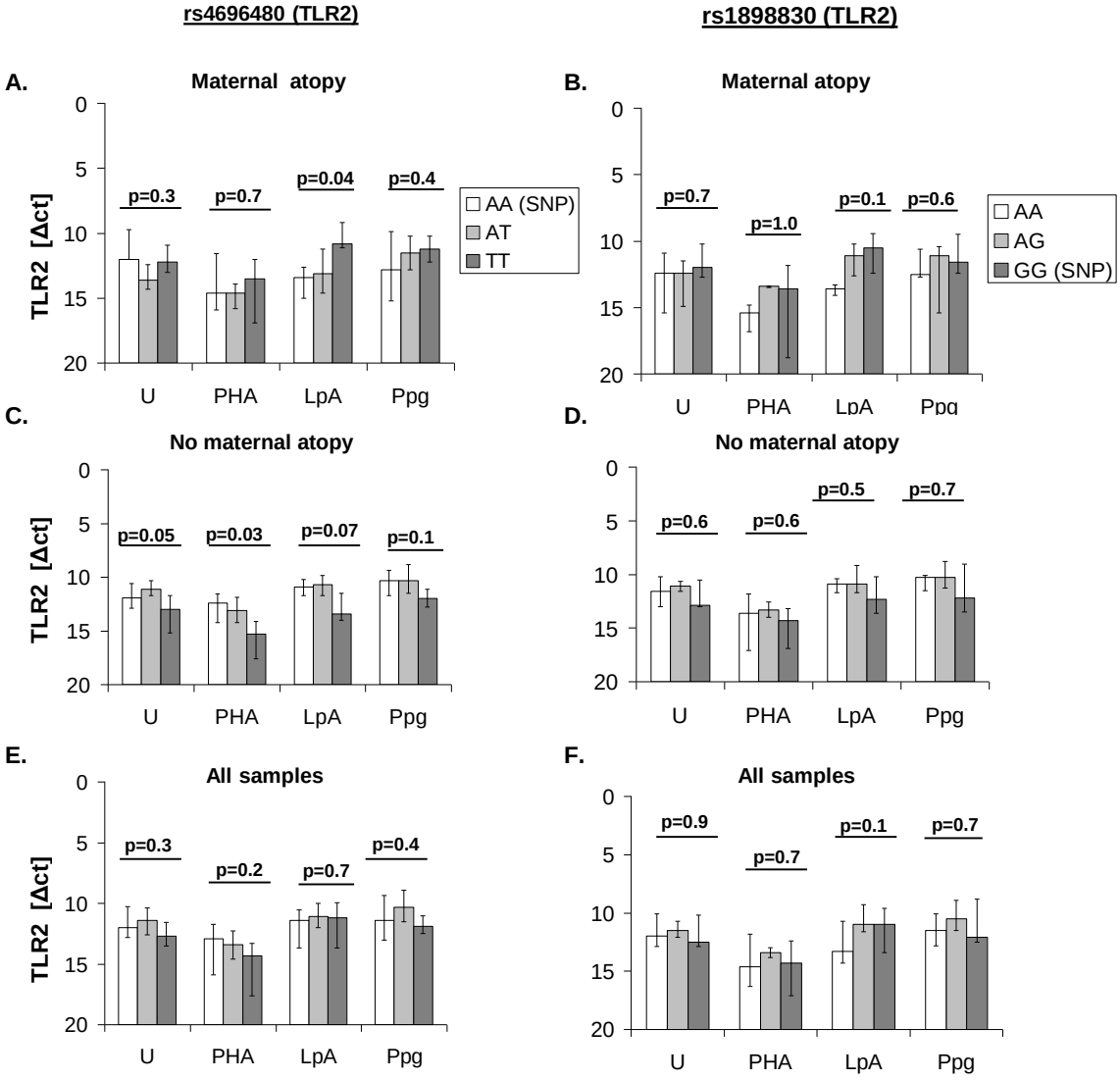

Figure 5. Impact of rs4696480 and rs1898830 on *TLR2* mRNA expression.
Values presented as median ΔCT (±interquartile range). Kruskal-Wallis test to compare SNP with heterozygous and wildtype genotype. **rs4696480:** (A) n (maternal atopy) = 10 (AA), 7 (AT), 8 (TT). (C) n (no maternal atopy) = 9 (AA), 17 (AT), 8 (TT). (E) all. **rs1898830:** (B) n (maternal atopy) = 10 (AA), 5 (AG), 4 (GG). (D) n (no maternal atopy) = 8 (AA), 7 (AG), 3 (GG). (F) all (Liu and Raedler *et al.* 2011).

3.1.2 Role of IL-10 SNPs in adaptive immunity in cord blood

Besides polymorphisms in *TLRs*, as characteristic receptors of the innate immune system, we furthermore investigated genetic variants of *IL-10*. IL-10 is a pleiotropic cytokine produced by a variety of cells of the adaptive immune system, including Th1, Th2, a subset of Th17 cells, mast cells, B cells and also Tregs (O'Garra *et al.* 2007; Taylor *et al.* 2006). IL-10 inhibits cytokine production by Th1 and Th2 cells, phagocytes and natural killer cells and besides

down-regulates pro-inflammatory cytokines including IL-1β, IL-6, IL-8, TNF-α and GM-CSF and was therefore formerly designated as "cytokine synthesis-inhibiting factor" (Fiorentino *et al.* 1991; Grutz G., 2005). *IL-10* was described as a candidate gene in the pathophysiologic mechanism of autoimmune and inflammatory diseases since it can regulate cellular and humoral immunity (Lyon *et al.* 2004).

3.1.2.1 *IL-10* SNP characteristics

Selection of the tagging SNPs of *IL-10* was based on the CEU and TSI population genotyped in the HapMap project and is depicted in the LD-plot in Figure 6.

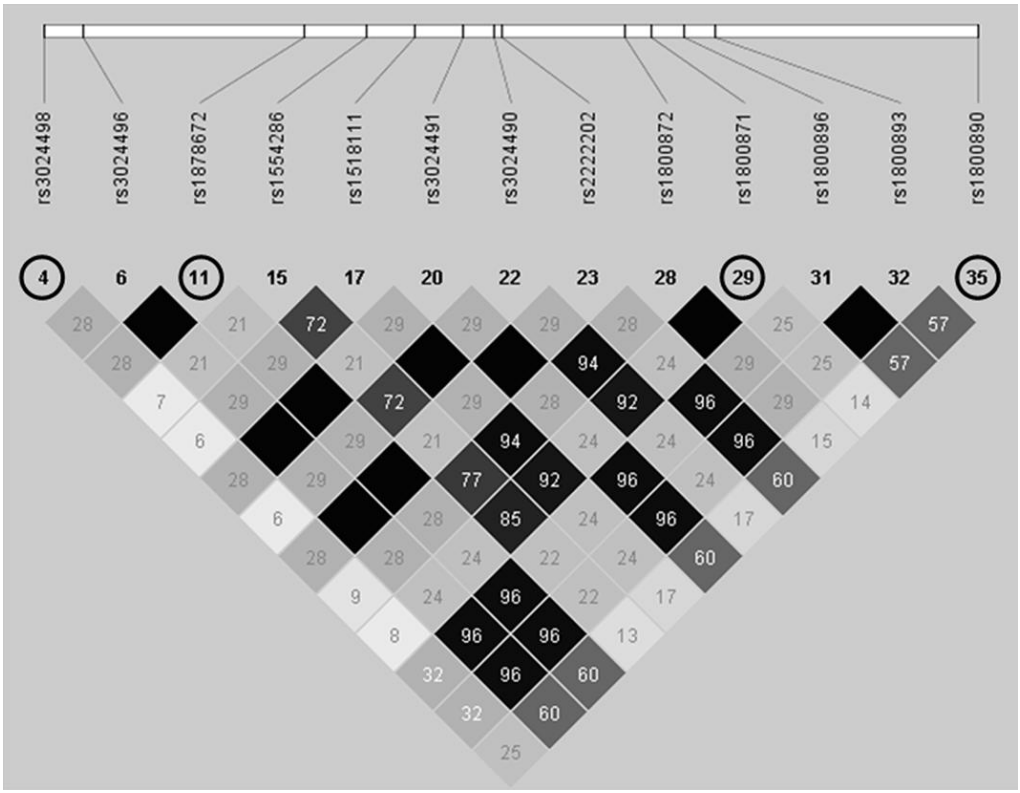

Figure 6. LD (R^2 plots) for 13 frequent *IL-10* polymorphisms within the American (CEU) and European (TSI) population registered in the HapMap project (minor allele frequency >0.1 in both populations). All SNPs were located from the proximal promoter (-4000 bp) to the 3´ untranslated region of *IL-10*. Blocks of tagging SNPs were defined by R^2 values of greater than 0.80. Shades in LD plot (Haploview): white (R^2=0), gray (0<R^2<1), and black (R^2=1). Tagging SNPs are marked with circles. The numbers describe the location of the genetic variations in this *IL-10* region; in total, the region comprises 35 genetic variations (Raedler *et al.* 2012).

Besides the four tagging SNPs (rs1800890, rs1800871, rs1878672, rs3024498) from Figure 6, we have selected two further SNPs, rs1800893 and rs3024496, which are in close LD with rs1878672 and two putative functional variations, rs79309463 (deletion) and rs10494879, located in the *IL-10* promoter (-7616 and -6424) (Kube *et al.* 2003; Rieth *et al.* 2004; Wilk *et al.* 2007). The eight genetic variants of the *IL-10* gene were assessed with a genotyping success rate of >90% (Table 3).

Table 3: Biological characteristics of the analysed genetic variants of *IL-10*

SNP/LD-Block	SNP	Rel. Position*	Alleles	Location	ΔAA	ΔTF	MF
Distal promoter SNP 1	rs79309463	-7616	AGG/-	promoter			0.21
Distal promoter SNP 2	rs10494879	-6365	C/G	promoter			0.36
Block 1	rs1800890	-3526	A/T	promoter	-	-	0.32
Block 2	rs1800871	-795	G/A	promoter	-	+	0.25
Block 3	rs1800893	-1328	C/T	promoter	-	+	0.44
	rs1878672 (tagging SNP)	2127	G/C	intron	-	+	0.44
	rs3024496	3976	A/G	3´UTR	-	+	0.44
Block 4	rs3024498	4311	T/C	3´UTR	-	+	0.24

* Relative position, based on *SNPper* (CHIP bioinformatics Tools); MAF, minor allele frequency within our study population; ΔAA, predicted result of amino acid change in protein domain based on FastSNP analyses; ΔTF, predicted result of transcription factor binding sites change based on FastSNP analyses. LD blocks are represented by one tagging SNP (R^2 >0.80) (Raedler *et al.* 2012).

The two SNPs rs1800890 and rs1878672 slightly deviated from HWE, but allele frequencies were similar compared to the HapMap reference population Centre d'Etude du Polymorphisme Humain (CEPH). Figure 7 depicts the LD-plot of the investigated *IL-10* polymorphisms within our cord blood study population.

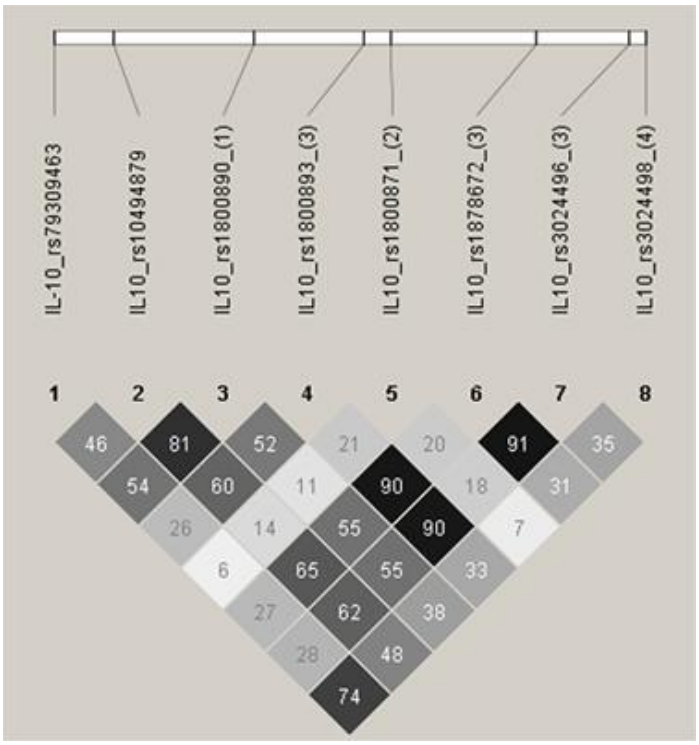

Figure 7. Linkage disequilibrium (R^2 plot) for the eight investigated *IL-10* polymorphisms within our study population. *IL-10*_rs number (LD block); shades in LD plot (Haploview): white (R^2=0), gray (0<R^2<1), black (R^2=1). (Raedler *et al.* 2012).

3.1.2.2 Impact of *IL-10* SNPs on cytokine secretion and mRNA expression of Treg-associated genes and *IL-10*

In homozygous carriers of all investigated *IL-10* SNPs except rs1800871 (Block2), the Th2 cytokine IL-5 was significantly down-regulated (Table 4). No consistent association was detected for IL-13 as another Th2 cytokine and mediator of allergic inflammation. The Th1 cytokine IFN-γ was, inverse to IL-5, up-regulated in homozygous carriers of the promoter SNP rs10494879 and the SNPs from Block 1 and 3. However, it has to be mentioned that highest secretion was detected for heterozygous carriers of the SNP. Concentrations of a further Th1 cytokine, IL-12, were not significantly different in the investigated *IL-10* SNPs. There was no consistently different pattern for the characteristic Th17 cytokine IL-17. Secretion of the pro-inflammatory cytokines TNF-α and GM-CSF was positively correlated in all stimulation conditions and significantly reduced in carriers of both distal promoter SNPs and the SNPs from LD block 3 and 4 (Table 4). The pro-inflammatory cytokine IL-6 was not differentially regulated depending on the *IL-10* genotype. To investigate the impact of the *IL-10* genotype on

Treg cells, we measured the gene expression of the Treg-associated genes *FOXP3*, *LAG3* and *GITR*, which are highly correlated with each other.

Table 4: Influence of genetic variants of *IL-10* on IL-5, IFN-γ, TNF-α, and GM-CSF cytokine secretion and gene expression of Treg associated genes

LD-Block/ SNP	IL-5		IFN-γ		TNF-α		GM-CSF		Treg-marker genes	
rs79309463	↓	U*		n.d.	↓	U*	↓	PHA*	↓	**LAG3**	U
		LpA* #				LpA #		LpA #			LpA #
		D #				Ppg* #		D* #			Ppg
						D*#		D+LpA #		**GITR**	U*
						D+LpA #					
rs10494879	↓	LpA	↑	U*	↓	LpA* #	↓	PHA		n.d.	
		D* #				Ppg* #		D #			
						D #		D+LpA* #			
						D+LpA* #					
Block 1 rs1800890	↓	D* #	↑	U	↑	U*		n.d.	↓	**GITR**	U*
					↓	Ppg #					
Block 2 rs1800871		n.d.		n.d.		n.d.		n.d.	↓	**FOXP3**	LpA*
										(fd)	Ppg*
									↑	**GITR**	D
										LAG3	D*
Block 3 rs1878672	↓	LpA*	↑	D+LpA*	↓	LpA #	↓	D* #	↓	**GITR**	Ppg
		D*				Ppg* #				(fd)	
						D* #					
						D+LpA*					
Block 3 rs1800893	↓	LpA*	↑	D+LpA*	↓	LpA #		n.d.	↓	**LAG3**	D
		Ppg*				Ppg*					
		D*	↓	Ppg*		D* #					
		D+LpA*				D+LpA #					
Block 3 rs3024496	↓	LpA*	↑	D+LpA	↓	LpA* #	↓	D	↓	**FOXP3**	U
		Ppg				Ppg* #				**GITR**	U*
		D*				D* #				(fd)	Ppg*
		D+LpA				D+LpA*				**LAG3**	U
Block 4 rs3024498	↓	LpA*		n.d.	↓	LpA* #	↓	PHA #	↓	**FOXP3**	Ppg*
		D* #				Ppg*		LpA* #		**GITR**	Ppg
						D* #		D*		**LAG3**	Ppg*
						D+LpA #		D+LpA* #			

↑: secretion/expression up-regulated in homozygous carriers of the minor allele; ↓: secretion/expression down-regulated in homozygous carriers of the minor allele; U, unstimulated; PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan; D, *Dermatophagoides pteronyssinus*; (fd), gene expression as fold difference; n.d., no difference; p-value from Kruskal-Wallis test. *, p≤0.05; all other presented results are p≤0.1. # highest/lowest secretion or gene-expression in carriers with increasing number of the minor allele (SNP-HT-WT); total n=200; frequencies of single SNPs were variable depending on MAF (Raedler *et al.* 2012).

At least one of the three Treg-associated genes was down-regulated in homozygous carriers of the SNPs from all four LD blocks (Table 4). An inconsistent regulation was observed for carriers of rs1800871, as *FOXP3* was significanlty down-regulated while *GITR* and *LAG3* were up-regulated. However, *FOXP3* currently represents the most specific Treg marker, and carriers of the SNPs from block 2, 3 and 4 showed decreased *FOXP3* mRNA expression.

We investigated the influence of the *IL-10* genotype on IL-10 at protein and mRNA level. The investigated *IL-10* SNPs did not show any significant differences in IL-10 levels apart from two SNPs. In homozygous carriers of the deletion rs79309463, IL-10 protein but not mRNA was decreased without stimulation ($p=0.03$), however at very low levels. An increase in IL-10 protein and mRNA was observed for carriers of rs3024496 (block 3) following Derp1-stimulation ($p=0.05$ for both, data not shown).

As we detected an effect of maternal atopy on T-cell immune responses early in life, we stratified the data for the atopy status of the mother (Liu and Raedler *et al*. 2011) and detected a minor influence of maternal atopy on the immune response of *IL-10* SNP carriers (data not shown), with primarily decreased IL-5 and IL-13 levels in CBMCs of non-atopic mothers and increased IFN-γ levels in CBMCs of atopic mothers. *FOXP3* expression was increased in carriers of the *IL-10* SNP rs1878672 following Derp1-stimulation without maternal atopy and decreased with maternal atopy. *FOXP3* expression was decreased in carriers of the rs1800871 SNP following LpA-stimulation without maternal atopy, while expression was increased with maternal atopy. This diametrical effect of *FOXP3* expression was already observed for *TLR2* SNPs, depending on maternal atopy (Liu and Raedler *et al*. 2011).

3.2 Immune regulation during the manifestation of atopic diseases in early childhood

To assess the impact of the SNPs on clinical outcomes, a detailed clinical follow-up was performed at the age of 3 years. Data were collected in questionnaires answered by the parents, assessing the outcomes atopic dermatitis (AD), wheeze, food allergy and asthma.

3.2.1 Prevalence of atopic diseases and wheeze in carriers of TLR SNPs

Clinical outcomes regarding atopy in general, AD, asthma, food allergy and wheeze were assessed at age 3 years with a follow-up rate of 88%. Several *TLR* SNPs were associated with risk for atopic diseases or wheeze at the age of 3 years. Carriers of the *TLR4* SNP rs6478317 were significantly associated with risk for food allergy (Table 5, p=0.0006) and *TLR4* rs4986790, rs4986791 and *TLR6* rs5743789 had an increased risk for AD (Table 5, all p≤0.09).

Table 5: Clinical outcomes of carriers of *TLR* SNPs at age 3 years

TLR	SNP	Outcome		WT % (n)		HT % (n)		SNP % (n)		p(MH)
TLR1	rs5743595	atopy	0	34	(20)	40	(14)	0	(0)	0.10
			1	66	(39)	60	(21)	100	(6)	
TLR2	rs1898830	wheeze	0	54	(20)	64	(30)	85	(11)	0.07
			1	46	(17)	36	(17)	15	(2)	
TLR2	rs1898830	asthma	0	76	(19)	92	(24)	100	(9)	0.14
			1	24	(6)	8	(2)	0	(0)	
TLR2	rs3804100	asthma	0	85	(74)	100	(12)	0	(0)	0.05
			1	15	(13)	0	(0)	100	(1)	
TLR4	rs4986790	AD_1	0	86	(77)	62.5	(5)	(x)		0.09
			1	14	(13)	37.5	(3)	(x)		
TLR4	rs4986791	AD_1	0	88	(29)	50	(2)	(x)		0.06
			1	12	(4)	50	(2)	(x)		
TLR4	rs6478317	food allergy	0	100	(40)	98	(53)	80	(8)	0.0006
			1	0	(0)	2	(1)	20	(2)	
TLR6	rs5743789	AD_2	0	71	(77)	71	(39)	44	(4)	0.09
			1	29	(31)	29	(16)	56	(5)	

% (n), percentage of children with respective genotype and outcome (number of cases); 0, negative for respective outcome; 1, positive for respective outcome; p(MH), p value from Mantel-Haenszel test; x, no SNP genotype in study population; atopy, doctor´s diagnosis or symptoms of atopy; wheeze, wheeze in the last 36 month; asthma, doctor´s diagnosis of asthma; AD_1, doctor´s diagnosis of atopic dermatitis; AD_2, atopic dermatitis in the last 36 months (symptoms); food allergy, doctor´s diagnosis of food allergy (Liu and Raedler *et al.* 2011).

For the two *TLR2* SNPs that showed different effects on Tregs depending on the maternal atopy status, results on outcomes were also stratified for maternal atopy (Table 6).

Homozygous carriers of *TLR2* SNP rs1898830 were trendwise protected from wheeze and atopy if mothers were not atopic (Table 6, p=0.07/0.11). Carriers of the *TLR2* SNP rs4696480 showed a trend for protection against any atopy until 3 years of age if mothers were atopic, while the risk for food allergy was increased without maternal atopy (p=0.11/0.10).

Table 6: Impact of the *TLR2* SNPs rs4696480 and rs1898830 on different clinical outcomes stratified for maternal atopy

SNP	atopy	outcome		WT		HT		SNP		p (MH)
				%	(n)	%	(n)	%	(n)	
rs1898830	0	wheeze	0	50	(8)	62	(21)	89	(8)	0.07
			1	50	(8)	38	(13)	11	(1)	
	1	wheeze	0	55	(11)	69	(9)	75	(3)	0.51
			1	45	(9)	31	(4)	25	(1)	
rs1898830	0	atopy	0	33	(2)	38	(8)	71	(5)	0.11
			1	67	(4)	62	(13)	29	(2)	
	1	atopy	0	39	(7)	40	(2)	0	(0)	0.29
			1	61	(11)	60	(3)	100	(2)	
rs4696480	0	atopy	0	48	(10)	30	(9)	29	(2)	0.54
			1	52	(11)	70	(21)	71	(5)	
	1	atopy	0	0	(0)	23.5	(4)	43	(9)	0.11
			1	100	(3)	76.5	(13)	57	(12)	
rs4696480	0	food allergy	0	97	(29)	93	(51)	84	(21)	0.10
			l	3	(1)	7	(4)	16	(4)	
	1	food allergy	0	100	(10)	83	(24)	87	(20)	0.90
			1	0	(0)	17	(5)	13	(3)	

% (n), percentage of children with respective genotype and outcome (number of cases); atopy, maternal atopy. 0, negative for respective outcome; 1, positive for respective outcome; p(MH), p value from Mantel-Haenszel test; wheeze, wheeze in the last 36 month; atopy, doctor´s diagnosis or symptoms of atopy; food allergy, skin manifestation (Liu and Raedler *et al.* 2011).

If carriers of both *TLR2* SNPs were stratified for maternal atopy, the genotypes with increased Treg numbers were associated with a lower prevalence of atopy (rs4696480 SNP and rs1898830 SNP; Table 6, both p=0.11) and wheeze (rs1898830 SNP; p=0.07), while the *TLR2* genotypes with decreased Tregs were associated with a higher risk for e.g. food allergy (rs4696480 SNP, p=0.10).

3.2.2 Prevalence of atopic dermatitis and wheeze in carriers of IL-10 SNPs

For *IL-10* SNPs, the clinical outcomes AD, wheeze and food allergy were assessed with a follow-up success rate of 88%. 37.5% of the children showed wheezy symptoms and in 29.5%, symptoms of atopy were present within the first 3 years of life. None of the investigated *IL-10* SNPs was associated with food allergy at age 3 years. Having the *IL-10* deletion rs79309463 or the SNP rs3024498 (block 4) presented as risk factor for wheeze (Table 7, p=0.07/0.02). Carriers of the SNP 1800890 were trendwise associated with an increased risk for AD (p=0.07). Carriers of both distal promoter SNPs and the SNPs from LD block 1, 3 and 4 were associated with an increased risk for AD, wheeze or both at age 3 years (p≤0.04). Moreover, the third SNP from block 3 showed a trend for an increased risk in AD, wheeze or both (p=0.11, not shown).

Table 7: Clinical outcomes of carriers of *IL-10* SNPs at age 3 years

SNP/ LD-Block	outcome		WT % (n)	HT % (n)	SNP % (n)	p (MH)
rs79309463	wheeze	0	60 (63)	69 (31)	30 (3)	0.07
		1	40 (42)	31 (14)	70 (7)	
	AD/wheeze	0	45 (47)	55 (24)	10 (1)	0.04
		1	55 (58)	45 (20)	90 (9)	
rs10494879	AD/wheeze	0	47 (31)	50 (33)	23 (5)	0.03
		1	53 (35)	50 (33)	77 (17)	
Block 1 rs1800890	AD/wheeze	0	47 (35)	51 (30)	24 (5)	0.04
		1	53 (39)	49 (29)	76 (16)	
	AD	0	72 (53)	75 (44)	52 (11)	0.07
		1	28 (21)	25 (15)	48 (10)	
Block 3 rs1878672	AD/wheeze	0	55 (30)	47 (30)	32 (12)	0.04
		1	45 (25)	53 (34)	68 (26)	
Block 3 rs1800893	AD/wheeze	0	54 (29)	46 (30)	29 (10)	0.02
		1	46 (25)	54 (35)	71 (25)	
Block 4 rs3024498	wheeze	0	61 (62)	69 (31)	29 (4)	0.02
		1	39 (39)	31 (14)	71 (10)	
	AD/wheeze	0	45 (45)	56 (25)	14 (2)	0.03
		1	55 (55)	44 (20)	86 (12)	

0, negative for respective outcome; 1, positive for respective outcome; AD, any symptoms of AD in the first 3 years of life; wheeze, any wheezy symptoms in the first 3 years of life; AD/wheeze, symptoms of AD, wheeze, or both in the first 3 years of life; HT, heterozygous carrier of the SNP; % (n), percentage of children with respective genotype and outcome (number of cases); p (MH), p-value of Mantel-Haenszel test; WT, wild-type (Liu and Raedler *et al.* 2011).

Although we observed a significant association of 6 SNPs with AD, wheeze or symptoms of both on single SNP level (Table 7), no association of the respective haplotypes containing all 6 minor alleles was detected.

The most frequent diplotypes of the six genetic *IL-10* variants that were associated with an outcome at age 3 years are depicted in Table 8. Compared to children having none of the six SNPs, who showed a wheeze prevalence of 39.1% and an AD prevalence of 29.4%, homozygous carriers of the SNPs rs1878672 (Table 8, SNP4) and rs1800893 (SNP5) showed similiar prevalences. Carriers of the aforementioned two SNPs from block 2 (SNP 4, 5) in addition to the SNPs rs10494879 (SNP 2) and rs1800890 (block 1, SNP 3) showed an increase in AD prevalence from 29.4% (diplotype 0) to 55.6% (diplotype 2).

Table 8: SNP diplotypes of the genetic variants of *IL10* associated with outcome at age 3 years

Diplo-type	SNP	SNP	SNP	SNP	SNP	SNP	SNP-pattern		wheeze		AD		AD/wheeze	
	1	2	3	4	5	6	N	%	n/N	%	n/N	%	n/N	%
0	-	-	-	-	-	-	125	76.7	43/110	39.1	32/109	29.4	57/109	52.3
1	-	-	-	x	x	-	11	6.8	3/10	30.0	3/10	30.0	5/10	50.0
2	-	x	x	x	x	-	10	6.1	2/9	22.2	5/9	55.6	6/9	66.7
3	x	x	x	x	x	x	10	6.1	7/10	70.0	5/10	50.0	9/10	90.0

AD, Any symptoms of AD in the first 3 years of life; AD/wheeze, symptoms of AD, wheeze, or both in the first 3 years of life; SNP 1, distal promoter SNP rs79309463 (deletion); SNP 2, distal promoter SNP rs10494879; SNP 3, rs1800890 (block 1); SNP 4, rs1878672 (block 3); SNP 5, rs1800893 (block 3); SNP 6, rs3024498 (Block 4); wheeze, any wheezy symptoms in the first 3 years of life; x, homozygous carrier of the respective SNP/deletion (Liu and Raedler *et al.* 2011).

Homozygous carriers of all SNPs (diplotype 3) showed besides a higher AD prevalence also an increase in the wheeze prevalence from 39.1% (diplotype 0) to 70%.

Both, genetic variations in Toll-like receptors and *IL-10* already had an impact on the neonatal immune regulation. *TLR2* SNPs affected gene expression of Treg marker genes, modulated by the atopy status of the mother. *IL-10* SNPs also influenced Treg-marker gene expression and besides Th1, Th2 and pro-inflammatory cytokine secretion. Both polymorphisms in *TLR2* and

IL-10 were associated with development of immune-mediated diseases such as atopic dermatitis and wheeze in early childhood.

3.3 Immune regulation during the manifestation of childhood asthma

In the first part of this thesis we investigated the influence of genetic variants on immune regulation in cord blood and could already detect an association of early immune maturation with a later development of wheeze and atopic diseases at age 3 years. In the second part, immune regulation later in childhood, namely during the manifestation of childhood asthma was investigated. The aim was to identify the immunological mechanisms which contribute to the pathophysiology of allergic and non-allergic asthma, the two main clinical asthma phenotypes in childhood (Raedler *et al.*, manuscript in preparation).

For this purpose, we assessed several dimensions of immune regulation: We studied different T helper cell subsets with a special focus on Tregs and Th17 cells. Besides a quantification of both cell types, we assessed suppressive capacity of Tregs and determined cytokine levels of further Th cell subsets. Gene expression was assessed genome-wide and on single gene level. To study the impact of epigenetic regulation on childhood asthma, we assessed the methylation status genome-wide and furthermore methylation and acetylation of single candidate genes. To study the role of genetics on allergic and non-allergic asthma in childhood, the study population was genotyped for 108 polymorphisms, including the *TLR2* and *IL-10* SNPs, which were reported to be associated with development of atopic disease and wheeze within the first part of this thesis.

From this comprehensive assessment of immune regulation, the main findings are presented in the following, whereas genome-wide methylation analysis and genotyping are currently performed and not yet included in the results below.

3.3.1 Characterization of the CLARA study population

From January 2009 on we recruited a study population of currently 236 children, while recruitment is still ongoing. Asthmatics were defined by at least 3 attacks of obstructive

bronchitis and/or a doctor´s diagnosis of recurrent episodes of obstructive bronchitis and/or a history of asthma medication and a typical lung function showing reversible pulmonary obstruction. The classification in allergic and non-allergic asthma was based on positive or negative specific allergic sensitization assessed by RAST test, respectively.

196 children from the CLARA population were included for this analysis, comprising 73 AA, 13 NA and 81 healthy children (HC). 12 children were excluded due to further pulmonary or autoimmune diseases or infections. 4 children suffered from allergy without asthma. 13 children showed an overlap of phenotypes and were not included.

Regarding phenotypic differences, eosinophils, IgE, FeNO and delta FEV1 were significantly different between the three groups, clearly delineating the three phenotypes (Table 9).

Table 9: Clinical parameters of the CLARA study population

Phenotype / Parameter	HC	AA	NA	p-value [KW]
N	81	73 (+12)	13 (+1)	
Eosinophils [%]	2.8 (±0.25)	8.1 (±0.54)	5.3 (±1.2)	<0.0001
IgE [IU/ml]	81.2 (±13.7)	729.4 (±140.8)	82.6 (±20.1)	<0.0001
FENO [ppg]	8.6 (±2.1)	51.1 (±5.9)	11.6 (±3.9)	<0.0001
AHR (ΔFEV1)	4.5 (±1.3)	13.0 (±0.8)	11.9 (±1.1)	<0.0001

HC, healthy controls; AA, allergic asthmatics; NA, non-allergic asthmatics; N, number of cases (+ diagnosis to be confirmed); IgE, Immunglobulin E; FENO, fraction of exhaled nitric oxygen; AHR, airway hyperresponsiveness; ΔFEV1, difference of forced expiratory volume in 1 second before and after bronchodilatation; p-value [KW], p-value from Kruskal-Wallis test; data presented as mean (± standard error of the mean).

AA had significantly increased eosinophils, total IgE and exhaled nitric oxygen. Allergic and non-allergic children showed increased airway hyperresponsiveness (>10 %), determined by lung function tests before and after bronchodilatation with the short-acting β_2-adrenergic receptor agonist salbutamol. Detailed population characteristics are depicted in Table 10.

Table 10: Population characteristics of the CLARA study population

Parameter	HC (n=81)	AA (n=73)	NA (n=13)	p-value
Female sex, n (%)	35 (43.21)	23 (31.51)	7 (53.85)	0.171
Age (years), mean ±SD	10.0±3.1	9.3±3.1	7.5±1.8	**0.004**
Maternal diagnosis of asthma (ever), n (%)	5 (7.89)	10 (13.89)	2 (15.38)	0.231
Maternal diagnosis of hay fever (ever), n (%)	17 (22.37)	21 (29.17)	0 (0)	**0.051**
Paternal diagnosis of asthma (ever), n (%)	6 (7.89)	11 (15.07)	1 (7.69)	0.378
Paternal diagnosis of hay fever (ever), n (%)	17 (22.37)	19 (26.03)	1 (7.69)	0.416
Smoking at home, n (%)	16 (20.51)	5 (6.85)	4 (30.77)	**0.012**
Maternal education, (%)				
Elementary school	17.95	19.40	46.15	0.085
Middle school	30.77	14.93	23.08	
High school	24.36	19.4	15.38	
University	23.08	40.30	7.69	
other	3.84	5.85	7.69	
Paternal education, (%)				
Elementary school	22.37	20.9	46.15	**0.005**
Middle school	22.37	4.48	7.69	
High school	21.05	20.9	30.77	
University	30.26	50.75	7.69	
No/other	3.95	2.99	7.69	

The three groups differed significantly in their age, with the lowest mean age in NA. The number of younger and older siblings did not differ between the three phenotypes (not shown). The three phenotypes differed significantly regarding age, maternal diagnosis of hay fever, smoking at home and paternal education. All analyses did not significantly change after adjusting for the potential confounders and are consequently presented unadjusted.

3.3.2 Immune signature of allergic and non-allergic asthmatics in regards to Treg and Th17 cells, gene expression, protein secretion and epigenetic regulation

To better characterize allergic and non-allergic asthmatics and to understand the underlying pathophysiology, we investigated different T helper cell subsets with a special focus on Tregs and Th17 cells, suppressive capacity of Tregs, cytokine levels of further Th cell subsets, gene

expression on genome-wide and on single gene level and epigenetic regulation by analysing methylation and acetylation of single candidate genes, at the manifestation of disease.

3.3.2.1 Immune signature of allergic asthmatics

As previous results from our group on cord blood immune regulation already indicated an important role for Tregs in development of asthma later in life (Schaub *et al.* 2009), we were interested in the role of Tregs in development of allergic and non-allergic asthma in children age 4 to 15 years within this study. By quantification via flow cytometry, we detected increased numbers of Tregs in asthmatics and especially in allergic asthmatics, yet differences were only significant in the comparison with HC (Figure 8 A-C,E). To quantify Tregs, we analysed the $CD4^+CD25^+$ cells that were additionally either positive for the Treg TF FOXP3 (Figure 8A) or negative for the α-chain of the integrin VLA-4 (CD49d) (Figure 8B). The absence of CD49d was suggested as further Treg marker by Kleinewietfeld *et al.* (Kleinewietfelt *et al.* 2010).

To objectively quantify Treg cells, $CD4^+CD25^{high}$ cells were defined as the upper 10% of $CD4^+CD25^+$ ($CD4^+$ up.10%$CD25^+$). In Figure 8C and 8D, the $FOXP3^+$ and $CD49d^-$ negative cells within the upper 10% of $CD4^+CD25^+$ cells are depicted. The $CD4^+CD25^+$ cells, positive for FOXP3 and in parallel negative for CD49d ($CD4^+CD25^+FOXP3^+CD49d^-$) are depicted in Figure 8E.

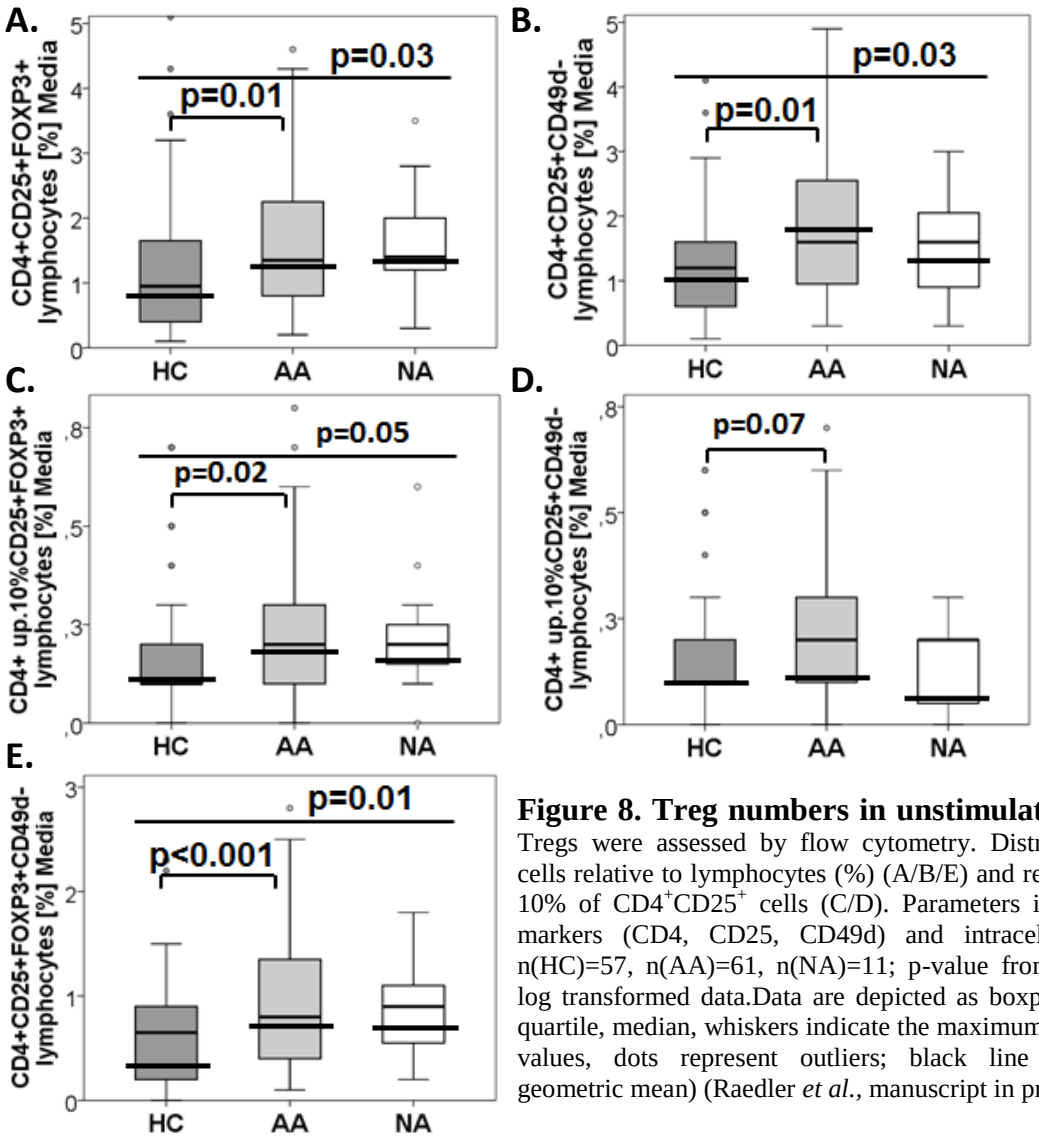

Figure 8. Treg numbers in unstimulated PBMCs. Tregs were assessed by flow cytometry. Distribution of the cells relative to lymphocytes (%) (A/B/E) and relative to upper 10% of $CD4^{+}CD25^{+}$ cells (C/D). Parameters include surface markers (CD4, CD25, CD49d) and intracellular FOXP3. n(HC)=57, n(AA)=61, n(NA)=11; p-value from ANOVA on log transformed data.Data are depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots represent outliers; black line indicates the geometric mean) (Raedler *et al.*, manuscript in preparation).

From a subgroup of samples we determined gene expression of the Treg transcription factor *FOXP3* and of *GARP*, which is specifically expressed on Tregs, by qRT-PCR. *FOXP3* and *GARP* mRNA were significantly increased in AA compared to HC (Figure 9A/B, both p=0.03) and *FOXP3* showed a trend for highest expression in AA compared to HC and NA independent of the stimulation condition, here entitled as "main effect" (Figure 9C, main effect, p=0.10/0.09).

These results were additionally confirmed by microarray experiments, showing significant higher expression of *FOXP3* and *CD25* in unstimulated PBMCs of AA vs. HC (not shown,

p=0.001/0.02) and significantly higher *FOXP3* and *GITR* levels in AA compared to NA (both p=0.02).

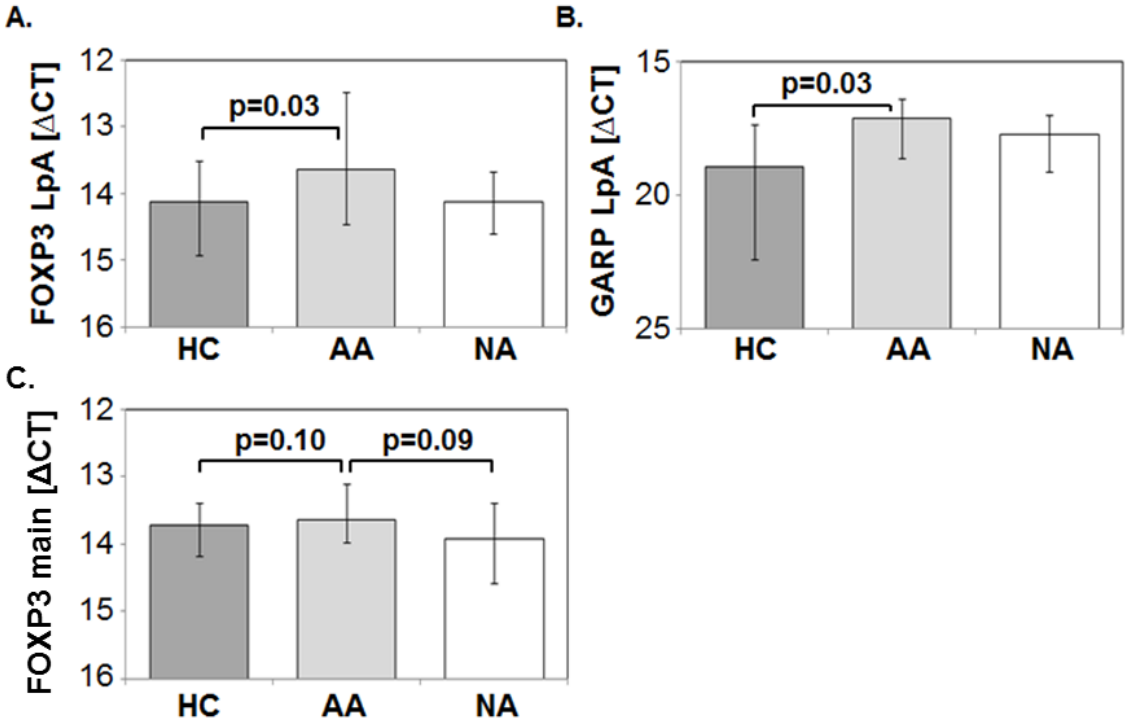

Figure 9. mRNA expression of *FOXP3* and *GARP* in HC, AA and NA.
(A) Expression of *FOXP3* and (B) *GARP* mRNA following LpA-stimulation; (C) *FOXP3* mRNA expression, independent of the stimulation condition (main effect); mRNA expression was assessed by qRT-PCR, PCR data depicted as median +/- CI ΔCT values; higher values reflect lower expression (axis vice versa); n(HC)=15, n(AA)=15, n(NA)=10; p-value from tobit on ranks analysis (Raedler *et al.*, manuscript in preparation).

FOXP3 demethylation in the Treg-specific demethylated region (TSDR) has been suggested as a novel and specific marker for natural Tregs in adults (Baron *et al.* 2007). Within our study, TSDR demethylation was assessed in PBMCs without and following anti-CD3/CD28-stimulation. The amount of Treg cells, determined by Epiontis via TSDR demethylation analysis, was increased in AA compared to HC without and following stimulation (Figure 10A/B, p=0.24/0.06). The number of Tregs did not significantly differ before and after anti-CD3/28-stimulation, indicating that TSDR demethylation is a specific marker for nTregs but not induced Tregs and activated T cells.

In order to assess if the expression of *FOXP3* was regulated by epigenetic mechanisms, we determined the acetylation status of the two histon proteins H3 and H4 in unstimulated $CD4^+$ cells (Figure 10C). Histon acetylation alters nucleosomal conformation and thereby increases the accessibility of transcriptional regulatory proteins to the chromatin template and results in higher levels of transcription (Struhl K, 1998). *FOXP3* acetylation at the histon protein H4 did not differ between AA and HC, while acetylation was increased at H3 in AA compared to HC, indirectly indicating a higher transcription rate of *FOXP3* in AA (Figure 10C, p=0.14).

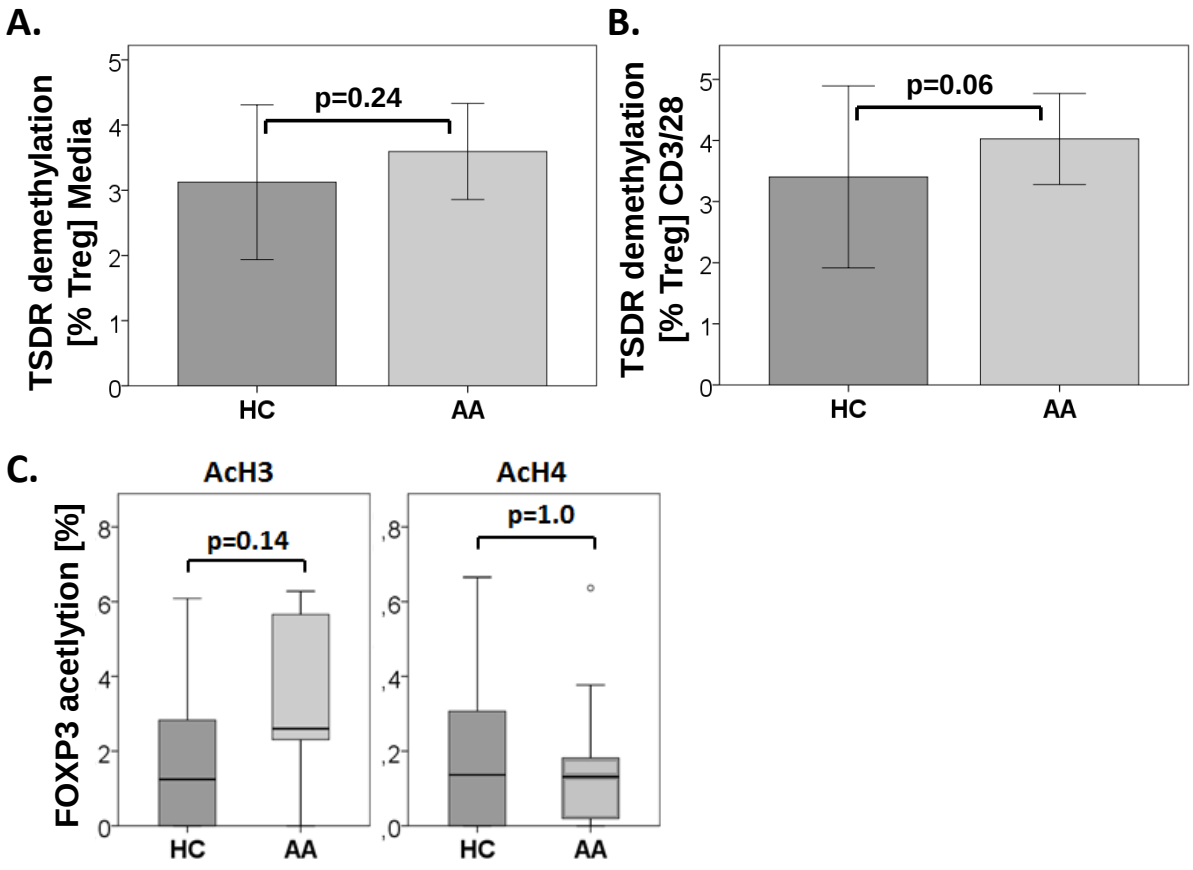

Figure 10. *FOXP3* TSDR demethylation and histon acetylation in HC and AA.
Number of nTregs [%] in PBMCs, determined by TSDR demethylation in (A) unstimulated and (B) anti-CD3/28-stimulated PBMCs, shown as mean±SD; n(HC)=11, n(AA)=11. (C) *FOXP3* acetylation at histone H3 (AcH3) and H4 (AcH4) in unstimulated $CD4^+$ cells. Data shown as percent enrichment to input controls, depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); n(HC)=15, n(AA)=9; p-value from Mann-Whitney test.

3. RESULTS

Quantification data from flow cytometry, qRT-PCR and microarray experiments suggest a higher amount of regulatory T cells in allergic asthmatics compared to non-allergic asthmatics and healthy children. By analysis of the methylation status of the Treg-specific demethylated region in AA and HC, the number of nTregs was confirmed to be increased in AA compared to HC, yet data were not significant. Expression of the Treg master regulator *FOXP3* was increased in AA, which might partially be explained by an increased histone H3 acetylation compared to HC.

While $FOXP3^+$ Tregs were reported to suppress aberrant immune responses and thereby contribute to the maintenance of immune tolerance and homeostasis, Tregs were increased in the AA phenotype. As this phenotype is characterized by allergic inflammation, Tregs may not be able to suppress the "excessive" immune reactions. To test the hypothesis that Treg function is impaired in AA, we analysed the suppressive capacity of Tregs in a subgroup of 11 AA and 10 HC and determined the effect of Tregs on cell division of effector cells (Figure 11) and the Treg suppressive capacity (Figure 12).

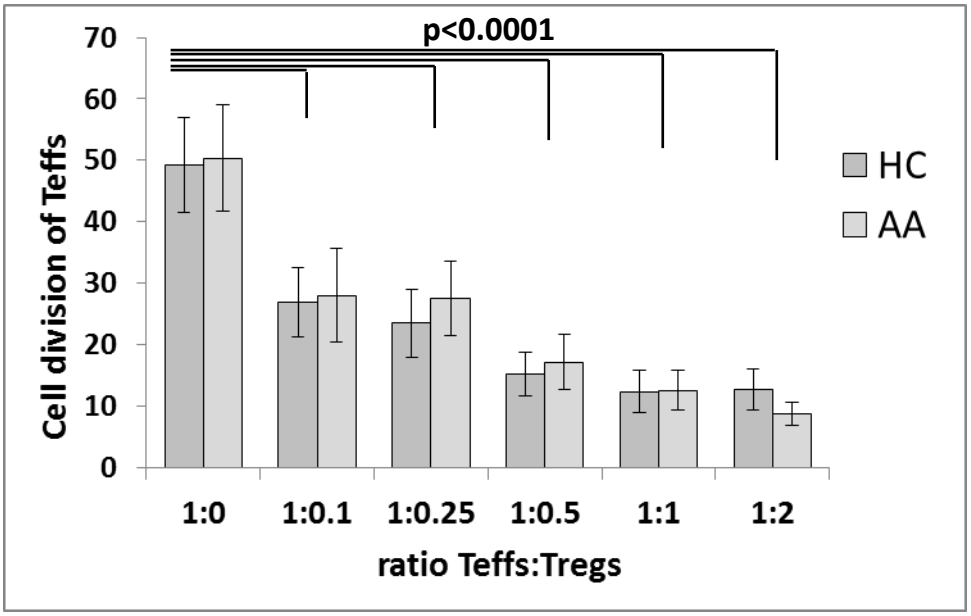

Figure 11. Cell divison of CFSE-stained effector T cells with increasing amounts of Tregs. Cell division of PHA-stimulated effector cells ($CD4^+CD25^-$) without Tregs (ratio Teffs:Tregs = 1:0) and with Tregs (Teffs:Tregs = 1:0.1, 1:0.25, 1:5, 1:1, 1:2), shown as mean±SD; p-value from linear mixed model; n(HC)=10, n(AA)=11.

Without Tregs about 50% of the CFSE-stained Teffs have divided after 72h of incubation. Addition of Tregs from both HC and AA was able to approximately half effector cell division at the ratio of Teff:Tregs = 1:0.1, whereas the decrease in cell division was more efficient with increasing numbers of Tregs (Figure 11, all $p \leq 0.0001$). The suppressive capacity of Tregs in AA was decreased compared to HC, however this effect was not significant (Figure 12).

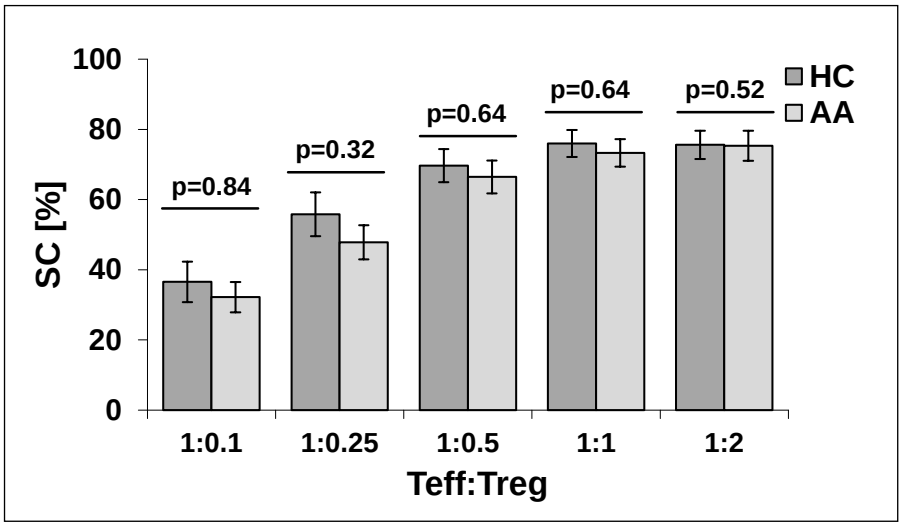

Figure 12. Suppressive capacity of Tregs of HC vs. AA. Suppressive capacity [SC] of Tregs; ratio Teffs:Tregs, ratio of effector cells to Tregs (1:0.1, 1:0.25, 1:0.5, 1:1, 1:2), shown as mean±SD; p-values from linear mixed model; n(HC)=10, n(AA)=11.

To investigate the underlying suppressive mechanism, we measured cytokines in the supernatants of PHA-stimulated effector cells alone and in coculture with Treg cells. Following addition of Tregs, cytokine levels of IL-5, IL-9, IL-13 and IFN-γ were significantly decreased in both HC and AA, however levels did not significantly differ between the two phenotypes before and after addition of Tregs, respectively (Figure 13, A/B/D/E, all $p \leq 0.007$).

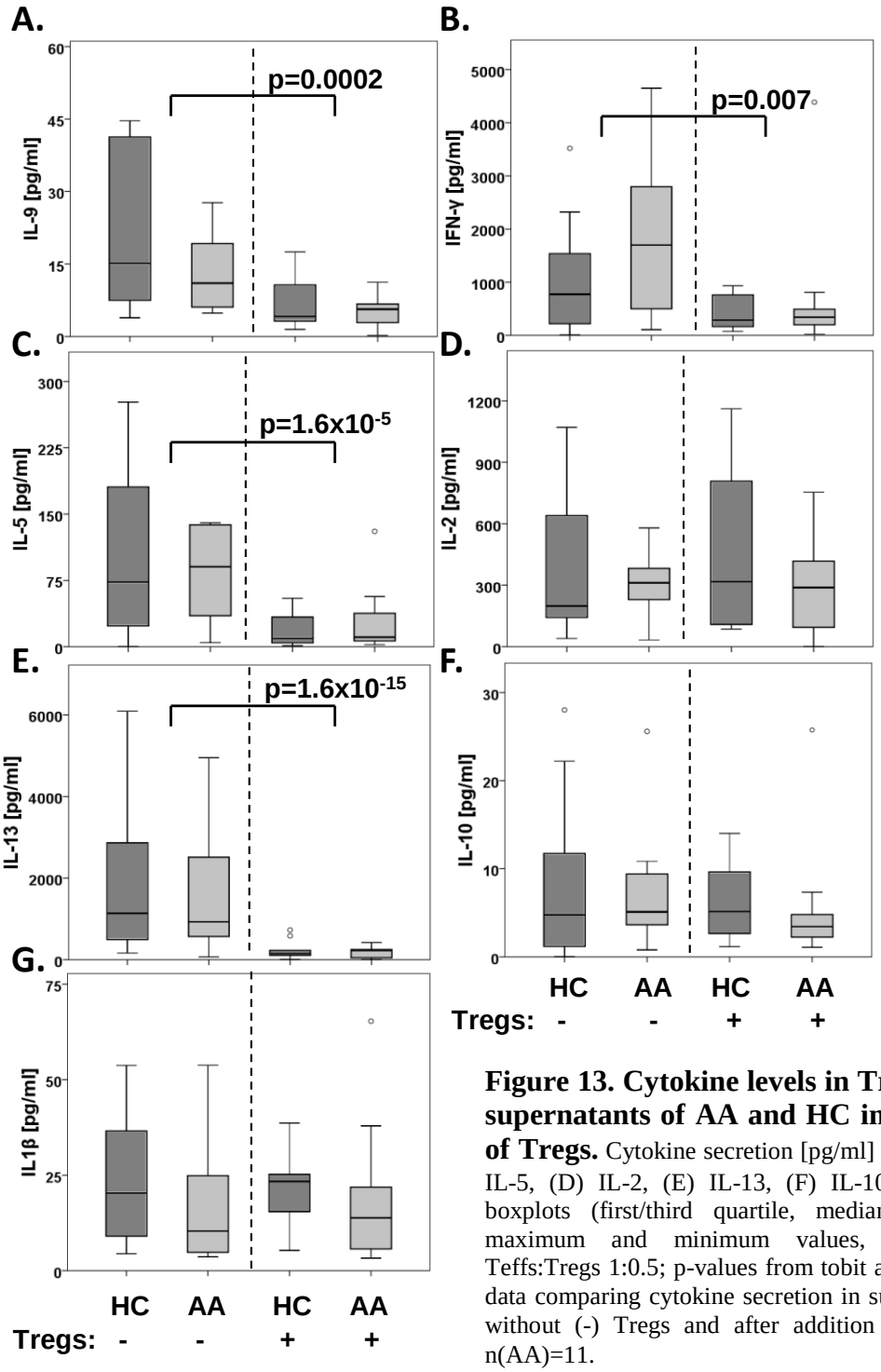

Figure 13. Cytokine levels in Treg suppression assay supernatants of AA and HC in presence or absence of Tregs. Cytokine secretion [pg/ml] of (A) IL-9, (B) IFN-γ, (C) IL-5, (D) IL-2, (E) IL-13, (F) IL-10, (G) IL-1β, depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); Teffs:Tregs 1:0.5; p-values from tobit analysis on log transformed data comparing cytokine secretion in supernatants of HC and AA without (-) Tregs and after addition of Tregs (+); n(HC)=10, n(AA)=11.

Cytokine regulation before and after Treg suppression was significantly different in both, HC and AA. However, the ratio of Th2/Th1 cytokines was significantly decreased in Teffs after Treg suppression only of HC but not AA (Figure 14, p=0.03).

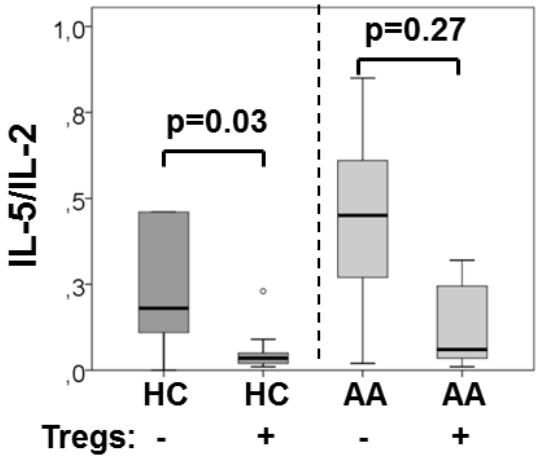

Figure 14. IL-5/IL-2 (Th2/Th1) cytokine ratio in Treg suppression assay supernatants of AA and HC in presence or absence of Tregs. Teffs:Tregs = 1:0.5; p-values from linear mixed model; cytokine ratio depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); n(HC)=10, n(AA)=11.

Children with AA were consequently characterized by an increase in Tregs with lower suppressive capacity for IL-5/IL-2 cytokine secretion.

3.3.2.2 Immune signature of non-allergic asthmatics

Highest levels of pro-inflammatory IL-1β protein levels were detected in NA. Levels were increased in NA compared to HC (Figure 15A-C, p(Media)=0.05; p(LpA)=0.003; p(Ppg)=0.07) and in NA compared to AA without stimulation (Figure 15A, p=0.09). *IL-1β* mRNA expression was significantly increased in NA compared to HC (Figure 15D, p(LpA)=0.03).

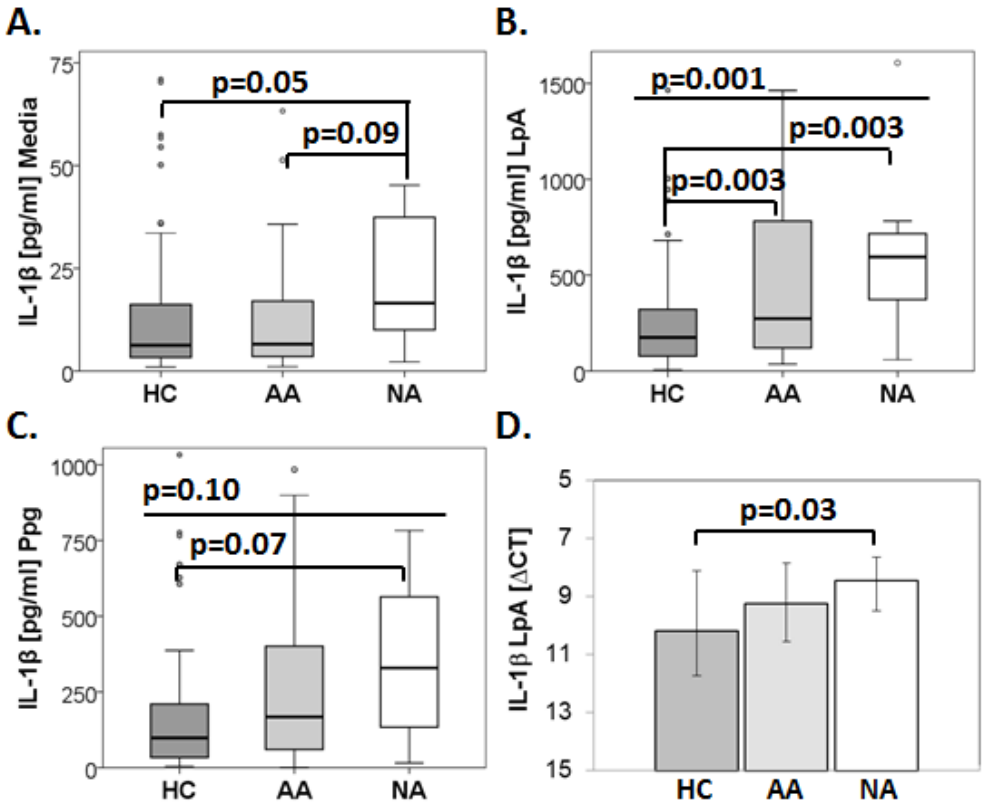

Figure 15. Pro-inflammatory IL-ß secretion and mRNA expression in HC, AA and NA. Protein levels of IL-1β following (A) no stimulation, (B) LpA-stimulation, (C) Ppg-stimulation; protein data depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); n(HC)=72, n(AA)=60, n(NA)=11. (D) *IL-1β* mRNA expression (LpA). PCR data presented as median +/- CI ΔCT values. Higher values reflect lower expression (axis vice versa); n(HC)=29, n(AA)=29, n(NA)=10 (Raedler *et al.*, manuscript in preparation).

While pro-inflammatory IL-1β was up-regulated in NA, the anti-inflammatory cytokine IL-37 was up-regulated in parallel within all stimulation conditions and significantly following anti-CD3/CD28-stimulation (Figure 16).

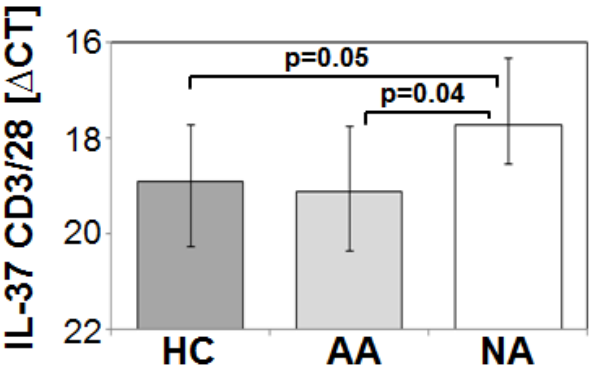

Figure 16. *IL-37* mRNA expression following anti-CD3/28-stimulation. PCR data shown as median +/- CI ΔCT values. Higher values reflect lower expression (axis vice versa); n(HC)=29, n(AA)=29, n(NA)=10 (Raedler *et al.*, manuscript in preparation).

While there was a trend for higher numbers of $IL17^{+}CD4^{+}$ cells detectable in AA compared to HC, there were no differences within the two asthma phenotypes (data not shown; p(M)=0.09, p(CD3/28)=0.06). At cytokine level, IL-17 protein was significantly different among HC, AA and NA following adaptive and innate stimulation (Figure 17, p=0.001/0.007). IL-17 was increased in asthmatics compared to HC, however did not differ significantly within AA and NA.

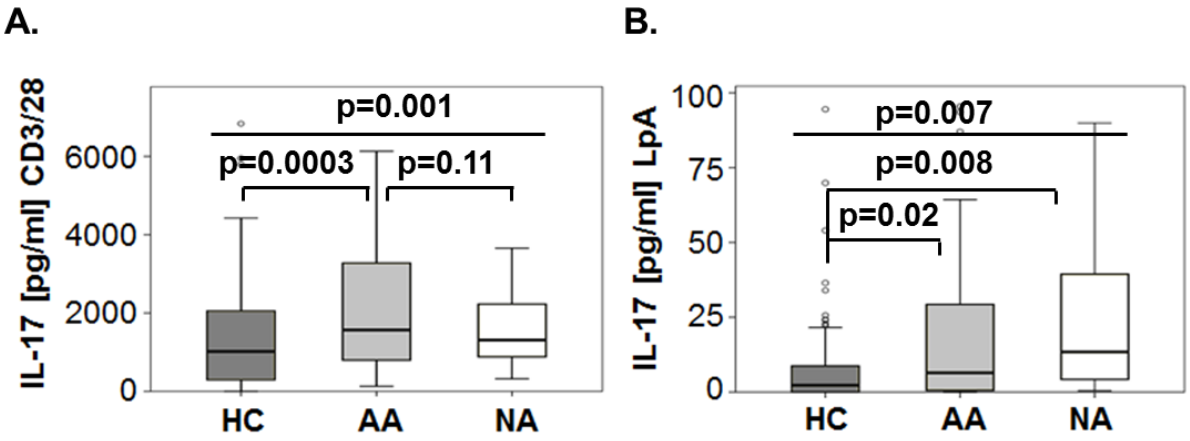

Figure 17. IL-17 cytokine levels in HC, AA and NA.
Protein levels of IL-17 following (A) anti-CD3/CD28- and (B) LpA-stimulation. Cytokine data depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); n(HC)=72, n(AA)=60, n(NA)=11; p-value from tobit analysis on log transformed data (Raedler *et al.*, manuscript in preparation).

A comparison of ratios of the characteristic Th17 cytokine IL-17 to pro-inflammatory, Th2 and Th1 cytokines revealed a Th17-shifted profile with highest ratios in NA compared to AA and HC (Figure 18). The ratios of IL-17 to IL-4, IL-13, IFN-γ, IL-1β and TNF-α were significantly increased in NA compared to HC (Figure 18A/C-F, all p<0.02). The ratio of IL-17/IFN-γ was significantly increased in NA compared to AA (Figure 18D, p=0.009).

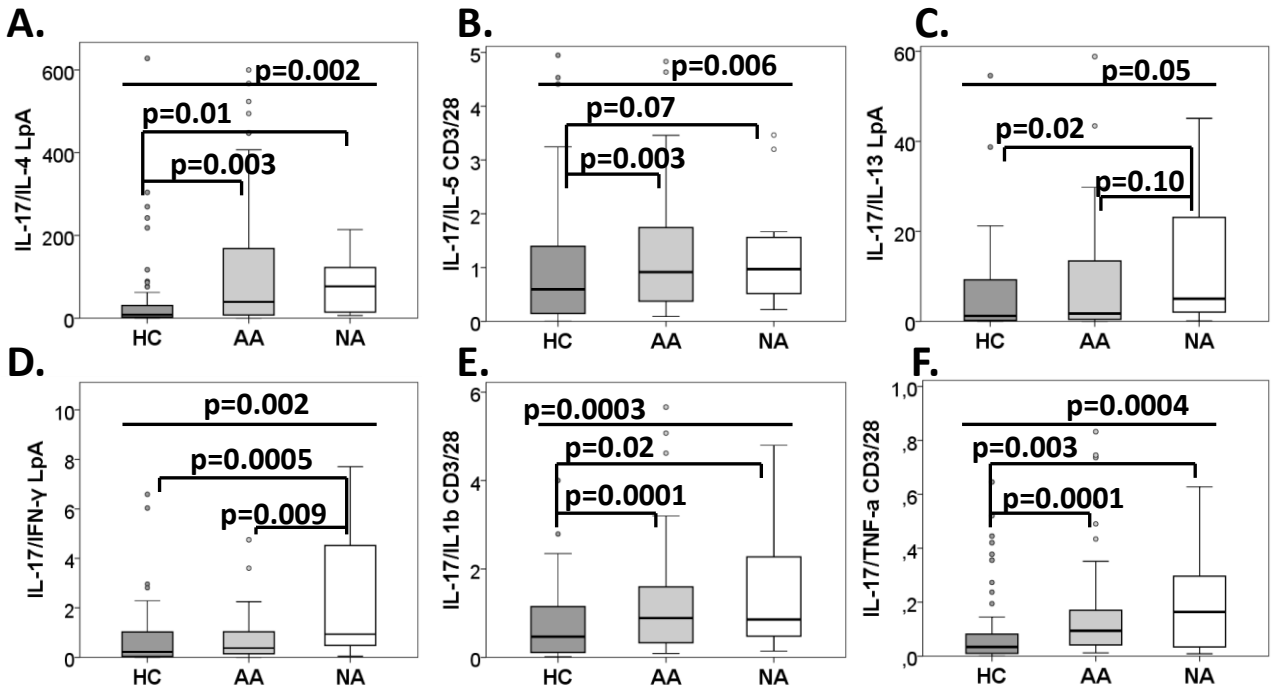

Figure 18. Cytokine ratios of IL-17 to Th2, Th1 and pro-inflammatory cytokines.
Cytokine ratios of IL-17 to the Th2 cytokines (A) IL-4 (LpA), (B) IL-5 (CD3/CD28), (C) IL-13 (LpA), (D) the Th1 cytokine IFN-γ (LpA), the pro-inflammatory cytokines (E) IL-1β (CD3/CD28) and (F) TNF-α (CD3/CD28), depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); n(HC)=72, n(AA)=60, n(NA)=11, p-values from linear models (Raedler *et al.*, manuscript in preparation).

Moreover, neutrophil counts in whole blood differed within the three phenotypes, with a higher percentage in NA compared to HC and AA (Figure 19, p=0.06/0.03).

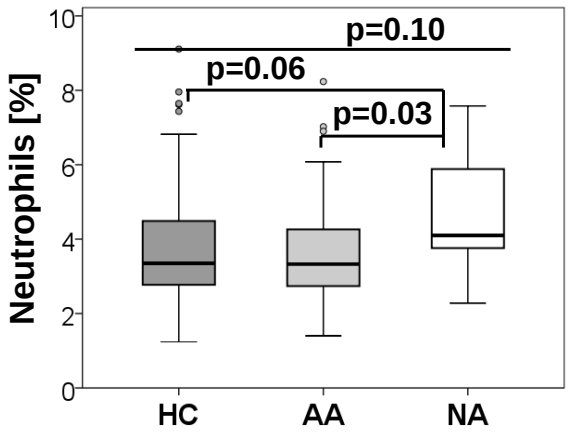

Figure 19. Percentage of neutrophils in whole blood of HC, AA and NA.
p-value from Kruskal-Wallis (3 groups) and Mann-Whitney test (2 groups); data depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); n(HC)=84, n(AA)=74, n(NA)=13 (Raedler *et al,*. manuscript in preparation).

3.3.2.3 Shared immunological profile of allergic and non-allergic asthmatics

As the pathogenesis of allergic asthma is believed to be in part mediated by an inadequate and excessive Th2 response (Kiley *et al.* 2007), we compared the Th2 cytokines IL-5 and IL-13 in AA, NA and HC. IL-5 and IL-13 concentrations differed significantly or trendwise among the three phenotypes (Figure 20). AA and NA shared a Th2-biased phenotype, as IL-5 levels were not only significantly increased in AA compared to HC (Figure 20A/B, p(M)=0.03, p(LpA)<0.001) but also showed increased levels in NA compared to HC (Figure 20A-C, p(M)=0.08, p(LpA)=0.001, p(Ppg)=0.10). For LpA- and Ppg-stimulation, IL-5 levels were highest in NA, yet did not significantly differ from AA. The Th2 cytokine IL-13 was significantly increased in AA compared to HC following anti-CD3/CD28-stimulation (Figure 20D, p=0.003).

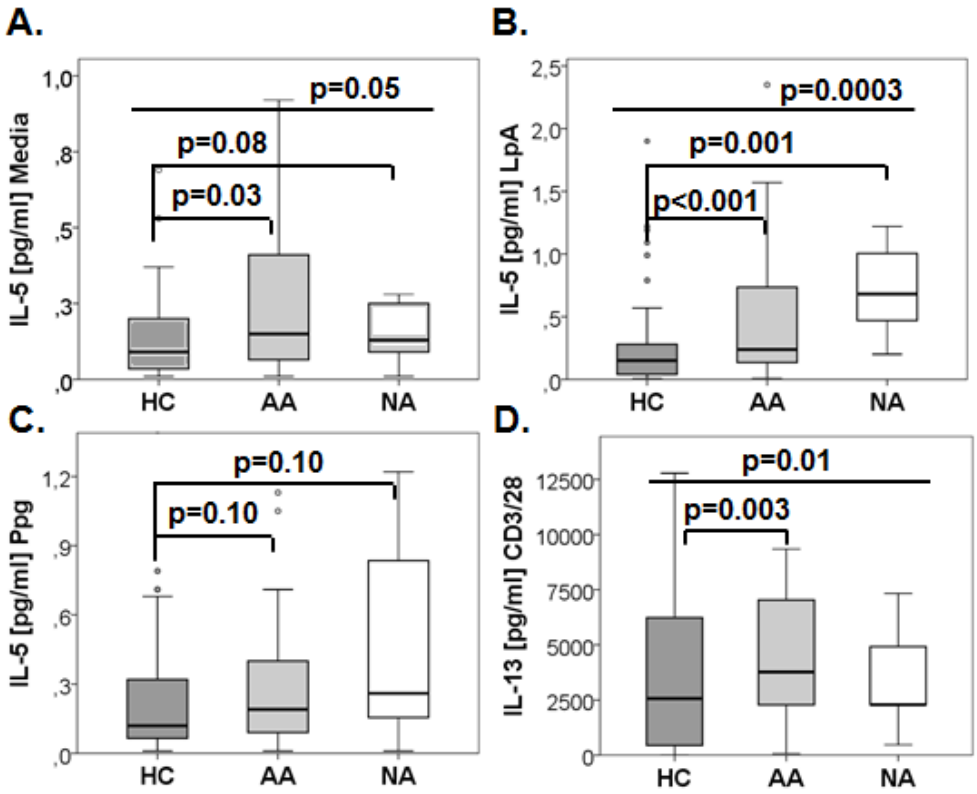

Figure 20. Th2 cytokine levels in HC, AA and NA.
Protein levels of IL-5 following (A) no stimulation, (B) LpA-stimulation, (C) Ppg-stimulation; (D) IL-13 protein level following anti-CD3/CD28-stimulation. n(HC)=72, n(AA)=60, n(NA)=11. Cytokine data are depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); p-value from Tobit analysis on log transformed data (Raedler *et al.*, manuscript in preparation).

Concentrations of the characteristic Th1 cytokine IFN-γ were highest in HC, but did not differ significantly in the three-group comparison of HC, AA and NA. IFN-γ levels were trendwise increased in HC compared to NA (Figure 21A/B, p=0.10/0.07) and were borderline significant decreased in NA compared to AA following LpA-stimulation (Figure 21A, p=0.05).

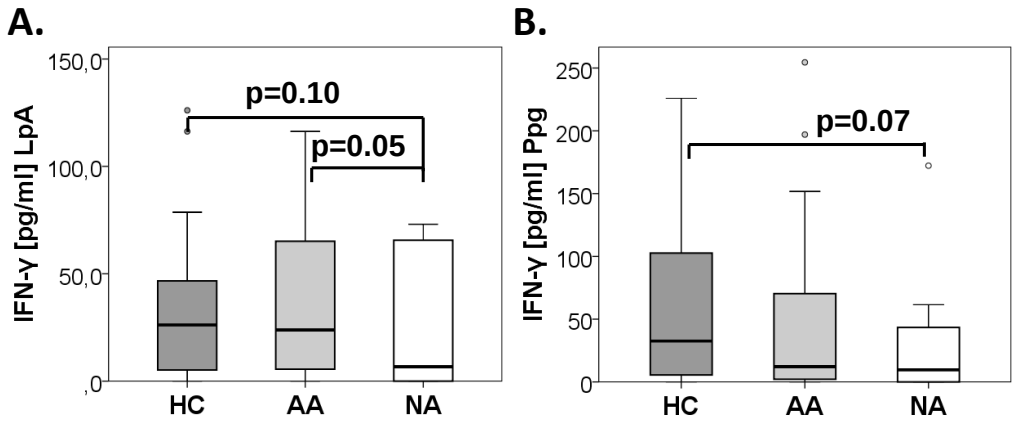

Figure 21. IFN-γ cytokine levels in HC, AA and NA.
Protein levels of IFN-γ following innate stimulation with (A) LpA or (B) Ppg. Cytokine data are depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); n(HC)=72, n(AA)=60, n(NA)=11; p-value from tobit analysis on log transformed data (Raedler *et al.*, manuscript in preparation).

As epigenetics were reported to be involved in T cell differentiation (Lee *et al.* 2006), we furthermore investigated *IFN-γ* promoter methylation and histon acetylation. Especially the *IFN-γ* locus was shown to undergo epigenetic changes during differentiation, which strongly control *IFN-γ* gene expression (White *et al.* 2006).

In cooperation with the group of Prof. Dr. Harald Renz in Marburg, we investigated the methylation status of 3 CpG sites in the proximal *IFN-γ* promoter, which were located downstream (+119, +125, +168) of the transcription start site (Figure 22).

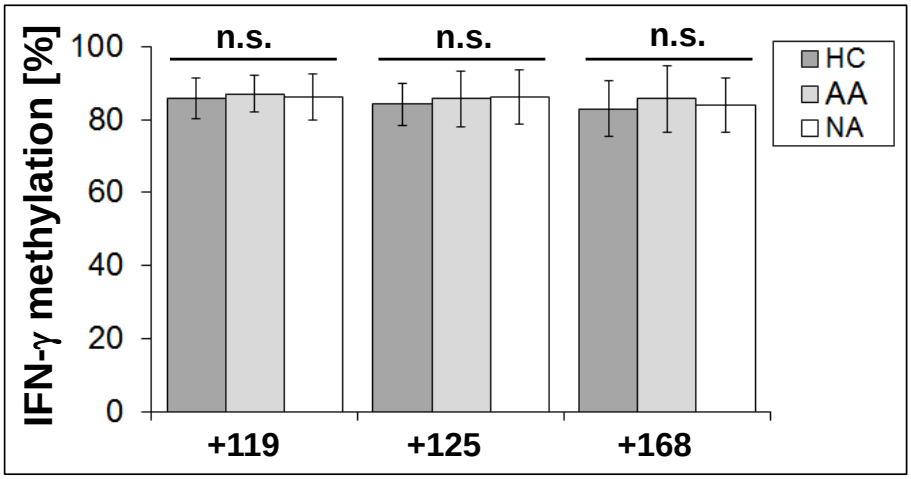

Figure 22. CpG methylation of the *IFN-γ* promoter in isolated CD4$^+$ cells.
Data presented as mean percentage of methylation ± SD at 3 CpG sites downstream (+) of the transcription start site (0); n(HC)=21, n(AA)=17, n(NA)=6; p-values from ANOVA analysis; n.s., not significant.

No significant differences in the methylation status of the analysed region within the *IFN-γ* promoter were observed between HC, AA and NA. Additionally, we studied *IFN-γ* histone acetylation, a further epigenetic mechanism which was, especially in mouse models, shown to be closely associated with *IFN-γ* mRNA expression (Brand *et al.* 2011) (Figure 23).

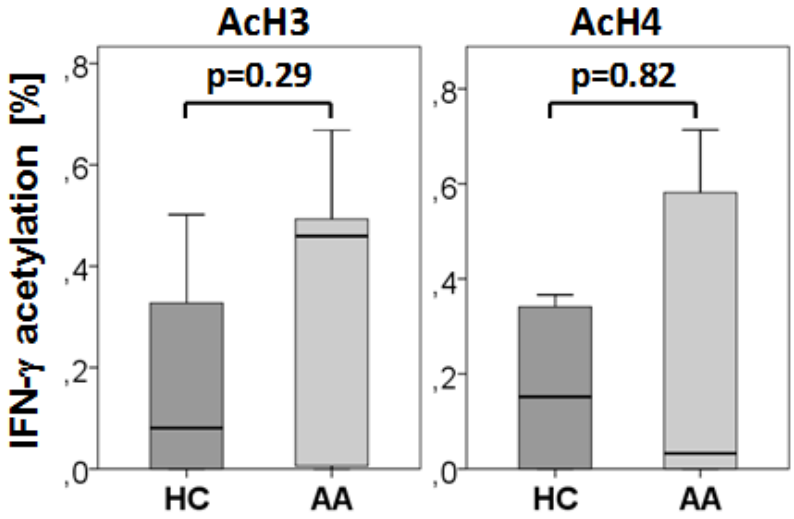

Figure 23. *IFN-γ* histone H3 and H4 acetylation in isolated CD4$^+$ cells.
IFNγ acetylation at histone H3 (AcH3) and H4 (AcH4) in unstimulated CD4$^+$ cells. Data shown as percent enrichment to input controls, depicted as boxplots (first/third quartile, median, whiskers indicate the maximum and minimum values, dots indicate outliers); n(HC)=15, n(AA)=9; p-value from Mann-Whitney test.

Acetylation of H3 and H4 did not differ between HC and AA (Figure 23).

In summary, the Th2 cytokines IL-5 and IL-13 were increased in asthmatics, while the Th1 cytokine IFN-γ was decreased (not significant). This decrease in IFN-γ was not mediated via epigenetic regulation by methylation or acetylation. Neither Th2 cytokines, nor the Th1 cytokine IFN-γ could help to clearly discriminate between the two asthmatic phenotypes.

3.4 Allergic and non-allergic childhood asthma: Identification of novel differentially regulated biomarker genes and pathways by microarray analysis

To be able to better characterize the two childhood asthma phenotypes we studied genome-wide gene expression and regulation applying microarray technology. The intention was to find differentially regulated genes and pathways within the three phenotypes that could serve as potential biomarkers and help to better understand the underlying pathophysiological mechanisms in allergic and non-allergic asthma.

3.4.1 Differentially expressed genes within allergic asthmatics, non-allergic asthmatics and healthy children

In order to identify differentially expressed genes that can contribute to better immunologically distinguish between HC, AA and NA, gene expression was investigated in PBMCs and isolated $CD4^+$ cells. In Figure 24, the differentially expressed genes within the three 2-group comparisons are depicted for PBMCs.

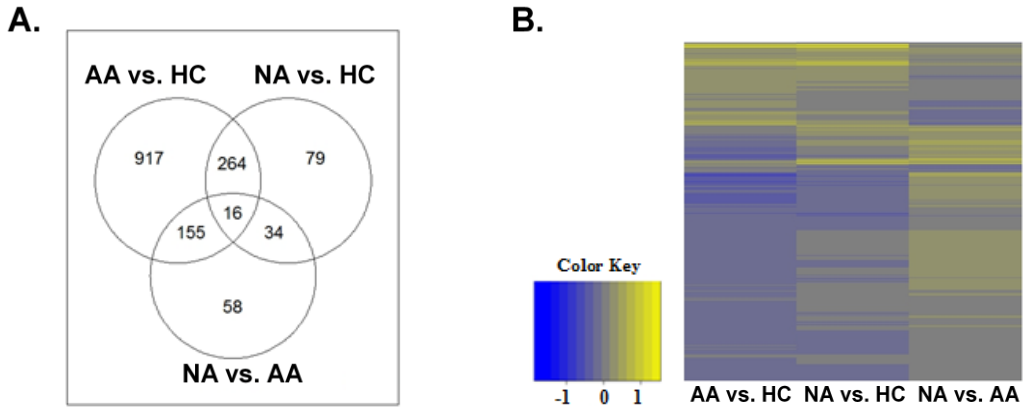

Figure 24. Differentially expressed genes within AA, NA and HC.
(A) Venn diagramm of the contrasts showing the numbers of differentially expressed genes; intersections are given in the overlapping circles. (B) Cluster analysis of significantly differential expressed genes is displayed in a heatmap (Raedler and Da Costa *et al.*, manuscript in preparation).

Table 11 depicts the top 100 significantly up-/down-regulated genes in each of the 3 two-group comparisons, independent of the stimulation condition (main effect).

Table 11: Top 100 genes from microarray 2-group comparisons (part I)

	AA vs. HC				NA vs. HC				NA vs. AA			
Nr.	**Aff. ID**	**Gene**	**logFC**	**adj.P.Val**	**Aff. ID**	**Gene**	**logFC**	**adj.P.Val**	**Aff. ID**	**Gene**	**logFC**	**adj.P.Val**
1	8089249	RPL24	-0.749	2.3E-11	7954310	RN18S1	-1.395	5.6E-05	7994371	LOC100132247	-0.930	1.3E-04
2	8071920	SNRPD3	-0.679	3.0E-10	7940070	C11orf31	-0.276	2.4E-04	7898988	**CLIC4**	0.475	9.0E-04
3	8165309	EDF1	-0.585	3.0E-10	8086752	SNORD13	1.491	3.4E-04	8166072	TMSB4X	0.260	1.2E-03
4	7912670	UQCRH	-0.588	1.3E-09	8034512	SNORD41	1.466	3.4E-04	7904469	SRGAP2	0.389	1.2E-03
5	8077663	JAGN1	-0.414	1.8E-09	8138647	C7orf31	0.286	3.5E-04	8133442	LAT2	0.380	1.4E-03
6	8082911	NCK1	-0.417	1.9E-09	8065359	**CD93**	0.693	3.8E-04	8112746	WDR41	0.383	1.5E-03
7	7989611	FAM96A	-0.461	2.4E-09	8145793	SNORD13	1.248	3.8E-04	7926677	OTUD1	0.468	1.5E-03
8	8043100	TMSB10	-0.459	3.0E-09	7907830	QSOX1	0.441	3.8E-04	7912040	TNFRSF25	-0.375	3.7E-03
9	8166072	TMSB4X	-0.310	3.6E-09	8073194	GRAP2	-0.369	3.8E-04	8043100	TMSB10	0.354	3.7E-03
10	8050695	SF3B14	-0.446	4.6E-09	8092905	LSG1	-0.396	3.8E-04	8075182	**XBP1**	0.349	3.7E-03
11	8040036	RPS7	-0.457	1.3E-08	7974066	PNN	-0.279	3.8E-04	8089249	RPL24	0.496	3.7E-03
12	7936727	RPL21	-0.497	2.4E-08	8044961	RNU4ATAC	1.090	3.8E-04	7899480	SNORA73A	0.549	3.7E-03
13	7898875	RPL11	-0.442	2.4E-08	7952056	CD3D	-0.369	4.1E-04	8134777	PVRIG	-0.359	4.0E-03
14	8063903	RPS21	-0.531	3.8E-08	8156610	HABP4	-0.348	9.7E-04	8172358	UXT	0.274	4.3E-03
15	8131661	RPL21	-0.527	4.9E-08	8030362	SNORD33	0.756	9.7E-04	8073194	GRAP2	-0.327	4.6E-03
16	8126952	WTAP	-0.311	9.4E-08	7922040	CD247	-0.355	1.2E-03	8121886	HINT3	0.361	4.7E-03
17	8131949	CBX3	-0.293	1.0E-07	8073603	RNU12	0.581	1.2E-03	7935337	PIK3AP1	0.423	4.9E-03
18	8143957	RHEB	-0.512	1.1E-07	7905171	PRPF3	-0.279	1.2E-03	7915101	**INPP5B**	0.342	4.9E-03
19	8086698	CCDC12	-0.394	1.1E-07	8060503	SNORD57	0.920	1.2E-03	7959023	MAP1LC3B2	0.291	5.0E-03
20	7915733	PRDX1	-0.442	1.4E-07	7914887	TRAPPC3	-0.263	1.2E-03	8152222	AZIN1	0.298	5.6E-03
21	8049574	UBE2F	-0.457	1.5E-07	7935337	PIK3AP1	0.441	1.2E-03	7981290	WARS	0.513	5.6E-03
22	8020149	NAPG	-0.424	1.5E-07	8041168	SNORD53	0.716	1.7E-03	7961900	ITPR2	0.364	5.6E-03
23	7983606	EID1	-0.331	1.6E-07	8093039	SDHAP1	-0.290	1.9E-03	8020149	NAPG	0.349	5.6E-03
24	7972044	COMMD6	-0.454	1.6E-07	8030199	SNRNP70	-0.289	1.9E-03	8166442	FAM3C	0.285	6.0E-03
25	8024194	GPX4	-0.335	1.7E-07	7914216	SNORA16A	0.934	1.9E-03	7911730	PANK4	-0.222	6.0E-03
26	8023063	ATP5A1	-0.370	1.7E-07	7902235	LRRC7	0.199	2.0E-03	8002322	PDXDC2P	-0.350	6.0E-03
27	7943158	SCARNA9	-0.756	1.8E-07	8139411	TMED4	-0.212	2.2E-03	8093294	**CCR2**	0.952	6.4E-03
28	8020898	ZNF271	-0.324	1.8E-07	8066262	SNORA71D	0.790	2.3E-03	8137264	TMEM176A	1.082	6.6E-03
29	7925691	ZNF124	-0.373	2.1E-07	7921237	FCRL5	0.807	2.3E-03	7905571	**S100A9**	0.762	6.6-03
30	8005877	RPS7	-0.439	2.2E-07	8059854	ARL4C	-0.423	2.3E-03	7989611	FAM96A	0.321	6.6E-03
31	8164067	PSMB7	-0.386	2.7E-07	7948902	SNORD29	0.771	2.6E-03	7924309	ESRRG	-0.175	6.6E-03
32	7911568	SSU72	-0.347	2.7E-07	8116297	HNRNPH1	-0.180	2.7E-03	8140433	POMZP3	-0.286	6.9E-03
33	8017421	CCDC47	-0.373	2.8E-07	8084917	LOC440993	-0.530	2.8E-03	7948894	RNU2-1	0.914	6.9E-03
34	8036710	**GMFG**	-0.304	2.9E-07	8023392	SNORA37	0.720	2.8E-03	8049574	UBE2F	0.364	6.9E-03
35	8042052	RPS27A	-0.496	3.0E-07	7934215	SPOCK2	-0.327	2.8E-03	8023043	PSTPIP2	0.628	6.9E-03
36	8110415	SUDS3	-0.412	3.1E-07	8025584	SNORD105	0.904	2.9E-03	7915468	CCDC23	0.569	6.9E-03
37	7983274	PDIA3	-0.345	3.2E-07	8096669	TET2	0.258	2.9E-03	8044417	TMEM87B	0.273	6.9E-03
38	8034021	CDC37	-0.331	3.2E-07	8126303	**TREM1**	0.908	2.9E-03	8050352	NTSR2	-0.215	6.9E-03
39	8152096	YWHAZ	-0.280	3.3E-07	7905571	**S100A9**	0.777	3.0E-03	8023450	TXNL1	0.247	6.9E-03
40	8086752	SNORD13	1.445	3.4E-07	7995539	**NOD2**	0.384	3.0E-03	8000537	PDXDC2P	-0.316	6.9E-03
41	8145793	SNORD13	1.235	4.1E-07	8030360	SNORD32A	0.845	3.2E-03	8023246	C18orf32	0.220	7.1E-03
42	8085263	TMEM111	-0.416	4.2E-07	7981242	BCL11B	-0.361	3.5E-03	8143790	TMEM176B	0.943	7.3E-03
43	7903582	RPL17	-0.299	4.3E-07	7918869	NGF	0.217	3.7E-03	8158240	TMSB4X	0.227	7.3E-03
44	8006812	PSMB3	-0.400	4.3E-07	8103508	MARCH1	0.511	3.8E-03	7912343	CASZ1	-0.343	7.6E-03
45	8148263	TRMT12	-0.402	4.3E-07	8164607	FNBP1	-0.184	4.0E-03	8011826	C17orf87	0.816	7.6E-03
46	8163930	NDUFA8	-0.434	4.5E-07	8066258	SNORA71A	0.689	4.0E-03	7945132	FLI1	0.203	8.2E-03
47	7964076	CNPY2	-0.382	4.6E-07	7932530	PIP4K2A	-0.246	4.0E-03	7924144	RPL21P28	0.533	8.3E-03
48	8172358	UXT	-0.302	5.1E-07	8053775	ZNF514	-0.365	4.0E-03	8017186	HEATR6	0.348	8.3E-03
49	7978201	NEDD8	-0.329	6.2E-07	8131996	CREB5	0.340	4.0E-03	7921237	FCRL5	0.741	8.3E-03
50	8023855	CYB5A	-0.398	6.5E-07	7915612	PTCH2	0.758	4.0E-03	8068593	ETS2	0.476	8.3E-03

Table 11: Top 100 genes from microarray 2-group comparisons (part II)

	AA vs. HC				NA vs. HC				NA vs. AA			
Nr.	**Aff. ID**	**Gene**	**logFC**	**adj.P.Val**	**Aff. ID**	**Gene**	**logFC**	**adj.P.Val**	**Aff. ID**	**Gene**	**logFC**	**adj.P.Val**
51	7936091	USMG5	-0.553	6.9E-07	7973221	TRAJ17	-0.321	4.5E-03	7992463	SLC9A3R2	-0.225	8.3E-03
52	7955817	PCBP2	-0.247	6.9E-07	8117746	SNORD32B	0.437	4.6E-03	7976243	CPSF2	0.258	8.3E-03
53	8029969	SEPW1	-0.342	6.9E-07	8011499	P2RX1	0.436	4.7E-03	8071392	MED15	-0.213	8.3E-03
54	7978754	C11orf58	-0.322	7.2E-07	8158829	BAT2L1	-0.228	4.7E-03	8154785	CHMP5	0.463	8.3E-03
55	7920873	SNORA42	0.975	7.4E-07	7908397	RGS13	0.178	5.1E-03	8102214	PAPSS1	0.374	8.3E-03
56	7924144	RPL21P28	-0.639	7.6E-07	8061364	RPL41	0.388	5.2E-03	8113433	EFNA5	-0.211	8.3E-03
57	8130952	PSMB1	-0.426	7.7E-07	8036479	YIF1B	0.281	5.3E-03	8084917	LOC440993	-0.493	8.3E-03
58	8063509	C20orf43	-0.233	8.2E-07	7924897	C1orf96	-0.202	5.4E-03	7916862	WLS	0.446	9.2E-03
59	7975113	CHURC1	-0.400	8.4E-07	7987279	GOLGA8B	-0.334	5.5E-03	7919578	JAGN1	-0.328	9.2E-03
60	8048195	RPL37A	-0.323	8.4E-07	8176972	**IL9R**	-0.228	5.7E-03	7946516	SBF2	0.320	9.2E-03
61	7905171	PRPF3	-0.290	8.4E-07	8010078	SNORD1C	0.893	5.9E-03	7958019	DRAM1	0.615	9.2E-03
62	8153223	PTK2	-0.335	8.9E-07	7981601	IGHA2	0.332	6.2E-03	7967563	UBC	0.152	9.4E-03
63	8061772	MAPRE1	-0.260	9.0E-07	7997940	SNORD68	0.907	6.3E-03	8161458	KGFLP2	-0.470	9.4E-03
64	8173713	MAGT1	-0.269	9.1E-07	8070083	TMEM50B	-0.337	6.3E-03	8042391	PLEK	0.595	9.4E-03
65	8044133	NCK2	0.308	9.1E-07	8093294	**CCR2**	0.903	6.3E-03	8174076	GLA	0.379	9.4E-03
66	8094271	MED28	-0.287	9.4E-07	8130578	SNORA20	1.071	6.3E-03	7937287	PSMD13	0.380	1.0E-02
67	7898894	C1orf128	-0.298	9.6E-07	7911568	SSU72	-0.272	6.3E-03	7943288	SRSF8	-0.216	1.0E-02
68	8152222	AZIN1	-0.330	9.7E-07	7921298	FCRL2	0.458	6.7E-03	8050695	SF3B14	0.297	1.0E-02
69	7937476	RPLP2	-0.366	9.7E-07	8018803	SRSF2	-0.225	7.9E-03	7993185	NUBP1	0.319	1.0E-02
70	7952549	RPUSD4	-0.335	1.1E-06	8161024	RMRP	0.569	8.1E-03	7960280	FBXL14	-0.234	1.0E-02
71	8079074	SS18L2	-0.281	1.1E-06	7904303	CD101	0.578	8.1E-03	7954055	APOLD1	-0.254	1.0E-02
72	7970999	SPG20	-0.370	1.1E-06	7981988	SNORD116-20	0.776	8.7E-03	7920244	S100A8	0.958	1.0E-02
73	8084986	FYTTD1	-0.387	1.2E-06	8009241	SNORD104	0.794	8.7E-03	8033987	**ICAM3**	0.278	1.0E-02
74	8171834	RPL9	-0.286	1.2E-06	7975459	SIPA1L1	0.230	8.8E-03	8039905	TMEM167B	0.237	1.0E-02
75	7906852	UHMK1	-0.280	1.2E-06	8146216	VDAC3	-0.256	9.1E-03	7899361	STX12	0.309	1.1E-02
76	7979931	MED6	-0.309	1.3E-06	8084904	SDHAP2	-0.250	9.6E-03	7917338	BCL10	0.247	1.1E-02
77	7904881	PDIA3P	-0.383	1.3E-06	7975113	CHURC1	-0.319	1.0E-02	7951030	SNORD6	0.531	1.1E-02
78	8142878	CDC26	-0.344	1.3E-06	8008087	NFE2L1	0.277	1.0E-02	8179041	HLA-A	0.228	1.1E-02
79	8124144	DEK	-0.384	1.3E-06	8008493	LUC7L3	-0.287	1.1E-02	7952549	RPUSD4	0.273	1.1E-02
80	8039796	CHMP2A	-0.293	1.4E-06	8030860	FPR2	0.782	1.1E-02	7989493	RPS27L	0.258	1.1E-02
81	8031669	ZNF470	-0.296	1.4E-06	8140196	STAG3L2	-0.201	1.1E-02	7945803	CARS	0.287	1.1E-02
82	8097782	RPS3A	-0.402	2.1E-06	8143188	CREB3L2	0.286	1.1E-02	8161147	HINT2	0.439	1.1E-02
83	7992987	HMOX2	-0.361	2.3E-06	7898655	CDA	0.642	1.1E-02	8091327	PLSCR1	0.646	1.1E-02
84	7904948	FAM108A1	0.706	2.3E-06	8142120	NAMPT	0.489	1.1E-02	7953564	C12orf57	0.377	1.1E-02
85	7935462	EXOSC1	-0.305	2.4E-06	8126018	STK38	-0.292	1.1E-02	7995539	**NOD2**	0.347	1.2E-02
86	8017634	DDX5	-0.247	2.4E-06	8016982	SUPT4H1	-0.230	1.1E-02	8028104	HCST	0.419	1.2E-02
87	8088384	PDHB	-0.249	2.4E-06	8003217	COX4NB	-0.346	1.1E-02	8048195	RPL37A	0.257	1.2E-02
88	8042211	B3GNT2	-0.325	2.4E-06	8007803	C17orf69	-0.340	1.1E-02	8057713	MSTN	-0.157	1.2E-02
89	7972810	CARS2	-0.326	2.5E-06	7987449	SRP14	-0.227	1.1E-02	7909175	SRGAP2	0.383	1.2E-02
90	7942839	PCF11	-0.273	2.5E-06	7906767	FCGR2C	0.752	1.1E-02	8015445	NT5C3L	-0.33	1.3E-02
91	8020919	C18orf21	-0.371	2.6E-06	7953981	ETV6	0.229	1.1E-02	8057394	SESTD1	0.388	1.3E-02
92	8161242	EXOSC3	-0.394	2.7E-06	7946933	SERGEF	-0.210	1.2E-02	8155096	CREB3	0.261	1.3E-02
93	8089993	WDR5B	-0.432	2.7E-06	8102321	PLA2G12A	-0.281	1.2E-02	8045499	**HNMT**	0.406	1.3E-02
94	8153449	EEF1D	-0.247	2.9E-06	8026698	C19orf62	-0.192	1.2E-02	8085660	DPH3	0.297	1.3E-02
95	8000003	THUMPD1	-0.340	3.0E-06	8133106	SNORA22	1.347	1.2E-02	8121814	NKAIN2	-0.192	1.3E-02
96	8041168	SNORD53	0.720	3.0E-06	7921319	FCRL1	0.469	1.2E-02	8070826	ITGB2	0.406	1.3E-02
97	8147228	DECR1	-0.316	3.4E-06	8066260	SNORA71C	0.490	1.2E-02	7992656	AMDHD2	-0.296	1.3E-02
98	8078834	WDR48	-0.376	3.4E-06	7983606	EID1	-0.239	1.2E-02	7997712	**IRF8**	0.410	1.4E-02
99	8138776	HIBADH	-0.445	3.8E-06	7950796	CREBZF	-0.262	1.2E-02	8030908	ZNF480	-0.327	1.4E-02
100	8109222	RPL7	-0.338	3.9E-06	8080781	PXK	0.332	1.3E-02	8099850	TMEM156	0.371	1.4E-02

Aff. ID, Affymetrix gene ID; logFC, log2-fold change; adj.P.Val, adjusted p-value. Genes chosen for confirmation by qRT-PCR printed in boldface (Raedler and Da Costa *et al.* Manuscript in preparation).

From these 300 genes, 13 were chosen for confirmation by qRT-PCR, based on their ranking, adjusted p-value and a possible biological role in immune regulatory processes (Table 12).

Table 12: **Gene selection for validation of microarray gene expression by qRT-PCR**

Gene	Literature	Reference
CCR2	o Involved in recruitment and activation of mononuclear cells o Linked to pulmonary fibroblasts and to remodelling diseases of the lung	Zhu *et al.* 2002
CD93	o Involved in adhesion and clearance of apoptotic cells o Inflammation triggers release of sCD93 in vivo o Regulation of processes involved in innate immunity (angiogenesis, phagocytosis of apoptotic cells, tissue repair and homeostasis)	Greenlee *et al.* 2009 Greenlee *et al.* 2008
CLIC4	o Chloride channel; regulates stabilization of cell membrane potential o Transepithelial transport, maintenance of intracellular pH o Regulation of cell volume o Positive regulator of LPS signalling o Enhances TGF-α signalling	 He *et al.* 2011 Shukla *et al.* 2009
GMFG	o Expressed in inflammatory cells o Regulating neutrophil chemotaxis by modulating actin cytoskeleton reorganization o Lower expression in asthmatic vs. healthy control airway epithelium	Aerbajinai *et al.* 2011 Kicic *et al.* 2010
HNMT	o Thr105Ile polymorphism in *HNMT* gene associated with asthma o HNMT plays dominant role in histamine metabolism in human bronchial epithelium o Functional SNPs in *HNMT* decrease its enzyme activity among asthmatics o Histamine involved in pathophysiology of asthma	Szczepankiewicz *et al.* 2010 Yan *et al.* 2000 Akagi M., 1998
ICAM3	o Expressed by resting T cells; important in first contact with DCs o Expressed in allergic lung diseases	Geijtenbeek *et al.* 2000 Popper *et al.* 2002
IL9R	o Expressed in asthmatic airways but not in healthy controls o Induction of hCLCA1, which has a potential role in mucus overproduction in asthmatics	Bhathena *et al.* 2000 Hauber *et al.* 2005
INPP5B	o Involved in cellular calcium signalling o Negative regulator of IgE-Ag-initiated mast cell degranulation	 Zhang *et al.* 2010
IRF8	o Novel intrinsic transcriptional inhibitor of Th17 cell differentiation o Important role in differentiation of myeloid cells, promoting monocyte over granulocyte differentiation o Role in inflammatory polarization of macrophages	Ouyang *et al.* 2011 Xu *et al.* 2012
NOD2	o Role in the immune response to intracellular bacterial lipopolysaccharides o Mutations associated with autoimmune diseases o Influences Th1/Th2 balance and thereby modulates the adaptive immune response to bacteria o Important in the development of atopy and asthma	Reijmerink *et al.* 2010
S100A9	o Pro-inflammatory protein, expressed abundantly in the cytosol of neutrophils and monocytes o Increased during early airway response in asthma (rat model of asthma) o Amplifier of inflammation in autoimmunity o High levels found in Crohn's disease, rheumatoid arthritis and cystic fibrosis o Regulation of calcium dependent intracellular processes	 Yin *et al.* 2008 Simard *et al.* 2010 Tirkos *et al.* 2006
TREM1	o Amplifies neutrophil- and monocyte-mediated inflammatory responses	Schenk *et al.* 2007
XBP1	o Transcription factor; regulates genes important for immune system o IRE1/XBP-1 pathway is relevant for airway epithelial inflammation-mediated cytokine secretion o TREM-1-activated neutrophils enhance their respiratory burst activity, degranulation and IL-8 release	Lambrecht and Hammad, 2012 Radsak *et al.* 2004

CCR2, chemokine (C-C motif) receptor 2; CD93, CD93 molecule; CLIC4, chloride intracellular channel 4; GMFG, glia maturation factor gamma; HNMT, histamine N-methyltransferase; ICAM3, intercellular adhesion molecule 3; IL9R, interleukin 9 receptor; INPP5B, inositol polyphosphate-5-phosphatase; IRF8, interferon regulatory factor 8; NOD2, nucleotide-binding oligomerization domain containing 2; S100A9, S100 calcium binding protein A9; TREM1, triggering receptor expressed on myeloid cells 1; XBP1, X-box binding protein 1.

From these 13 differentially expressed genes, significant differences in mRNA expression could be confimed by qRT-PCR for the genes *CD93*, *TREM1*, *S100A9*, *CLIC4*, *INPP5B* and *IRF8* (Table 13).

Table 13: Comparison of microarray and qPCR results for main effects

	Microarray				qRT-PCR		
	Microarray population				Microarray population		
Gene	**p-value**	**Rank**	**2-group comparison**	**logFC**	**p-value PCR finding**	**2-group comparison**	**Difference in ΔCT medians**
GMFG	$2.86x10^{-7}$	34	AA vs. HC	-0.30	n.s.		
CD93	$3.80x10^{-4}$	6	NA vs. HC	0.69	**0.002**	NA vs. HC	-1.68
	0.01	149	NA vs. AA	0.51	**$9.1 x10^{-6}$**	NA vs. AA	-1.79
TREM1	$2.90x10^{-3}$	38	NA vs. HC	0.91	**0.09**	NA vs. HC	-1.34
	0.03	346	NA vs. AA	0.71	**0.005**	NA vs. AA	-1.80
S100A9	$3.00x10^{-3}$	39	NA vs. HC	0.78	**0.01**	NA vs. HC	-1.14
	$5.80x10^{-3}$	29	NA vs. AA	0.76	**0.03**	NA vs. AA	-0.99
IL9R	$5.70x10^{-3}$	88	NA vs. HC	-0.23	n.s.		
					0.08	AA vs. HC	0.6
CCR2	$6.30x10^{-3}$	65	NA vs. HC	0.90	n.s.		
	$6.10 x10^{-3}$	27	NA vs. AA	0.95	n.s.		
CLIC4	$9.30 x10^{-4}$	2	NA vs. AA	0.48	n.s.		
	0.02	5326	AA vs. HC	-0.20	**0.03**	AA vs. HC	1.00
XBP1	$2.30 x10^{-3}$	10	NA vs. AA	0.35	n.s.		
INPP5B	$4.40 x10^{-3}$	18	NA vs. AA	0.34	**0.03**	NA vs. AA	-0.56
ICAM3	0.01	73	NA vs. AA	0.28	n.s.		
HNMT	0.01	93	NA vs. AA	0.41	0.10	NA vs. AA	-0.73
IRF8	0.02	98	NA vs. AA	0.41	**0.002**	NA vs. AA	-0.79
	0.17	13674	AA vs. HC	-0.14	**0.006**	AA vs. HC	0.43
NOD2	$3.00 x10^{-3}$	40	NA vs. HC	0.38	n.s.		
	0.01	85	NA vs. AA	0.35	n.s.		

Gene selection of 13 genes was based on significantly different 2-group comparisons of the microarray analyses; a higher delta CT value (ΔCT) refers to a lower gene expression; bold p-values, microarray findings that could be confirmed by qRT-PCR; n.s., difference in gene expression assesed by qRT-PCR is not significant (Raedler and Da Costa *et al.* Manuscript in preparation).

CD93 and *TREM1* were confirmed to be up-regulated in NA compared to HC and besides showed a significantly increased expression in NA compared to AA (Table 13, $p=9.1x10^{-6}$/ 0.005, Figure 25A/B/C). *S100A9* was significantly higher expressed in NA compared to HC and AA (Table 13, both $p\leq0.03$; Figure 25A/D) and *IRF8* mRNA expression was significantly increased in NA compared to AA and decreased in AA compared to HC (Table 13, both $p\leq0.006$; Figure 25A/E). Expression of *CLIC4* was decreased in AA compared to HC (Figure 24A, p=0.03, expression not shown). Expression of *INPP5B* and *HNMT* was increased in NA compared to AA (Figure 25A/F/G; p=0.03/0.10).

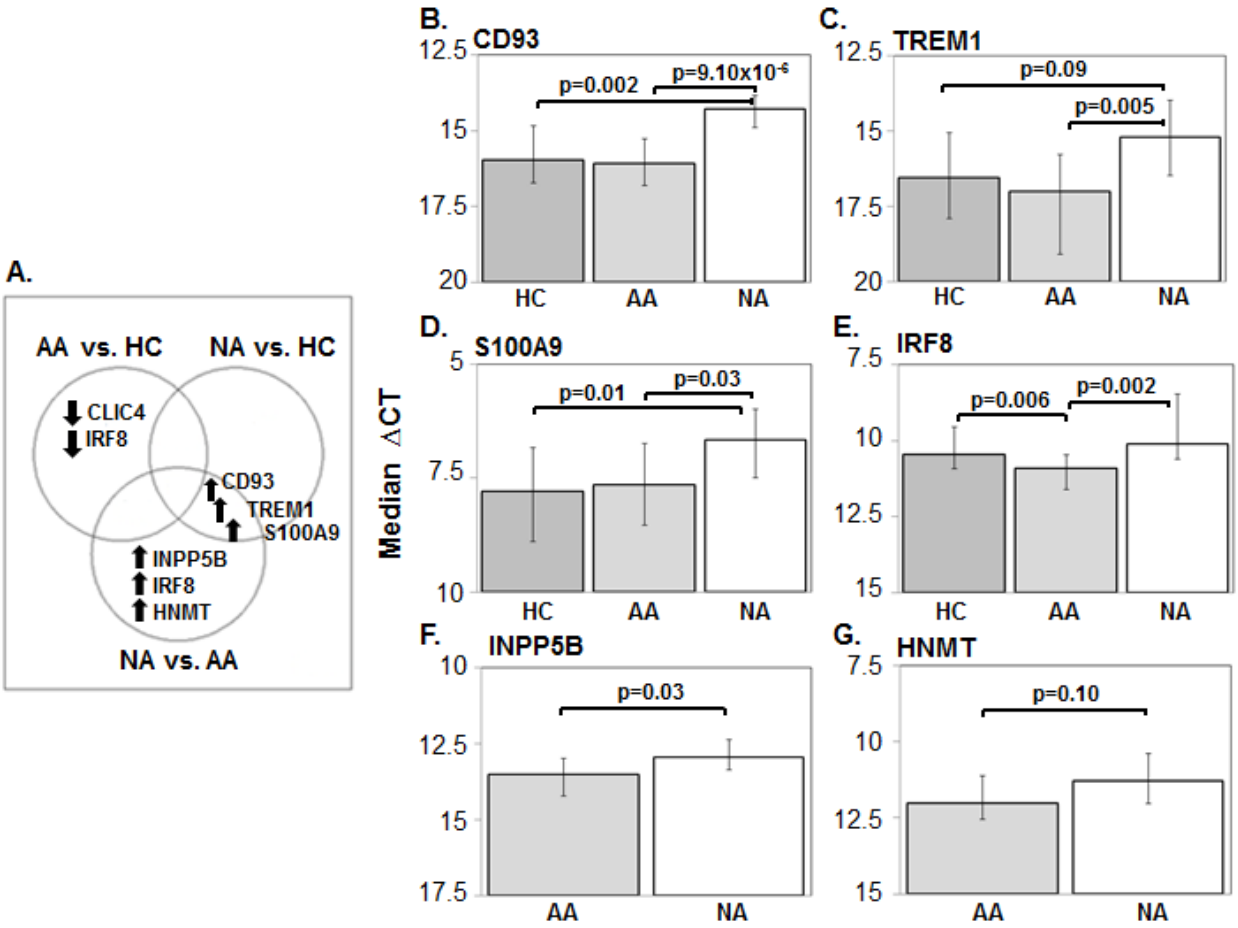

Figure 25. Confirmation of microarray gene expression results by qRT-PCR.
Differentially expressed genes assessed by qRT-PCR in the microarray population. Data shown as median +/- CI ΔCT values. The y-axes are depicted vice-vera as a higher ΔCT refers to a lower gene expression. (A) Venn diagramm with three 2-group comparisons; ↑/↓, up-/down-regulation of the gene in the respective 2-group comparison(s); gene expression of (B) *CD93*, (C) *TREM1*, (D) *S100A9*, (E) *IRF8*, (F) *INPP5B*, (G) *HNMT;* p-value from tobit regression on log transformed data; n(HC)=14; n(AA)=14; n(NA)=8. (Raedler and Da Costa *et al.*, manuscript in preparation).

In summary, the three phenotypes showed distinct gene expression profiles. Expression of *CD93*, *TREM1* and *S100A9* was significantly increased in non-allergic asthmatic children compared to allergic asthmatic and healthy children.

3.4.2 Differentially regulated pathways within allergic asthmatics, non-allergic asthmatics and healthy children

Besides single differentially expressed genes, important cellular pathways were investigated by GlobalAncova analysis regarding their regulation in AA, NA and HC. Two different sources of information were used: First, already annotated cellular pathways from the KEGG pathway database, a collection of online databases that connect information on molecular interaction networks. Second, characteristic genes of different immune cells including transcription factors, cytokines, chemokines and receptors, relevant for the respective cell type (Table 14).

Significant differences in KEGG pathways and expression of immune cell-characteristic genes are shown in Table 15. Differences in regulation of several KEGG pathways were mainly seen before and after innate LpA-stimulation. Following anti-CD3/CD28-stimulation, no differences were detected except for the TLR and mTOR pathway. mTOR regulation differed significantly between AA and HC within all stimulation conditions and between NA and AA after anti-CD3/CD28-stimulation (Table 15, mTOR, $p \leq 0.03$). TGF-β signalling differed significantly in PBMCs of NA and HC (Table 15; TGF-β, Media, $p=0.02$) and AA compared to HC following LpA-stimulation (Table 15, TGF-β, $p<0.0001$; Figure 26). Following innate stimulation, TLR signalling differed significantly in AA or NA compared to HC and also among the two asthma phenotypes (Table 15, TLR, p=0.02/0.02/0.04).

Regarding microarray gene expression of characteristic genes of immune cells, AA and NA differed regarding their expression of characteristic Th2 genes from HC (Table 15, Th2, LpA: p=0.05/0.03), with increased expression of Th2 genes in asthmatic subjects (data not shown).

Expression of Treg genes was significantly increased in AA compared to HC and NA without stimulation (Table 15, Treg, p=0.03/0.04). In PBMCs of NA, expression of characteristic macrophage genes was increased compared to HC and AA, without (Table 15, MP, p=0.006/0.05) and following LpA-stimulation (Table 15, MP, p=0.03/0.006).

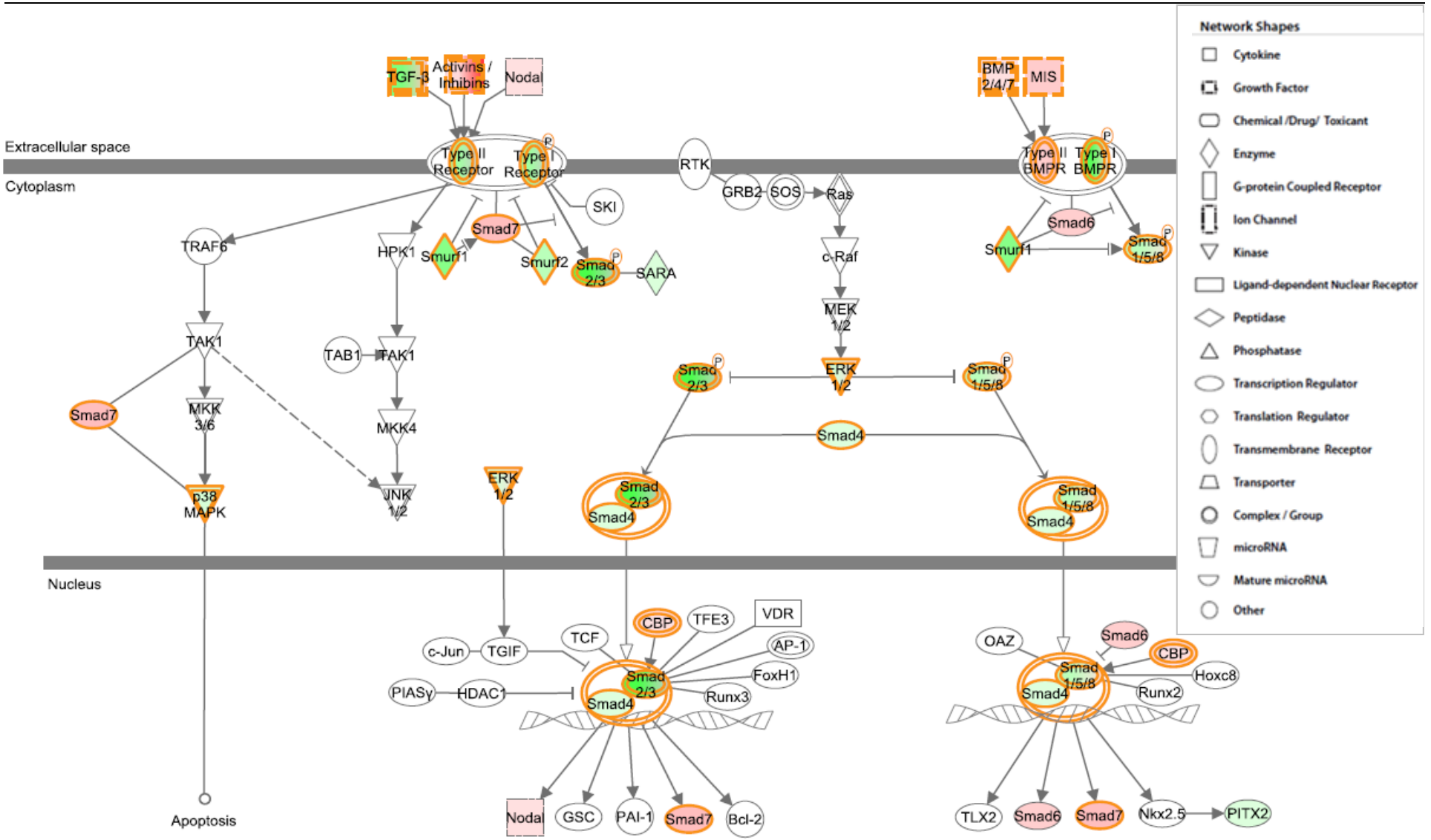

Figure 26. TGF-β signalling pathway in LpA-stimulated PBMCs of AA compared to HC.

Ingenuity pathway of the KEGG gene selection for TGF-β signalling; n(AA)=14, n(HC)=14. Color shading corresponds to the type of regulation: red for up-regulated genes, green for down-regulated genes. The shape of the node indicates the major function of the protein (see network shapes); a line denotes binding of the products of the two genes while a line with an arrow denotes 'acts on'.

Table 14: Characteristic genes of different immune cells

Cell type											
nTh		Tfh		NK		Th1		Th2		Th9	
Gene	Aff. ID	Gene	Aff. ID	Gene	Aff. ID	Gene	Aff. ID	Gene	Aff. ID	Gene	Aff. ID
CD62L	7931914	CD200	8081657	KLRB1	7961059	STAT4	8057771	GATA3	7964360	PU.1	7947861
CXCR4	7922219	TNFSF8	8163629	NKG2	7961187	TBX21	8008029	STAT6	7926105	IL9	8114256
CCR7	8055465	CD40LG	8170187	IL4	8107977	Hlx	7909890	CCR3	8079383	IRF4	8116559
IL2	8015031	CD44	7939341	IFNG	7964787	IRF1	8114010	CCR4	8078442	STAT6	7926105
CD45RA	8102697	IL6R	7905789	NCR1	8031387	CXCR6	8079377	CCR8	8078888	GATA3	7964360
		BTLA	8089519	NCR2	8119435	CD195	8093298	PTGDR2	7974363		
		PDCD1	8060294	NCR3	8124955 8178517 8179773	IL12RB1	8035380	IL4	8107977		
		ICOS	8047702	NLRK1	7961151	IL12RB2	7902205	IL5	8114023		
		IL21R	7994292	KLRC1	7961187	IL18R1	8044035	IL6	8131803		
		CD57	7952884	IL22	7964803	IFNG	7964787	IL13	8107970		
		CXCR5	7944335	IFNG	7964787	LTA	8177976 8118137 8179258	IL9	8114256		
		OX40	7911413	IL10	7923907	PRF1	7934161				
				IL21R	7994292	GNLY	8043236				
				IL1R1	8043995						
Cell type											
Th17		Th22		Treg		B		DC		MP	
Gene	Aff. ID	Gene	Aff. ID	Gene	Gene	Gene	Aff. ID	Gene	Aff. ID	Gene	Aff. ID
RORC	7920082	AHR	8131614	FOXP3	FOXP3	CD19	7994487	HLA-DRA	8179481	CD14	8114612
IL-23R	7902189	IL22	7964803	CD25	CD25	CD20	7940287	CD40	8063156	CD40	8063156
CCR6	8123364	IL26	7964795	CTLA-4	CTLA-4	CD22	8027837	CD86	8082035	CD11b	7995096
CCR4	8078442	IL13	8107970	GITR	GITR	CD40	8063156	CD83	8116983	EMR1	8025103
KLRB1	7961059	CCR4	8078442	GARP	GARP	OCT2	8037123	CD11c	7995128	CD68	8004510
IL23R	7902189	CCR6	8123364	CCR7	CCR7	Pax5	8161211	LAMP3	8092348	CSF1R	8115076
IL17A	8120210	CCR10	8015681	CCR4	CCR4	CD79A	8029136	IFNA	8154627	IL4R	7994280
IL17F	8127024	STAT3	8015607	TNFRSF9	TNFRSF9	BLK	8144625	MH±I	8115147		
IL21	8102707			TNFRSF4	TNFRSF4	POU2AF1	7951596	ICAM-1	8025601		
IL22	7964803			PDCD1	PDCD1			CD123	8165752 8176323		
IL26	7964795			RUNX1	RUNX1			CD64	7905047		
CCL20	8048864			IKZF2	IKZF2			CD32	7906757		
IL12RB2	7902205			ENTPD1	ENTPD1						
				GPR83	GPR83						

Aff. ID, Affymetrix gene ID; nTh, naïve T helper cell; Tfh, follicular T helper cell; NK, natural killer cell; Th1, T helper cell type 1; Th2, T helper cell type 2; Th9, T helper cell type 9; Th17, T helper cell type 17; Th22, T helper cell type 22; Treg, regulatory T cell; B, B cell; DC, dendritic cell; MP, macrophage (Raedler and Da Costa *et al.*, manuscript in preparation).

Table 15: Regulation of KEGG pathways and characteristic genes of immune cells in PBMCs

Analysis / KEGG	Nr. of genes	PBMCs								
		AA vs HC (14 vs 14)			NA vs HC (8 vs 14)			NA vs AA (8 vs 14)		
		U	CD3/28	LpA	U	CD3/28	LpA	U	CD3/28	LpA
MAPK	282	0.085	0.471	**0.011**	0.111	0.226	0.055	0.102	0.096	**0.028**
TGF-β	91	0.108	0.312	**0.000**	**0.017**	0.214	0.115	0.225	0.395	0.051
Apoptosis	88	**0.035**	0.169	**0.015**	0.142	0.212	0.129	0.097	0.195	0.239
NK cyto	142	**0.006**	0.445	0.123	0.090	0.276	0.227	0.071	0.380	0.131
Cyto-cyto	270	0.282	0.653	**0.049**	0.394	0.398	0.072	0.404	0.164	0.153
p53	69	0.175	0.438	0.385	0.059	0.245	0.723	0.490	0.092	0.619
TLR	102	0.149	0.286	**0.024**	0.181	0.251	**0.015**	0.057	**0.043**	**0.040**
Comp	72	0.393	0.583	0.141	0.383	0.200	0.088	0.135	0.093	0.102
TCR	111	**0.015**	0.196	**0.042**	0.079	0.156	0.065	0.141	0.673	0.061
NOD	59	0.057	0.473	**0.033**	0.055	0.271	**0.018**	0.088	0.245	0.193
Chemo	199	0.214	0.821	0.067	0.542	0.565	0.063	0.444	0.339	**0.050**
Thyroid	68	0.325	0.247	0.407	0.526	0.622	0.906	0.457	0.930	0.724
JAK	159	**0.037**	0.284	**0.023**	0.122	0.102	0.095	0.185	0.103	0.193
Calcium	185	**0.017**	0.270	**0.017**	0.128	0.150	**0.017**	**0.037**	0.283	**0.005**
mTOR	58	**0.015**	**0.006**	**0.034**	0.405	0.148	0.089	0.202	**0.003**	0.188
Cell type		**U**	**CD3/28**	**LpA**	**U**	**CD3/28**	**LpA**	**U**	**CD3/28**	**LpA**
nTh	5	0.079	0.586	0.092	0.689	0.738	0.303	0.267	0.223	0.844
Tfh	12	0.430	0.549	0.290	0.576	0.293	0.162	0.733	0.232	0.176
NK	14	0.527	0.366	0.337	0.149	0.563	0.187	0.183	0.619	0.487
Th1	13	0.153	0.429	0.282	0.098	0.538	0.491	0.535	0.916	0.422
Th2	11	0.244	0.492	**0.047**	0.310	0.162	**0.026**	0.339	0.108	0.728
Th9	5	0.643	0.241	0.637	0.093	0.702	0.170	0.135	0.400	0.271
Th17	13	0.156	0.634	0.425	0.088	0.163	0.515	0.148	0.069	0.547
Th22	8	0.198	0.304	0.278	0.066	0.410	0.353	0.082	0.051	0.094
Treg	14	**0.034**	0.273	0.099	0.428	0.319	0.507	**0.036**	0.857	0.798
B	9	0.152	**0.020**	0.250	0.086	0.157	0.103	0.271	0.109	0.343
DC	11	0.337	0.768	0.327	0.113	0.163	0.138	0.223	0.117	0.082
MP	7	0.291	0.826	0.12	**0.006**	0.237	**0.027**	**0.050**	0.234	**0.006**

KEGG, annotated pathways from the Kyoto Encyclopedia of Genes and Genomes. Cell type defined by characteristic genes for the respective cell subset. MAPK, mitogen-activated protein kinase signalling pathway; TGF-β, transforming growth factor beta signalling pathway; NK cyto, natural killer cell mediated cytotoxicity; Cyto-cyto, cytokine-cytokine receptor interaction; TLR, toll-like receptor signalling pathway; Comp, complement and coagulation cascades; TCR, T cell receptor signalling pathway; NOD, NOD-like receptor signalling pathway; Chemo, chemokine signalling pathway; Thyroid, autoimmune thyroid disease; JAK, Jak-STAT signalling pathway; Calcium, calcium signalling pathway; mTOR, mTOR signalling pathway. $p \leq 0.05$ considered as significant; significant p-values presented in boldface. U, unstimulated. nTh, naïve T helper cell; Tfh, follicular T helper cell; NK, natural killer cell; Th1, T helper cell type 1; Th2, T helper cell type 2; Th9, T helper cell type 9; Th17, T helper cell type 17; Th22, T helper cell type 22; Treg, regulatory T cell; B, B cell; DC, dendritic cell; MP, macrophage (Raedler and Da Costa *et al.*, manuscript in preparation).

We furthermore analysed a subpopulation of PBMCs, the $CD4^+$ T lymphocytes, to study whethere genes and pathways within isolated $CD4^+$ cells are more specific or sensitive compared to PBMCs. Due to limitations in cell numbers, this analysis was performed in a subgroup of 5 AA and 3 HC. In order to compare results for the isolated $CD4^+$ cells with PBMCs, we did a further analysis of PBMC data from the same subgroup of children (Table 16, 5AA vs. 3HC).

Table 16: Regulation of KEGG pathways and characteristic genes of immune cells in PBMCs and $CD4^+$ cells

Analysis KEGG	Nr. of genes	PBMC s		CD4+
		AA vs. HC_unstimulated		
		(14 vs 14)	(5 vs 3)	(5 vs 3)
MAPK	282	0.085	**0.0179**	0.179
TGF-β	91	0.108	0.286	0.732
Apoptosis	88	**0.035**	**0.018**	0.714
NK cyto	142	**0.006**	**0.019**	0.732
Cyto-cyto	270	0.282	0.732	0.732
p53	69	0.175	0.732	0.732
TLR	102	0.149	0.196	0.714
Comp	72	0.393	0.732	1.000
TCR	111	**0.015**	**0.000**	0.179
NOD	59	0.057	0.714	0.714
Chemo	199	0.214	0.732	0.732
Thyroid	68	0.325	0.554	0.286
JAK	159	**0.037**	**0.000**	0.196
Calcium	185	**0.017**	**0.018**	0.732
mTOR	58	**0.015**	**0.018**	0.196
Cell type				
nTh	5	0.079	0.467	**0.018**
Tfh	12	0.430	0.300	0.179
NK	14	0.527	1.000	0.982
Th1	13	0.153	0.400	0.714
Th2	11	0.244	0.567	0.196
Th9	5	0.643	0.333	0.982
Th17	13	0.156	0.400	0.714
Th22	8	0.198	0.167	0.196
Treg	14	**0.034**	0.300	0.196
B	9	0.152	**0.000**	1.000
CD	11	0.337	0.267	0.732
MP	7	0.291	0.333	0.714

KEGG, annotated pathways from the Kyoto Encyclopedia of Genes and Genomes; cell type defined by characteristic genes for the respective cell subset; $p \leq 0.05$ considered as significant; significant p-values presented in boldface. MAPK, mitogen-activated protein kinase signalling pathway; TGF-β, transforming growth factor beta signalling pathway; NK cyto, natural killer cell mediated cytotoxicity; Cyto-cyto, cytokine-cytokine receptor interaction; TLR, toll-like receptor signalling pathway; Comp, complement and coagulation cascades; TCR, T cell receptor signalling pathway; NOD, NOD-like receptor signalling pathway; Chemo, chemokine signalling pathway; Thyroid, autoimmune thyroid disease; JAK, Jak-STAT signalling pathway; Calcium, calcium signalling pathway; mTOR, mTOR signalling pathway. nTh, naïve T helper cell; Tfh, follicular T helper cell; NK, natural killer cell; Th1, T helper cell type 1; Th2, T helper cell type 2; Th9, T helper cell type 9; Th17, T helper cell type 17; Th22, T helper cell type 22; Treg, regulatory T cell; B, B cell; DC, dendritic cell; MP, macrophage (Raedler and Da Costa *et al.*, manuscript in preparation).

The results for the comparison of PBMCs from 14 AA with 14 HC are similar to the comparison of PBMCs from 5 AA to 3 HC. In the subgroup analysis, regulation of MAPK additionally reached significance (Table 16, MAPK, p=0.02). In the $CD4^+$ cells, we could not observe significant differences in the regulation of KEGG pathways and characteristic genes of immune cells except for nTh (Table 16, nTh, p=0.02). Differences in the expression of Treg genes were no further significant in the PBMC subgroup, while B cell genes were significantly different expressed in the subgroup of 5 AA vs. 3 HC.

In summary, we could identify TLR, calcium and mTOR signalling by GlobalAncova analysis to be significantly different regulated within PBMCs of the three phenotypes.

4. DISCUSSION

The prevalence of atopic diseases in childhood increased dramatically over the past decades. In the results presented in this thesis, we investigated two different phases of immune maturation in the development of atopic disease: First, the early influence of genetic variation on immune regulation was studied in cord blood and children were followed until age 3 years to assess the development of atopic diseases. The aim was to study if the genotype of the fetus allows any prediction for immune maturation regarding atopy development later in life. Second, several dimensions of immune regulation were studied in children later in life, at the time of clinical manifestation of an allergic or non-allergic asthma phenotype, to identify currently unknown underlying immunological mechanisms.

Immune regulation in early childhood

Exogenous influences such as gene-environment interactions and their impact on immune regulation become more and more important for the development of atopic diseases in childhood. Genetic predisposition is considered as one key risk factor for development of atopic diseases (Moffatt *et al.* 2007), and several published reports have shown an association between the presence of certain genetic variants of Toll-like receptors or specific Interleukin-10 genotypes and development of atopic diseases in both children and adults. In the experiments presented herein, we studied the impact of certain *TLR* and *IL-10* SNPs on various aspects of the immune response in a German birth cohort at the University Children's Hospital in Munich, Germany. Importantly, as opposed to numerous other published studies employing patient populations of childhood and adult age, we obtained and analysed cord blood, therefore allowing us to perform our studies at the earliest possible time point of immune maturation. Furthermore, we were able to follow these children to precisely assess atopic phenotypes and wheeze until the age of 3years.

4. DISCUSSION

Among the *TLR* polymorphisms that were studied, genetic variants of *TLR2* were shown to influence Treg marker gene expression, with expression levels being modulated by maternal atopy. In carriers of *IL-10* SNPs within the same study population, we observed a distinct pattern of T cell lineage fate, with decreased expression of Treg markers, decreased levels of TNF-α, GM-CSF and IL-5 and a concomitant increase in IFN-γ levels. Genetic variants of *TLRs* and *IL-10* were shown to act as determinants for the development of immune-mediated diseases including atopic dermatitis and wheeze in early childhood at 3 years of age.

In the second part of this work, we addressed immune regulation later in childhood, studying the underlying immunoregulatory and pathophysiological mechanisms of the two main phenotypes in childhood asthma, allergic and non-allergic asthma, at the onset of disease. While both allergic and non-allergic asthmatic children shared a Th2 dominated phenotype, they clearly differed in specific immunological aspects. Our experiments showed an increase in total numbers of Tregs in children with asthma of allergic origin compared to non-allergic asthmatics and healthy controls. This increase might be explained as a physiological response in order to balance exuberant immune responses and to compensate for the decreased suppressive capacity of Tregs in patients with allergic asthma. This might enable the child to more efficiently control the ongoing inflammatory response. Although, the decrease in suppressive capacity of Tregs from AA compared to HC did not reach statistical significance, mild differences in suppressive capacity together with changes in cytokine secretion may have a biological impact.

In contrast, patients with asthma of non-allergic origin were found to have increased levels of the pro-inflammatory cytokine IL-1β and showed a shift towards a Th17 phenotype with elevated numbers of neutrophils in peripheral blood.

Employing a genome-wide microarray expression analysis, we were able to characterize childhood allergic and non-allergic asthma based on a newly described specific gene

expression profile, including *CD93*, *TREM1* and *S100A9* which were significantly increased in non-allergic asthmatic compared to allergic asthmatic children and also healthy controls.

The prevalence of atopic diseases in childhood increased dramatically over the past decades, especially in countries with a westernized lifestyle and high hygiene standards (Matricardi *et al.* 2000). The so-called "hygiene hypothesis" proposes that a lack of early childhood exposure to various antigens (infectious agents, microorganisms, parasites among others) increases susceptibility to atopic disease by suppressing natural development of the immune system. This process is in part mediated by TLRs, a family of conserved innate immune receptors that recognize pathogen-associated molecular patterns of microbes (Raedler and Schaub, 2013). Published work suggests that genetic polymorphisms and associated gene-environment interactions play a prominent role in disease development (Le Souef P.N., 2009; Niebuhr *et al.* 2008; Pinto *et al.* 2008; Vercelli D., 2008).

In the experiments shown herein, the *TLR2* SNPs rs4696480 and rs1898830 influenced Treg marker gene expression, a finding supported by recent reports suggesting a direct or indirect modulation of Tregs by *TLRs* (Dai *et al.* 2009; Liu *et al.* 2006; Nyirenda *et al.* 2009).

Of note, the two above mentioned *TLR2* SNPs showed a diametrical effect on Treg marker expression, which was dependent on maternal atopy. This might point to an immunomodulatory effect caused by the presence or absence of maternal atopy, which could already have an impact on neonatal immune maturation. However, the underlying mechanisms are incompletely understood. Direct intrauterine immunological pathways, epigenetic regulation or currently unknown genetic and immune-mediated modulation have been suggested (Liu and Raedler *et al.* 2011). Since we did not detect an accumulation of the *TLR2* risk alleles in atopic mothers, an influence of maternal genotypes on Treg modulation of the fetus is unlikely.

In children with *TLR2* SNP rs4696480 (AA), Tregs at birth were found to be decreased in the absence of maternal atopy. In contrast, children with the same SNP, but whose mothers were known to have atopic disease, had elevated numbers of Tregs and showed a shift towards a Th2 phenotype with predominance of the Th2 cytokines IL-5 and IL-13 and the pro-inflammatory cytokine TNF-α. Physiological up-regulation of Treg numbers during early childhood might potentially counterbalance a present Th1/Th2 disparity in this subgroup, and has been implicated with manifestation of atopic disease in childhood (Van der Velden *et al.* 2001). In another study published by Eder *et al.*, carriers of the WT allele (T) of *TLR2* rs4696480 among a population of farmers´ children were shown to have a lower risk to develop atopic diseases (Eder *et al.* 2004). In a study published by Kormann *et al.* (Kormann *et al.* 2008), carriers of *TLR2* SNP rs1898830 showed a trend towards developing non-atopic asthma, although this correlation was not found to be significant. In our study, carriers of the *TLR2* SNP rs1898830 (GG) who had atopic mothers, were found to have lower expression levels of Treg markers, indirectly indicating that those patients are likely to have lower total Treg numbers. Of note, Treg markers such as FOXP3 had to be used as an indirect mean to estimate total number of Tregs, since counting total cells by flow cytometry was not feasible due to a limited amount of cells available from each patient for this assessment. A decrease of *FOXP3* expression could indicate lower numbers of total Tregs and as a consequence potentially less immunosuppression. Consistent with this hypothesis is a report by Lazarus *et al.*, showing an association between decreased *FOXP3* levels in children with the *TLR10* SNP rs4129009 and clinically diagnosed asthma (Lazarus *et al.* 2004). In our study, following non-specific innate stimulation with LpA and Ppg, we also observed decreased *FOXP3* expression in homozygous carriers of both, the *TLR10* SNP rs4129009 and the *TLR1* SNP rs4833095, yet we did not observe an association with risk for atopic disease or wheeze at the age of 3 years. The ligand spectrum of TLR10 might actually be more complex due to a possible heterodimerization of

TLR1 with TLR2, and the fact that TLR10 is highly homologous to TLR1 and TLR6, which are both able to form heterodimers with TLR2 (Liu and Raedler *et al.* 2011; Takeuchi *et al.* 2002).

If both *TLR2* SNPs (rs4696480 and rs1898830) were stratified for maternal atopy, clinical outcomes assessed at the age of 3 years might be explained by cord blood immune responses. Consistent with this finding, increased expression of Treg markers at birth generally correlated with protection against wheeze (rs1898830) and atopy (rs1898830, rs4696480), whereas decreased expression of Treg markers was associated with increased risk for developing food allergy (rs4696480).

Together the data indicate that a certain *TLR* genotype in combination with presence or absence of maternal atopy allow a further prediction for immune maturation regarding development or protection against atopic diseases in early childhood.

It has to be acknowledged that these findings have to be verified in the next follow-up of this study population at the age of 6 years, where additional atopic phenotypes such as asthma can be objectively assessed, and as well in additional cohort studies employing cord blood T cells, as only this can predict later effects during immune maturation (Liu and Raedler *et al.* 2011).

In addition, we studied the impact of genetic variation in the *IL-10* gene on Th1/Th2 lineage and pro-inflammatory cytokines as well as Treg marker genes in cord blood. IL-10 is known to be a key mediator of adaptive immunity. Due to its pleiotrophic effects and its role in regulation of both cellular and humoral immunity, it became a candidate risk gene in the regulation of various inflammatory and autoimmune diseases (Lyon *et al.* 2004). Several *IL-10* SNPs were previously associated with inflammatory disorders, such as asthma (Bossé *et al.* 2009; Hunninghake *et al.* 2008; Chatterjee *et al.* 2005), lupus erythematosus (Gibson *et al.*

2001), psoriasis (Asadullah *et al.* 2001), rheumatoid arthritis (Paradowska-Gorycka *et al.* 2010) and certain infectious diseases (Shin *et al.* 2000). All of these conditions are likely to be at least in part T cell-mediated and are considered to have their clinical onset during early immune maturation. We observed a distinct pattern of T cell linage fate in cord blood of *IL*-10 SNP carriers, with decreased gene expression of Treg markers, decreased levels of TNF-α, GM-CSF and IL-5, and a concomitant increase in IFN-γ levels. The observed decrease in Treg marker gene expression could indicate lower numbers of Tregs, what has been described for children with wheeze at the age of 8-20 months (Borrego *et al.* 2009).

Despite the fact that *IL-10* was originally discovered as "cytokine synthesis inhibitory factor" capable of suppressing the Th1 response in a study by Fiorentino and colleagues, within our study, IFN-γ secretion was increased in homozygous carriers of *IL-10* SNPs (Fiorentino *et al.* 1991). Of note, the results have to be evaluated carefully, considering the fact that in this study, cytokine levels were assessed in bulk culture supernatants. As a consequence the origin of IFN-γ cannot be attributed exclusively to Th1 cells. The finding that IL-5, TNF-α and GM-CSF were decreased in homozygous carriers of *IL-10* SNPs rather indicates an atopy-protective state of immune development (Raedler *et al.* 2012). Despite this observed shift from a Th2 towards a Th1 phenotype and reduced pro-inflammatory cytokines, several *IL-10* SNPs were found to be risk factors for AD, wheeze or both in the follow-up at 3 years of age.

In the past, contradictory results regarding *IL-10* SNPs and their association with atopy or asthma have been reported. This can probably be attributed to differences in study design, different assessment of the clinical phenotype or failure to adequately model gene-environment interactions among others (Hunninghake *et al.* 2008; Raedler *et al.* 1012). The minor allele of rs3024498 proofed to be a risk factor for AD and wheeze in our study population as well as a risk factor for childhood asthma in the study population of the Canadian Asthma Primary Prevention Study (CAPPS; Bossé *et al.* 2009*)*. In contrast, presence of the same allele was not

associated with increased risk for developing asthma in the Canadian Saguenay-Lac-Saint-Jean (SLSJ) study population or in a German asthma family study (Bossé *et al.* 2009). In children representing the Study of Asthma Genes and the Environment cohort, presence of the minor allele of rs3024498 even seemed to protect from development of atopic disease (Bossé *et al.* 2009). Regarding the *IL-10* SNP rs1800871 we did not observe an association with a disease outcome, whereas the same SNP was associated with adult asthma in the Canadian SLSJ study population (Bossé *et al.* 2009). Homozygous carriers of the *IL-10* SNP rs3024496 showed a higher prevalence of AD, wheeze or both within our study population, a finding that is consistent with a study from Hunninghake and colleagues, showing augmented levels of IgE and increased risk for development of asthma at high levels of dust mite exposure in the Childhood Asthma Management Program study population (Hunninghake *et al.* 2008).

Interestingly, within our study, *IL-10* SNPs affected both AD and wheeze, two potentially distinct diseases.

While six single *IL-10* SNPs were associated with clinical outcomes at 3 years of age, we did not detect a high-risk haplotype, containing all six minor alleles. This might be explained by the fact that the number of heterozygous SNP carriers was higher, the majority of which showed a lower prevalence of AD and wheeze compared to homozygous SNP carriers.

Using diplotype analysis, the *IL-10* SNP rs3024498 and/or the deletion rs79309463 could be shown to be involved in the development of wheeze, whereas the distal promoter SNPs rs10494879 and/or rs1800890 might play a role in the development of AD in childhood. The effect of these two SNP/SNP or SNP/deletion combinations could not be disentangled, as these two homozygous SNP/deletion genotype combinations alone did not occur within this study population (Raedler *et al.* 2012).

Hee and colleagues proposed *IL-10* to be controlled at transcriptional level. The authors suggested *IL-10* SNPs to affect the binding of regulatory sequences to the *IL-10* promoter, therefore resulting in changes in IL-10 production (Hee *et al.* 2007). In our study population we did not observe an influence of the *IL-10* genotype on IL-10 mRNA or protein levels. Supporting our findings, Hayden *et al.* did not observe an association between *IL-10* promoter SNPs and IL-10 levels in Derp1-stimulated PBMCs of children (Hayden *et al.* 2011). Also consistent with our findings, they did however describe an association of the *IL-10* SNPs with Th2 cytokine levels, indicating a crucial role for IL-10 in Th2 cytokine regulation (Hayden *et al.* 2011; Raedler *et al.* 2012). Since the effects of *IL-10* SNPs were not mediated by changes in IL-10 levels, a modulation in binding of the transcription factor AP-1 (activator protein 1), which influences *IL-10* promoter activation as well as Treg regulation would be possible (Kube *et al.* 1995; Wu *et al.* 2006; Raedler *et al.* 2012). However, the exact mechanism still has to be determined.

In summary, polymorphisms in *IL-10* seem to favour an atopy-prone immune development and to affect early immune maturation prior to onset of clinical disease, whereas Tregs might play a more fundamental role at this stage of immune development compared to Th1/Th2 cytokine lineages (Raedler *et al.* 2012).

Our results show that both *TLR* and *IL-10* polymorphisms influence immune regulation in cord blood and act as determinants for development of atopic diseases or wheeze later in life.

Based on these findings, in ongoing studies we are currently analyzing the role of the polymorphisms in *TLRs*, *IL-10* and additional candidate genes (identified e.g. within GWAS in asthma) in the development of allergic and non-allergic asthma later in childhood (Moffat *et al.* 2007; Moffat *et al.* 2010).

Immune regulation at school-age

In a second set of experiments, we investigated the mechanisms of immune regulation in asthmatic children at the onset of disease. From a clinical perspective, there are two main phenotypes of childhood asthma: allergic and non-allergic asthma. Despite intensive research over the past decades, the underlying pathophysiological mechanisms remain incompletely understood. Importantly, current treatment protocols for asthmatic children follow a "one-size-fits-all approach" including the administration of a short-term β-agonist and an inhalative corticosteroid, and therefore do not take potential phenotypic differences into account.

Employing very strict inclusion criteria, we were able to recruit a unique study population including AA, NA and healthy children. Since asthmatic children were recruited at the first visit to the asthma and allergy outpatient clinic of the children´s hospital in Munich, we were able to include the children at disease onset or early after disease development, a timepoint at which they were still in a steroid-naïve state. After retrieving a detailed patient history, patients underwent physical examination and blood testing for specific immunological markers. In order to reliably distinguish between the allergic and non-allergic phenotype of asthmatic disease, data on specific and total immunoglobulins, fractional exhaled nitric oxide (FeNO) as a measure of airway inflammation and radioimmunoassays for detection of specific IgE antibodies were all taken into account. With the allergic phenotype being more common than the non-allergic type and representing about 90-95% of the total number of asthmatic cases in children (Craig *et al.* 2010), this unequal distribution of both phenotypes was also reflected in our patient population, with the frequency of AA being five times higher than that of NA.

Our results imply that AA and NA can be characterized by distict immunological profiles (Raedler *et al.,* manuscript in preparation). Patients with AA had higher levels of Tregs

compared to patients with NA and HC, as comprehensively assessed by flow cytometric measurement, gene expression analysis by qRT-PCR and microarrays and epigenetic analysis of *FOXP3* TSDR. In order to quantify Tregs by flow cytometry, we measured the transcription factor FOXP3, which currently represents the most specific marker for Tregs, and added CD49d as an additional surface marker. CD49d has previously been reported to be absent on immunosuppressive Tregs by Kleinewietfeld and colleagues (Kleinewietfeld *et al.* 2010). Since the total number of $CD4^+CD25^+CD49d^-$ cells was shown to correlate well with the total number of $CD4^+CD25^+FOXP3^+$ cells, the use of anti-CD49d (which binds to an integrin alpha subunit) could facilitate the isolation of "untouched" suppressive regulatory T cells by negative selection as suggested by Kleinewietfeld *et al*. In accordance to our flow cytometry data, microarray experiments revealed increased Treg gene expression in AA compared to NA and HC.

To distinguish between natural, thymus-derived and induced Tregs, which are induced in the periphery, we analysed the methylation status of the Treg-specific demethylated region (TSDR). Demethylation in this region was suggested to be characteristic for nTregs in adults, whereas this region is methylated in effector cells and induced Tregs (Baron *et al.* 2007). As the demethylation status of TSDR did not differ between unstimulated and anti-CD3/CD28-stimulated cells, we could demonstrate that TSDR demethylation is a specific marker to quantify nTregs, but not stimulated iTregs, in PBMCs of children. Treg numbers assessed by TSDR demethylation analyses were trendwise increased in AA compared to HC, in accordance to our data from flow cytometric measurements, microarray and qRT-gene expression analyses.

To study the role of epigenetic regulation on the expression of *FOXP3*, we assessed the acetylation status of the histone proteins H3 and H4. Acetylation of histones alters the chromatin condensation and thereby increases the accessibility of transcription factors that

thereafter regulate gene expression (Struhl K., 1998). Zhang *et al.* suggested that *FOXP3* expression and protein activity are controlled by acetylation of *FOXP3* (Zhang *et al.* 2012). While acetylation did not differ at H4 within our study population, acetylation was trendwise higher at histone H3 and might be associated with increased *FOXP3* mRNA and protein levels in AA. Consistent with our data, Cavassani *et al.* demonstrated an association of acetylation at histone H3, but not H4, with *FOXP3* expression (Cavassani *et al.* 2010).

While the total number of Tregs was increased in children with allergic asthma, their relative suppressive capacity, especially for Th2/Th1 cytokine secretion, was found to be decreased.

In previous cord blood studies from our group, we could show a decrease in Treg numbers in children at risk for asthma (Schaub *et al.* 2008), while Treg numbers were increased in children protected from asthma (Schaub *et al.* 2009). Interestingly, the effects were different in cord blood and in peripheral blood of children at school age. While children at risk for asthma had decreased Treg numbers in cord blood, Treg numbers were increased in peripheral blood of asthmatic children within our study. In a 2012 review by Langier *et al.,* Tregs were reported to be altered in number and function in allergic asthmatics; however the results in the literature vary between children and adults and blood cells and airway tissue (Langier *et al.* 2012).

Lower numbers of $CD4^+CD25^+$ cells were found in blood and BAL of asthmatic children compared to HC (Lee *et al.* 2007; Hartl *et al.* 2007). Of note, measurement of $CD4^+CD25^+$ cells (without analysis of intracellular FOXP3 levels) does not allow to reliably characterize regulatory T cells. In PBMCs of allergic children with persistent moderate to severe asthma, $CD4^+CD25^+FOXP3^+$ cells and *IL-10* mRNA levels were increased compared to mild asthmatics, and Treg numbers showed a positive correlation with total IgE levels (Lee *et al.* 2007). Thunberg *et al.* observed an increase in Treg numbers in mild asthmatics following allergen inhalation, suggesting an association of number of Tregs with the clinical degree of

airway inflammation in asthma (Thunberg *et al.* 2010). Consistent with reports from Thunberg *et al*., our data suggest a counter-regulatory process in children with AA, during which total numbers of Tregs increase in order to compensate for their reduced relative functional capacity. Consistent with our suppression data, yet not significant, Yamamoto *et al.* also described an impaired suppressive capability of Tregs in asthmatic children (Yamamoto *et al.* 2011).

Regarding the underlying pathophysiological mechanism of suppression excerted by Treg cells, Yamaguchi *et al.* suggested two main categories of suppression, depending on the degree of inflammation present. Under physiological, non-inflammatory conditions, the authors suggest a Treg-dependent deprivation of activation signals, including the inhibition of CD28 co-stimulatory signals, CTLA-4 dependent down-regulation of CD80/CD86, IL-2 absorption and CD39/CD73-dependent ATP-degradation, in order to keep the responder cells in a naïve state. In contrast, in the presence of severe inflammation, conventional T cells are suppressed via an inactivating mechanism, including secretion of IL-10, TGF-β, IL-35, granzyme, perforin and cAMP (Yamaguchi *et al.* 2011). First results of our study concerning the molecular mechanism of Treg suppression indicate reduced suppression of Th2 responses in AA compared to HC. In our ongoing studies, we are currently studying the molecules that were previously suggested by others to contribute to the suppressive mechanism on mRNA level in isolated Tregs.

In summary, Treg numbers assessed by flow cytometry, mRNA levels of Treg marker genes determined via microarray and qRT-PCR and epigenetic analysis of *FOXP3* acetylation and methylation at the Treg-specific demethylated region, indicate increased numbers of Tregs in AA compared to HC and NA, while suppressive capacity was decreased in AA compared to HC.

In contrast to patients with AA, NA were characterized by significantly increased levels of the pro-inflammatory cytokine IL-1β, a finding that was observed both at gene and protein level. A possible role of IL-1β in asthma pathology has already been implicated in a report from 1994, in which intratracheal administration of IL-1β in rats was associated with AHR and increased neutrophil counts in BAL (Tsukagoshi *et al.* 1994). In a different study, *IL-1β* levels were found to be increased in bronchial epithelium from asthmatic patients compared to healthy controls. Epithelial *IL-1β* expression was positively correlated with the number of subepithelial macrophages, indicating a possible association between *IL-1β* expression and the degree of ongoing inflammation in bronchial asthma (Sousa *et al.* 1996).

Consistent with previous reports by Tsukagoshi and colleagues employing a mouse model, we measured elevated IL-1ß as well as increased neutrophil counts in whole blood of NA within our study population. In a study from 2000, NA had already been shown to have increased numbers of neutrophils compared to AA and HC; however these investigators analysed tissue obtained from bronchial biopsies (Amin *et al.* 2000), as opposed to peripheral blood in our study.

In addition to elevated levels of the pro-inflammatory cytokine IL-1β, we detected an up-regulation of *IL-37* gene expression in NA. IL-37, which is a member of the IL-1 cytokine family, has anti-inflammatory properties and has been shown to suppress innate immune responses (Nold *et al.* 2010). Furthermore a role in suppression of adaptive immunity has been proposed previously (Kim and Kroneberg, 2011). IL-37 was shown to directly inhibit expression, production and function of pro-inflammatory cytokines and the chemokine CXCL2, a neutrophil chemoattractant (Tete *et al.* 2012; Kim and Kroneberg, 2011). Considering our current results, we suggest that *IL-37* gene expression is physiologically up-regulated in patients with NA, in order to counteract the IL-1β-driven, predominantly neutrophilic inflammation. Consistent with this hypothesis is the finding by others, that *IL-37*

undergoes extensive posttranscriptional regulation. IL-37 might be part of a feedback mechanism during termination of the inflammatory response, since stability of *IL-37* mRNA and expression levels of *IL-37* have been shown to depend on the presence of inflammatory stimuli (Kim and Kroneberg, 2011).

Studying cytokine profiles, we observe a Th17-shifted profile in NA compared to AA and HC. Th17 cells are involved in activation and proliferation of neutrophils and were therefore proposed to play a prominent role in the pathogenesis of asthma; non-allergic asthma in specific, since it was associated with a neutrophil-driven airway inflammation (Louten *et al.* 2009; Molet *et al.* 2001; Laan *et al.* 1999). In addition to the measurement of single Th1, Th2, Th17 and pro-inflammatory cytokines, we also assessed the ratios between the different cytokines, which has previously been suggested by Wong *et al*. in order to better reflect cytokine homeostasis (Wong *et al.* 2001; Colavita *et al.* 2000). In our experiments NA had higher ratios of Th17/Th1, Th17/Th2 and Th17/pro-inflammatory cytokine producing cells than AA or HC. We believe that using cytokine ratios, as opposed to comparison of single cytokines, provides a better reflection of cytokine balance present in the individual patient in a more physiological context. Studying cytokine ratios in our experiments revealed a clear Th17 predominance in NA compared to AA and HC.

Employing microarrays followed by validation of the results by qRT-PCR, we could identify three genes (*CD93*, *S100A9* and *TREM1)*, which were significantly higher expressed in NA compared to HC and AA. *CD93* has a role in several cellular regulation processes, including tissue repair and might therefore be important during tissue remodelling in patients with asthma. The pro-inflammatory molecule S100A9 has already been shown to be up-regulated during the early airway response in several animal models of atopic diseases. In addition to its function as a mediator of inflammation in autoimmunity, S100A9 also seems to be involved in the pathogenesis of other diseases in which inflammation has a prominent pathophysiological

role, including Crohn´s disease or rheumatoid arthritis (Yin *et al.* 2008; Simard *et al.* 2010). Both CD93 and S100A9 are calcium binding molecules and might therefore be involved in the regulation of calcium signalling. Of note, in our study population regulation of calcium signalling differed significantly among the three groups (AA, NA and HC). A dysregulation of calcium responses has been associated with pathophysiological processes of autoimmune and inflammatory diseases (Feske *et al.* 2007), yet a possible role for calcium signalling, including the underlying molecular mechanisms, in the development of childhood asthma remains controversial, and is subject of ongoing studies within our study population.

TREM1 has previously been shown to be a mediator of the neutrophil-driven inflammatory response (Schenk *et al.* 2007). This finding is consistent with the Th17-shifted neutrophilic inflammation that we observed in NA. TREM-1 has previously been shown to play a crucial role during the acute inflammatory response following certain forms of bacterial infection and has also been shown to co-localize with TLR4 upon stimulation of human neutrophils (Fortin *et al.* 2007). Consistent with these reports is our finding, that the expression of *TLRs*, including *TLR4*, was increased in NA compared to AA and HC. TLRs are considered as key players in immunity and serve as mediators of both innate and adaptive immune responses. Genetic variants of *TLRs* have been shown to have an impact on the development of various immune-mediated diseases, including asthma, AD and wheeze, as shown in our current work (Schwartz and Cook, 2005; Liu, Raedler *et al.* 2011).

The afore-presented data imply that the pathophysiology of non-allergic asthmatics is characterized by a Th17-shifted neutrophilic inflammation with significantly increased levels of pro-inflammatory IL-1β. Furthermore, expression of the two calcium binding molecules *CD93* and *S100A9* was significantly increased in NA compared to AA and HC and could therefore serve as biomarkers to immunologically distinguish between asthmatics of allergic and non-allergic origin. As transcriptional regulation of the calcium signalling differed

significantly within the three phenotypes, we hypothesize a possible role for calcium signalling and its underlying molecular mechanisms in asthma pathogenesis.

There is increasing evidence that, besides the Th1/Th2 paradigm, additional cell types are involved in the pathogenesis of asthma. Nevertheless, dysregulation of Th1 and Th2 cells is still considered to play a major role in asthma (Colavita *et al.* 2000). Since a maladapted Th2 response was reported to be the crucial factor in the pathophysiology of allergic asthma (Kiley *et al.* 2007; Bosnjak *et al.* 2011), we measured cytokine levels of the characteristic Th2 cytokines IL-5 and IL-13 in cell supernatants of our study population. Th2 cytokine levels were increased in asthmatics, both AA and NA, compared to HC. Particularly IL-5 was significantly elevated in AA and NA compared to HC and secretion was even more elevated in NA following adaptive stimulation, although the latter finding was not statistically significant. Consistent with these findings, our microarray experiments revealed significant differences in Th2 regulation between AA or NA compared to HC, with higher Th2 gene expression in asthmatics, further indicating a common Th2-biased immune phenotype in both allergic and non-allergic asthmatic children.

Levels of the classical Th1 cytokine IFN-γ were decreased in cell supernatants of asthmatics compared to HC. This decrease has already been reported in PBMCs of moderate atopic asthmatic children by Hoekstra and colleagues (Hoekstra *et al.* 1997). Within our study population, levels of IFN-γ were similar between AA and NA, with a tendency towards lower levels in the non-allergic phenotype, compared to the allergic phenotype.

Since epigenetic regulation is considered an important factor during effector T cell development and epigenetic changes in $CD4^+$ T cells have been associated with autoimmune diseases (Perl A., 2010), we determined levels of DNA methylation within the IFN-γ promoter region, as well as histone H3 and H4 acetylation in $CD4^+$ cells. While OVA

sensitization/challenge led to an increase in *IFN-γ* promoter methylation in $CD4^+$ cells in a mouse model (Brand *et al.* 2012), we did not detect any differences in methylation status within the human *IFN-γ* promoter region. Furthermore, *IFN-γ* acetylation at the histone proteins H3 and H4 did not differ in HC and AA, which is contradictory to findings in mouse models, in which the protective effect of *Acinetobacter lwoffii F78* was at least in part mediated via protection against the loss of H4 acetylation (Brand *et al.* 2011).

T helper cells, as component of the adaptive immune system and moreover cells of the innate immune system are regulated by TGF-β, which suppresses immune responses by inhibiting the function of inflammatory cells and by promoting the suppressive capacity of Treg cells (Wan Y.Y., 2008). TGF-β was reported to play a major role in asthma, especially in airway remodelling, a process associated with decreased pulmonary function of asthmatics (Makinde *et al.* 2007). While elevated levels of TGF-β have been observed in asthmatic airways, especially in severe asthmatics (Makinde *et al.* 2007), expression of characteristic genes of the TGF-β signalling pathway, including *Smad2/3* and *TGF-β* itself, were decreased in both childhood asthma phenotypes compared to HC within our study population, and *Smad6* and *Smad7,* two negative regulators of TGF-β signalling, were up-regulated in AA compared to HC (Makinde *et al.* 2007), however microarray expression results still have to be validated by qRT-PCR.

In contrast, increased TGF-β signalling and *Smad2/3* expression have been shown in asthmatic airways (Bossé and Rola-Pleszczynski, 2007; Torrego *et al.* 2007). These different findings might be explained by the different biological material which was investigated and Bossé and Rola-Pleszczynski suggest that TGF-β activity is not directly related to gene expression, but might be increased at other points of control (Bossé and Rola-Pleszczynski, 2007). As a measure of transcriptional activity, Sagara *et al.* determined the phosphorylation status of

Smad2 and could thereby show increased TGF-β signalling in asthmatics and an association with airway remodelling in asthma (Sagara *et al.* 2002). To determine the amount of phosphorylated Smad2 and Smad3, which translocate to the nucleus and are directly involved in regulation of TGF-β-dependent gene expression, would be necessary to clarify the role of TGF-β signalling within allergic and non-allergic children within our study population.

In summary, AA and NA together share a Th2-biased phenotype, while Th1 cells are reduced in numbers in both phenotypes. The lower levels of IFN-γ in AA compared to HC could not be attributed to epigenetic regulation via *IFN-γ* promoter methylation or histone H3 or H4 acetylation. Neither the measurement of Th1 nor Th2 cytokine levels allowed a discrimination between allergic and non-allergic asthmatic children.

Due to the complexity of the asthma phenotypes, we decided to additionally investigate $CD4^+$ cells by microarrays, which were suggested to play a major role in asthma pathogenesis (Robinson *et al.* 2010). The isolated $CD4^+$ cells from the subgroup of AA and HC solely differed in nTh gene expression but not within any KEGG pathways. These results indicate that the regulation of asthma seems to be more complex and not mainly mediated by a dysregulation of $CD4^+$ cells but potentially an interplay between several cell types from the PBMC population, including $CD4^+$, $CD8^+$, B and natural killer cells, basophils and dendritic cells.

By assessing several dimensions of immune regulation, we have shown that the underlying pathophysiological mechanisms of allergic and non-allergic asthma share some degree of homology but differ in cytokine profiles as well as T cell regulation.

5. SUMMARY

The prevalence of atopic diseases in childhood increased dramatically over the past decades, especially in industrialized countries. However, little is known about the underlying mechanisms of immune regulation during infancy and at the onset of atopic diseases.

In my thesis, I have studied the regulation of different T cell subpopulations and the impact of genetic predisposition and epigenetic regulation on the development of atopic diseases at two different time points: early in life (cord blood) and later in childhood (age 4-15 years).

In contrast to numerous other published studies examining atopic diseases in children and adults, we assessed immune regulation in cord blood, therefore allowing us to perform our studies at the earliest possible time point of immune maturation. In addition, we assessed immune responses later in childhood during manifestation of disease, which offered the unique opportunity to identify important regulatory pathways that are key determinants for the development of childhood asthma.

In my experiments I obtained the following results:

1) Among the innate *TLR* polymorphisms addressed in our birth cohort study, genetic variants of *TLR2* were shown to impact T regulatory cell (Treg) marker gene expression in cord blood, with expression levels being modulated by maternal atopy.

   In carriers of *IL-10* SNPs within the same study population, we observed a distinct pattern of cord blood T cell lineage fate, with decreased expression of Treg markers, decreased levels of pro-inflammatory TNF-α, GM-CSF and IL-5 (Th2 cytokine), and a concomitant increase in IFN-γ (Th1 cytokine) levels. Importantly, genetic variants of *TLRs* and *IL-10* were shown to be determinants for the development of atopic diseases in early childhood until 3 years of age.

2) The two main clinical phenotypes of childhood asthma (allergic and non-allergic asthma) differed regarding their underlying pathophysiological mechanisms when studied during manifestation of disease in childhood: Children with allergic compared to non-allergic asthma and healthy children, were found to have increased total numbers of regulatory T cells (Tregs) in parallel with reduced suppressive capacity of Tregs.

   The increase in total numbers of natural, thymus-derived Tregs (nTregs) could additionally be confirmed by epigenetic regulation of the Treg-specific demethylated region (TSDR), with higher demethylation found in allergic asthmatics.

   The increase in Tregs could represent a physiological response to the ongoing allergic inflammation found in allergic asthmatics, in order to compensate for the pathologically enhanced inflammatory reaction. Of note, the children develop the allergic asthmatic phenotype despite increased numbers of Tregs, indicating that this control mechanism is still not sufficient, possibly explained by a reduced suppressive capacity of Tregs.

   Importantly, our study showed that children with asthma of non-allergic origin have increased levels of the pro-inflammatory cytokine IL-1β, increased *IL-37* expression and a Th17-shifted immune phenotype with elevated numbers of neutrophils in peripheral blood, compared to allergic asthmatics and healthy controls.

   Thus, the non-allergic phenotype was immunologically quite distinct from the allergic asthmatic phenotype, characterized by a Treg-dominated immune response, and showed a Th17-shifted pro-inflammatory immune milieu promoting neutrophilic inflammation.

   Interestingly, in our study both allergic and non-allergic asthmatic children shared a Th2-dominated phenotype.

3) Employing genome-wide microarray expression analysis, which was confirmed by qRT-PCR, we identified novel specific gene expression profiles for childhood allergic and non-allergic asthma. We observed increased expression of the two calcium binding molecules

CD93 and *S100A9* and of *TREM1*, associated with neutrophilic inflammation, in PBMCs of non-allergic asthmatics compared to allergic asthmatics and healthy children. Additionally, both asthma phenotypes differed significantly regarding their expression of genes of the TLR, calcium and mTOR signalling pathways.

Together our data imply that a modulated immune regulation in cord blood, influenced by the genotype, has an impact on the development of atopic diseases later in childhood.

At the time of manifestation of allergic and non-allergic asthma in steroid-naïve, untreated children at age 4–15 years, both clinical phenotypes could be distinguished by different pathophysiological mechanism.

Importantly, the results of our study can help to develop specific biomarkers for differentiation of allergic from non-allergic asthma and may lead to a more individualized clinical treatment of patients with childhood asthma of allergic vs. non-allergic origin.

6. ZUSAMMENFASSUNG

Das Auftreten atopischer Erkrankungen im Kindesalter hat vor allem in Industrieländern in den letzten Jahrzehnten drastisch zugenommen. Die zugrundeliegenden Mechanismen der Immunregulation im Kleinkindesalter sowie bei Manifestation der Erkrankung sind bisher allerdings weitgehend unbekannt.

In meiner Doktorarbeit habe ich zu zwei unterschiedlichen Zeitpunkten (im Säuglings- und im Kindesalter) die Regulation verschiedener T-Zell Subpopulationen sowie den Einfluss genetischer Prädisposition und epigenetischer Regulation auf die Entstehung atopischer Erkrankungen untersucht.

Während in zahlreichen anderen Studien atopische Erkrankungen bei Kindern und Erwachsenen untersucht wurden, haben wir die Immunregulation im Nabelschnurblut, und damit zum frühestmöglichen Zeitpunkt der Immunmaturation untersucht.

Die zusätzliche Untersuchung der Immunantwort bei Krankheitsmanifestation im späteren Kindesalter (4-15 Jahre) eröffnet zudem die einzigartige Möglichkeit, wichtige Regulationsmechanismen in der Entstehung atopischer Erkrankungen zu identifizieren.

Meine Dissertation lieferte folgende Ergebnisse:

1) Von den in unserer Geburtskohorte untersuchten *TLR* Polymorphismen konnte für *TLR2* SNPs ein Einfluss auf die Expression von Treg-assoziierten Genen gezeigt werden, wobei die Genexpression zusätzlich vom mütterlichen Atopiestatus abhängig war.

   In der gleichen Studienpopulation konnte für *IL-10* SNP Träger folgendes Muster in der T-Zellregulation im Nabelschnurblut gezeigt werden: Neben einer erniedrigten Expression von Treg-assoziierten Genen konnten wir eine erniedrigte TNF-α, GM-CSF (pro-inflammatorische Zytokine) und IL-5 Sekretion (Th2 Zytokin) bei gleichzeitig erhöhtem

IFN-γ (Th1 Zytokin) zeigen. Im frühen Kindesalter (bis zu 3 Jahren) traten bei Kindern mit *TLR* und *IL-10* SNPs vermehrt atopische Erkrankungen auf.

2) Das kindliche Asthma wird in zwei Haupt-Phänotypen unterteilt, das allergische und das nicht-allergische Asthma. In unserer Studie konnten beide Phänotypen hinsichtlich ihrer zugrundeliegenden Pathophysiologie wie folgt unterschieden werden: Kinder (im Alter von 4 bis 15 Jahren) mit allergischem Asthma wiesen, im Vergleich zu nicht-allergischen Asthmatikern und gesunden Kindern, eine höhere Anzahl an regulatorischen T-Zellen (Tregs), bei gleichzeitig verminderter suppressiver Kapazität der Tregs, auf. Eine erhöhte Anzahl an natürlich vorkommenden Tregs (nTregs) konnte zudem mittels epigenetischer Regulation der Treg-spezifischen demethylierten Region (TSDR), mit erhöhter Demethylierung in allergischen Asthmatikern, bestätigt werden.

   Beim Anstieg von Tregs in allergischen Asthmatikern könnte es sich um eine physiologische Antwort auf die allergische Entzündung handeln mit dem Ziel die pathologisch gesteigerte Entzündungsreaktion, zum Teil bedingt durch eine erniedrigte suppressive Kapazität der Tregs, zu kompensieren. Es bleibt anzumerken, dass die Kinder trotz oben beschriebener Regulationsmechanismen unter allergischem Asthma leiden, was auf eine insgesamt unzureichende Kontrolle der pathologischen Immunreaktion hindeutet.

   Zusätzlich zeigte die Studie, dass bei Kindern mit nicht-allergischem Asthma, im Vergleich zu allergischen Asthmatikern und gesunden Kontrollen, sowohl das pro-inflammatorische Zytokin IL-1β als auch die Expression von IL-37 erhöht war. Zudem zeigten nicht-allergische Asthmatiker einen nach Th17 verschobenen Immunphänotyp, sowie eine erhöhte Anzahl von Neutrophilen in peripherem Blut.

   Kinder mit nicht-allergischem Asthma unterschieden sich demnach anhand oben gezeigter immunologischer Mechanismen klar von Kindern mit allergischem Asthma, und zeigten

verglichen mit der Treg-dominierten Antwort bei allergischen Asthmatikern, eine gesteigerte Th17-dominierte neutrophile Entzündungsreaktion.

Interessanterweise zeigten in unserer Studie sowohl allergische als auch nicht-allergische Asthmatiker einen Th2-dominierten Phänotyp.

3) Mithilfe von Microarray-Expressionsanalyse und nachfolgender Validierung der Genexpression mittels qRT-PCR, konnten wir neue spezifische Genexpressionsprofile für allergische und nicht-allergische Asthmatiker identifizieren. Dabei war die Expression der beiden Calcium-bindenden Proteine *CD93* und *S100A9* und von *TREM1*, welches mit neutrophiler Entzündung assoziiert ist, in nicht-allergischen Asthmatikern im Vergleich zu allergischen Asthmatikern und gesunden Kontrollen signifikant erhöht. Zusätzlich konnten wir zeigen, dass sich die beiden Asthma-Phänotypen hinsichtlich ihrer Expression von Genen aus der TLR-, Calcium- sowie der mTOR-Signalkaskade unterscheiden.

Zusammenfassend zeigen unsere Daten, dass bereits zum Zeitpunkt der Geburt eine veränderte Immunregulation, beeinflusst durch den Genotyp, eine Auswirkung auf die Prävalenz atopischer Erkrankungen im Kindesalter hat.

Bei Erstmanifestation von allergischem und nicht-allergischem Asthma in steroid-naiven, vorher unbehandelten Kindern im Alter von 4 bis 15 Jahren, konnten die beiden Phänotypen durch verschiedene pathophysiologische Mechanismen unterschieden werden.

Zusammenfassend können unsere Studienergebnisse bei der Entwicklung spezifischer Biomarker zur besseren Differenzierung und gezielteren Therapie von allergischem versus nicht-allergischem Asthma beitragen.

7. REFERENCES

- Aerbajinai W, Liu L, Chin K, Zhu J, Parent CA, Rodgers GP. Glia maturation factor-gamma mediates neutrophil chemotaxis. *J Leukoc Biol*. 2011;90:529-38.
- Akagi M. [Histamine in the pathogenesis of asthma]. *Nihon Yakurigaku Zasshi*. 1998;111:217-22.
- Akdis M, Palomares O, van de Veen W, van Splunter M, Akdis CA. TH17 and TH22 cells: a confusion of antimicrobial response with tissue inflammation versus protection. *J Allergy Clin Immunol*. 2012;129:1438-49.
- Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol*. 2001;2:675-80.
- Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol*. 2004;4:499-511.
- Alcorn JF, Crowe CR, Kolls JK. TH17 cells in asthma and COPD. *Annu Rev Physiol*. 2010;72:495-516.
- Amin K, Ludviksdottir D, Janson C, Nettelbladt O, Bjornsson E, Roomans GM, et al. Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. *Am J Respir Crit Care Med*. 2000;162:2295-301.
- Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. Defining the human T helper 17 cell phenotype. *Trends Immunol*. 2012;33:505-12.
- Asadullah K, Eskdale J, Wiese A, Gallagher G, Friedrich M, Sterry W. Interleukin-10 promoter polymorphism in psoriasis. *J Invest Dermatol*. 2001;116:975-8.
- Ballenberger N, Lluis A, von Mutius E, Illi S, Schaub B. Novel statistical approaches for non-normal censored immunological data: analysis of cytokine and gene expression data. *PLoS One*. 2012;7:e46423.
- Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol*. 2007;37:2378-89.
- Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological).* 1995;57(1):289-300.
- Beydon N, Davis SD, Lombardi E, et al. An official American Thoracic Society/European Respiratory Society statement: pulmonary function testing in preschool children. *Am J Respir Crit Care Med.*2007;175:1304-1345.
- Beyer M, Schultze JL. Plasticity of T(reg) cells: is reprogramming of T(reg) cells possible in the presence of FOXP3? *Int Immunopharmacol*. 2011;11:555-60.
- Bhathena PR, Comhair SA, Holroyd KJ, Erzurum SC. Interleukin-9 receptor expression in asthmatic airways In vivo. *Lung*. 2000;178:149-60.
- Biedermann T and Rocken M. Th1/Th2 balance in atopy. Springer Semin Immunopathol 1999;21:295-316.
- Bisgaard H, Pipper CB, Bonnelykke K. Endotyping early childhood asthma by quantitative symptom assessment. *J Allergy Clin Immunol*. 2011;127:1155-64 e2.
- Borrego LM, Arroz MJ, Videira P, Martins C, Guimaraes H, Nunes G, et al. Regulatory cells, cytokine pattern and clinical risk factors for asthma in infants and young children with recurrent wheeze. *Clin Exp Allergy*. 2009;39:1160-9.
- Bosnjak B, Stelzmueller B, Erb KJ, Epstein MM. Treatment of allergic asthma: modulation of Th2 cells and their responses. *Respir Res*. 2011;12:114.
- Bossé Y, Rola-Pleszczynski M. Controversy surrounding the increased expression of TGF beta 1 in asthma. *Respiratory research*. 2007;8:66.
- Bossé Y, Lemire M, Poon AH, Daley D, He JQ, Sandford A, et al. Asthma and genes encoding components of the vitamin D pathway. *Respir Res*. 2009;10:98.
- Brand S, Teich R, Dicke T, Harb H, Yildirim AO, Tost J, et al. Epigenetic regulation in murine offspring as a novel mechanism for transmaternal asthma protection induced by microbes. *J Allergy Clin Immunol*. 2011;128:618-25 e1-7.

7. REFERENCES

- Brand S, Kesper DA, Teich R, Kilic-Niebergall E, Pinkenburg O, Bothur E, et al. DNA methylation of TH1/TH2 cytokine genes affects sensitization and progress of experimental asthma. *J Allergy Clin Immunol*. 2012;129:1602-10 e6.
- Busse WW and Lemanske RF, Jr. Asthma. N Engl J Med 2001;344:350-62.
- Cameron L, Webster RB, Strempel JM, Kiesler P, Kabesch M, Ramachandran H, et al. Th2 cell-selective enhancement of human IL13 transcription by IL13-1112C>T, a polymorphism associated with allergic inflammation. *J Immunol*. 2006;177:8633-42.
- Casaca VI, Illi S, Suttner K, Schleich I, Ballenberger N, Klucker E, et al. TBX21 and HLX1 polymorphisms influence cytokine secretion at birth. *PLoS One*. 2012;7:e31069.
- Cavassani KA, Carson WFt, Moreira AP, Wen H, Schaller MA, Ishii M, et al. The post sepsis-induced expansion and enhanced function of regulatory T cells create an environment to potentiate tumor growth. *Blood*. 2010;115:4403-11.
- Chang HC, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol*. 2010;11:527-34.
- Chatterjee R, Batra J, Kumar A, Mabalirajan U, Nahid S, Niphadkar PV, et al. Interleukin-10 promoter polymorphisms and atopic asthma in North Indians. *Clin Exp Allergy*. 2005;35:914-9.
- Ciprandi G, Fenoglio D, De Amici M, Quaglini S, Negrini S, Filaci G. Serum IL-17 levels in patients with allergic rhinitis. *J Allergy Clin Immunol*. 2008;122:650-1 e2.
- Colavita AM, Reinach AJ, Peters SP. Contributing factors to the pathobiology of asthma. The Th1/Th2 paradigm. *Clin Chest Med*. 2000;21:263-77, viii.
- Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F. Th17 cells: new players in asthma pathogenesis. *Allergy*. 2011;66:989-98.
- Craig TJ. Aeroallergen sensitization in asthma: prevalence and correlation with severity. *Allergy Asthma Proc*. 2010;31:96-102.
- Crapo RO, Casaburi R, Coates AL, et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med.* 2000;161:309-329.
- Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity*. 2009;30:626-35.
- Dai J, Liu B, Li Z. Regulatory T cells and Toll-like receptors: what is the missing link? *Int Immunopharmacol*. 2009;9:528-33.
- Development Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. 2005.
- Devereux G. The increase in the prevalence of asthma and allergy: food for thought. Nat Rev Immunol 2006;6:869-74.
- Ding C and Cantor CR. A high-throughput gene expression analysis technique using competitive PCR and matrix-assisted laser desorption ionization time-of-flight MS. Proc Natl Acad Sci U S A 2003;100:3059-64.
- Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol*. 2009;10:857-63.
- Dykewicz MS and Hamilos DL. Rhinitis and sinusitis. J Allergy Clin Immunol 2010;125:S103-15.
- Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. J Allergy Clin Immunol 2004;113:482-8.
- Feske S. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol*. 2007;7:690-702.
- Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol*. 2009;10:689-95.
- Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol*. 1991;147:3815-22.
- Fortin CF, Lesur O, Fulop T, Jr. Effects of TREM-1 activation in human neutrophils: activation of signalling pathways, recruitment into lipid rafts and association with TLR4. *Int Immunol*. 2007;19:41-50.

7. REFERENCES

- Frey U, von Mutius E. The challenge of managing wheezing in infants. *N Engl J Med.* May 14 2009;360(20):2130-2133.
- Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, et al. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*. 2000;100:575-85.
- Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 2004;5:R80.
- Gibson AW, Edberg JC, Wu J, Westendorp RG, Huizinga TW, Kimberly RP. Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. *J Immunol*. 2001;166:3915-22.
- Greenlee MC, Sullivan SA, Bohlson SS. CD93 and related family members: their role in innate immunity. *Curr Drug Targets*. 2008;9:130-8.
- Greenlee M, Sullivan S, Bohlson S. Detection and characterization of soluble CD93 released during inflammation. *Inflamm Res*. 2009;58:909-19.
- Grutz G. New insights into the molecular mechanism of interleukin-10-mediated immunosuppression. *J Leukoc Biol*. 2005;77:3-15.
- Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol*. 2007;119:1258-66.
- Hauber HP, Bergeron C, Tsicopoulos A, Wallaert B, Olivenstein R, Holroyd KJ, et al. Increased expression of the calcium-activated chloride channel hCLCA1 in airways of patients with obstructive chronic bronchitis. *Can Respir J*. 2005;12:143-6.
- Hayden CM, Zhang G, Judge PK, Khoo SK, Laing IA, Turner SW, et al. Regulatory role of IL10 genetic variations in determining allergen-induced T(H)2 cytokine responses in children. *J Allergy Clin Immunol*. 2011;128:237-9 e8.
- He G, Ma Y, Chou SY, Li H, Yang C, Chuang JZ, et al. Role of CLIC4 in the host innate responses to bacterial lipopolysaccharide. *Eur J Immunol*. 2011;41:1221-30.
- Hee CS, Gun SC, Naidu R, Gupta E, Somnath SD, Radhakrishnan AK. Comparison of single nucleotide polymorphisms in the human interleukin-10 gene promoter between rheumatoid arthritis patients and normal subjects in Malaysia. *Mod Rheumatol*. 2007;17:429-35.
- Hellings PW, Kasran A, Liu Z, Vandekerckhove P, Wuyts A, Overbergh L, et al. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol Biol*. 2003;28:42-50.
- Hoekstra MO, Hoekstra Y, De Reus D, Rutgers B, Gerritsen J, Kauffman HF. Interleukin-4, interferon-gamma and interleukin-5 in peripheral blood of children with moderate atopic asthma. *Clin Exp Allergy*. 1997;27:1254-60.
- Holgate ST. Pathogenesis of asthma. Clin Exp Allergy 2008;38:872-97.
- Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med*. 2012;18:673-83.
- Hollingsworth JW, Maruoka S, Boon K, Garantziotis S, Li Z, Tomfohr J, et al. In utero supplementation with methyl donors enhances allergic airway disease in mice. *J Clin Invest*. 2008;118:3462-9.
- Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol*. 2009;9:83-9.
- Hughes T, Webb R, Fei Y, Wren JD, Sawalha AH. DNA methylome in human CD4+ T cells identifies transcriptionally repressive and non-repressive methylation peaks. *Genes Immun*. 2010;11:554-60.
- Humbert M, Menz G, Ying S, Corrigan CJ, Robinson DS, Durham SR, et al. The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. Immunol Today 1999;20:528-33.
- Hunninghake GM, Soto-Quiros ME, Lasky-Su J, Avila L, Ly NP, Liang C, et al. Dust mite exposure modifies the effect of functional IL10 polymorphisms on allergy and asthma exacerbations. *J Allergy Clin Immunol*. 2008;122:93-8, 8 e1-5.

7. REFERENCES

- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4:249-64.
- Just J, Gouvis-Echraghi R, Couderc R, Guillemot-Lambert N, Saint-Pierre P. Novel severe wheezy young children phenotypes: boys atopic multiple-trigger and girls nonatopic uncontrolled wheeze. *J Allergy Clin Immunol*. 2012;130:103-10 e8.
- Jutel M, Akdis CA. T-cell subset regulation in atopy. *Curr Allergy Asthma Rep*. Apr 2011;11(2):139-145.
- Kay AB. Allergy and allergic diseases. First of two parts. *N Engl J Med*. Jan 4 2001;344(1):30-37.
- Kicic A, Hallstrand TS, Sutanto EN, Stevens PT, Kobor MS, Taplin C, et al. Decreased fibronectin production significantly contributes to dysregulated repair of asthmatic epithelium. *Am J Respir Crit Care Med*. 2010;181:889-98.
- Kiley J, Smith R, Noel P. Asthma phenotypes. *Curr Opin Pulm Med*. 2007;13:19-23.
- Kim G, Kronenberg M. Cooling the fires of inflammation. *Proc Natl Acad Sci U S A*. 2011;108:16493-4.
- Kleinewietfeld M, Starke M, Di Mitri D, Borsellino G, Battistini L, Rotzschke O, et al. CD49d provides access to "untouched" human Foxp3+ Treg free of contaminating effector cells. *Blood*. 2009;113:827-36.
- Klotz L, Burgdorf S, Dani I, Saijo K, Flossdorf J, Hucke S, et al. The nuclear receptor PPAR gamma selectively inhibits Th17 differentiation in a T cell-intrinsic fashion and suppresses CNS autoimmunity. *J Exp Med*. 2009;206:2079-89.
- Koga C, Kabashima K, Shiraishi N, Kobayashi M, Tokura Y. Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol*. 2008;128:2625-30.
- Kormann MS, Depner M, Hartl D, Klopp N, Illig T, Adamski J, et al. Toll-like receptor heterodimer variants protect from childhood asthma. J Allergy Clin Immunol 2008;122:86-92, e1-8.
- Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol*. 2009;27:485-517.
- Kube D, Hua TD, von Bonin F, Schoof N, Zeynalova S, Kloss M, et al. Effect of interleukin-10 gene polymorphisms on clinical outcome of patients with aggressive non-Hodgkin's lymphoma: an exploratory study. Clin Cancer Res 2008;14:3777-84.
- Kube D, Mormann M, Tomiuk J, Rieth H, Hua TD, Kremsner PG, et al. Simultaneous analysis of interleukin-10 gene microsatellites and single-nucleotide polymorphisms in parallel with tumour necrosis factor and interferon-gamma short tandem repeats by fluorescence-based polymerase chain reaction. Genes Immun 2003;4:459-68.
- Kube D, Platzer C, von Knethen A, Straub H, Bohlen H, Hafner M, et al. Isolation of the human interleukin 10 promoter. Characterization of the promoter activity in Burkitt's lymphoma cell lines. *Cytokine*. 1995;7:1-7.
- Laan M, Cui ZH, Hoshino H, Lotvall J, Sjostrand M, Gruenert DC, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol*. 1999;162:2347-52.
- Lambrecht BN, Hammad H. The airway epithelium in asthma. *Nat Med*. [10.1038/nm.2737]. 2012;18:684-92.
- Langier S, Sade K, Kivity S. Regulatory T cells in allergic asthma. *Isr Med Assoc J*. 2012;14:180-3.
- Lazarus R, Raby BA, Lange C, Silverman EK, Kwiatkowski DJ, Vercelli D, et al. TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. *Am J Respir Crit Care Med*. 2004;170:594-600.
- Lee GR, Kim ST, Spilianakis CG, Fields PE, Flavell RA. T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity*. 2006;24:369-79.
- Lee JH, Yu HH, Wang LC, Yang YH, Lin YT, Chiang BL. The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. *Clin Exp Immunol*. 2007;148:53-63.
- Le Souef PN. Gene-environmental interaction in the development of atopic asthma: new developments. *Curr Opin Allergy Clin Immunol*. 2009;9:123-7.

7. REFERENCES

- Leung DY, Boguniewicz M, Howell MD, Nomura I and Hamid QA. New insights into atopic dermatitis. J Clin Invest 2004;113:651-7.
- Li X, Ampleford EJ, Howard TD, Moore WC, Torgerson DG, Li H, et al. Genome-wide association studies of asthma indicate opposite immunopathogenesis direction from autoimmune diseases. *J Allergy Clin Immunol*. 2012;130:861-8 e7.
- Lin YL, Shieh CC, Wang JY. The functional insufficiency of human CD4+CD25 high T-regulatory cells in allergic asthma is subjected to TNF-alpha modulation. *Allergy*. 2008;63:67-74.
- Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, et al. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet*. 2004;363:608-15.
- Liu G, Zhao Y. Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells. *Immunology*. 2007;122:149-56.
- Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signalling modulates the functions of CD4+ CD25+ regulatory T cells. *Proc Natl Acad Sci U S A*. 2006;103:7048-53.
- Liu J, Lluis A, Illi S, Layland L, Olek S, von Mutius E, Schaub B. T regulatory cells in cord blood--FOXP3 demethylation as reliable quantitative marker.*PLoS One* .2010;5(10): e13267.
- Liu J, Raedler D, Illi S, Klucker E, Turan E, von Mutius E, et al. TLR2 polymorphisms influence neonatal regulatory T cells depending on maternal atopy. *Allergy*. 2011;66:1020-9.
- Lluis A, Schedel M, Liu J, Illi S, Depner M, von Mutius E, et al. Asthma-associated polymorphisms in 17q21 influence cord blood ORMDL3 and GSDMA gene expression and IL-17 secretion. *J Allergy Clin Immunol*. 2011;127:1587-94 e6.
- Louten J, Boniface K, de Waal Malefyt R. Development and function of TH17 cells in health and disease. *J Allergy Clin Immunol*. 2009;123:1004-11.
- Lyon H, Lange C, Lake S, Silverman EK, Randolph AG, Kwiatkowski D, et al. IL10 gene polymorphisms are associated with asthma phenotypes in children. *Genet Epidemiol*. 2004;26:155-65.
- Makinde T, Murphy RF, Agrawal DK. The regulatory role of TGF-beta in airway remodelling in asthma. *Immunol Cell Biol*. 2007;85:348-56.
- Martino DJ, Prescott SL. Silent mysteries: epigenetic paradigms could hold the key to conquering the epidemic of allergy and immune disease. *Allergy*. 2010;65:7-15.
- Matricardi PM, Rosmini F, Riondino S, Fortini M, Ferrigno L, Rapicetta M, et al. Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. *BMJ*. 2000;320:412-7.
- Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature*. 2007;448:470-3.
- Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. *N Engl J Med*. 2010;363:1211-21.
- Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol*. 2001;108:430-8.
- Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;136:2348-57.
- Niebuhr M, Langnickel J, Draing C, Renz H, Kapp A, Werfel T. Dysregulation of toll-like receptor-2 (TLR-2)-induced effects in monocytes from patients with atopic dermatitis: impact of the TLR-2 R753Q polymorphism. *Allergy*. 2008;63:728-34.
- Nielsen KG, Bisgaard H. Cold air challenge and specific airway resistance in preschool children. *Paediatr Respir Rev.*2005;6:255-266.
- Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P, Dinarello CA. IL-37 is a fundamental inhibitor of innate immunity. *Nat Immunol*. 2010;11:1014-22.
- Nyirenda MH, O'Brien K, Sanvito L, Constantinescu CS, Gran B. Modulation of regulatory T cells in health and disease: role of toll-like receptors. *Inflamm Allergy Drug Targets*. 2009;8:124-9.

7. REFERENCES

- O'Garra A, Vieira P. T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol*. 2007;7:425-8.
- Ou LS, Goleva E, Hall C, Leung DY. T regulatory cells in atopic dermatitis and subversion of their activity by superantigens. *J Allergy Clin Immunol*. 2004;113:756-63.
- Ouyang X, Zhang R, Yang J, Li Q, Qin L, Zhu C, et al. Transcription factor IRF8 directs a silencing programme for TH17 cell differentiation. *Nat Commun*. 2011;2:314.
- Papadopoulos NG, Arakawa H, Carlsen KH, et al. International consensus on (ICON) pediatric asthma. *Allergy.* Aug 2012;67(8):976-997.
- Paradowska-Gorycka A, Trefler J, Maciejewska-Stelmach J, Lacki JK. Interleukin-10 gene promoter polymorphism in Polish rheumatoid arthritis patients. *Int J Immunogenet*. 2010;37:225-31.
- Perl A. Pathogenic mechanisms in systemic lupus erythematosus. *Autoimmunity*. 2010;43:1-6.
- Pinto LA, Stein RT, Kabesch M. Impact of genetics in childhood asthma. *J Pediatr (Rio J)*. 2008;84:S68-75.
- Popper HH, Pailer S, Wurzinger G, Feldner H, Hesse C, Eber E. Expression of adhesion molecules in allergic lung diseases. *Virchows Arch*. 2002;440:172-80.
- Potter PC. Current guidelines for the management of asthma in young children. *Allergy Asthma Immunol Res*. Jan 2010;2(1):1-13.
- Pumputiene I, Emuzyte R, Siaurys A, Tamosiunas V, Valiulis A. CD4+CD25(high) Treg cells in peripheral blood during remission and exacerbation of allergic asthma in children. *Acta Paediatr*. 2011;100:1006-10.
- Radsak MP, Salih HR, Rammensee HG, Schild H. Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: differential regulation of activation and survival. *J Immunol*. 2004;172:4956-63.
- Raedler D, Illi S, Pinto LA, von Mutius E, Illig T, Kabesch M, et al. IL10 polymorphisms influence neonatal immune responses, atopic dermatitis, and wheeze at age 3 years. *J Allergy Clin Immunol*. 2012.
- Raedler D, Schaub B. Immunology and defence mechanisms. ERS Handbook of Paediatric Respiratory Medicine 2013. Chapter 1. (In press)
- Reijmerink NE, Bottema RW, Kerkhof M, Gerritsen J, Stelma FF, Thijs C, et al. TLR-related pathway analysis: novel gene-gene interactions in the development of asthma and atopy. *Allergy*. 2010;65:199-207.
- Rieth H, Mormann M, Luty AJ, Assohou-Luty CA, Roupelieva M, Kremsner PG, et al. A three base pair gene variation within the distal 5'-flanking region of the interleukin-10 (IL-10) gene is related to the in vitro IL-10 production capacity of lipopolysaccharide-stimulated peripheral blood mononuclear cells. *Eur Cytokine Netw*. 2004;15:153-8.
- Robinson DS. The role of the T cell in asthma. *J Allergy Clin Immunol*. 2010;126:1081-91.
- Romagnani S. T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol*. 2000;85:9-18.
- Romanet-Manent S, Charpin D, Magnan A, Lanteaume A, Vervloet D. Allergic vs nonallergic asthma: what makes the difference? *Allergy*. 2002;57:607-13.
- Rybojad M. Atopic dermatitis. *Arch Pediatr*. 2012 Aug;19(8):882-5.
- Sabin BR, Peters N, Peters AT. Chapter 20: Atopic dermatitis. *Allergy Asthma Proc*. 2012;33 Suppl 1:67-9.
- Sagara H, Okada T, Okumura K, Ogawa H, Ra C, Fukuda T, et al. Activation of TGF-beta/Smad2 signalling is associated with airway remodelling in asthma. *J Allergy Clin Immunol*. 2002;110(2):249-54.
- Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol*. 2010;10:490-500.
- Sato K, Suematsu A, Okamoto K, Yamaguchi A, Morishita Y, Kadono Y, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. *J Exp Med*. 2006;203:2673-82.
- Schaub B, Campo M, He H, Perkins D, Gillman MW, Gold DR, et al. Neonatal immune responses to TLR2 stimulation: influence of maternal atopy on Foxp3 and IL-10 expression. *Respir Res*. 2006;7:40.

7. REFERENCES

- Schaub B, Liu J, Hoppler S, Haug S, Sattler C, Lluis A, et al. Impairment of T-regulatory cells in cord blood of atopic mothers. *J Allergy Clin Immunol*. 2008;121:1491-9, 9 e1-13.
- Schaub B, Liu J, Hoppler S, Schleich I, Huehn J, Olek S, et al. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. *J Allergy Clin Immunol*. 2009;123:774-82 e5.
- Schedel M, Pinto LA, Schaub B, Rosenstiel P, Cherkasov D, Cameron L, et al. IRF-1 gene variations influence IgE regulation and atopy. *Am J Respir Crit Care Med*. 2008;177:613-21.
- Schenk M, Bouchon A, Seibold F, Mueller C. TREM-1--expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *J Clin Invest*. 2007;117:3097-106.
- Schwartz DA, Cook DN. Polymorphisms of the Toll-like receptors and human disease. *Clin Infect Dis*. 2005;41 Suppl 7:S403-7.
- Sehouli J, Loddenkemper C, Cornu T, Schwachula T, Hoffmuller U, Grutzkau A, et al. Epigenetic quantification of tumor-infiltrating T-lymphocytes. *Epigenetics*. 2011;6:236-46.
- Semlali A, Jacques E, Plante S, Biardel S, Milot J, Laviolette M, et al. TGF-beta suppresses EGF-induced MAPK signalling and proliferation in asthmatic epithelial cells. *Am J Respir Cell Mol Biol*. 2008;38:202-8.
- Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity*. 2006;25:195-201.
- Shi HZ, Li S, Xie ZF, Qin XJ, Qin X, Zhong XN. Regulatory CD4+CD25+ T lymphocytes in peripheral blood from patients with atopic asthma. *Clin Immunol*. 2004;113:172-8.
- Shin HD, Winkler C, Stephens JC, Bream J, Young H, Goedert JJ, et al. Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. *Proc Natl Acad Sci U S A*. 2000;97:14467-72.
- Shukla A, Malik M, Cataisson C, Ho Y, Friesen T, Suh KS, et al. TGF-beta signalling is regulated by Schnurri-2-dependent nuclear translocation of CLIC4 and consequent stabilization of phospho-Smad2 and 3. *Nat Cell Biol*. 2009;11:777-84.
- Simard JC, Girard D, Tessier PA. Induction of neutrophil degranulation by S100A9 via a MAPK-dependent mechanism. *J Leukoc Biol*. 2010;87:905-14.
- Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.
- Smyth LJ, Eustace A, Kolsum U, Blaikely J, Singh D. Increased airway T regulatory cells in asthmatic subjects. *Chest*. 2010;138:905-12.
- Sousa AR, Lane SJ, Nakhosteen JA, Lee TH, Poston RN. Expression of interleukin-1 beta (IL-1beta) and interleukin-1 receptor antagonist (IL-1ra) on asthmatic bronchial epithelium. *Am J Respir Crit Care Med*. 1996;154:1061-6.
- Suttner K, Ruoss I, Rosenstiel P, Depner M, Pinto LA, Schedel M, et al. HLX1 gene variants influence the development of childhood asthma. *J Allergy Clin Immunol*. 2009;123:82-8 e6.
- Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev*. 1998;12:599-606.
- Szczepankiewicz A, Breborowicz A, Sobkowiak P, Popiel A. Polymorphisms of two histamine-metabolizing enzymes genes and childhood allergic asthma: a case control study. *Clin Mol Allergy*. 2010;8:14.
- Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol*. 2002;169:10-4.
- Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. *Immunology*. 2006;117:433-42.
- Tete S, Tripodi D, Rosati M, Conti F, Maccauro G, Saggini A, et al. IL-37 (IL-1F7) the newest anti-inflammatory cytokine which suppresses immune responses and inflammation. *Int J Immunopathol Pharmacol*. 2012;25:31-8.
- Thunberg S, Gafvelin G, Nord M, Gronneberg R, Grunewald J, Eklund A, et al. Allergen provocation increases TH2-cytokines and FOXP3 expression in the asthmatic lung. *Allergy*. 2010;65:311-8.

7. REFERENCES

- Tirkos S, Newbigging S, Nguyen V, Keet M, Ackerley C, Kent G, et al. Expression of S100A8 correlates with inflammatory lung disease in congenic mice deficient of the cystic fibrosis transmembrane conductance regulator. *Respir Res*. 2006;7:51.
- Torrego A, Hew M, Oates T, Sukkar M, Fan Chung K. Expression and activation of TGF-beta isoforms in acute allergen-induced remodelling in asthma. *Thorax.* 2007;62(4):307-13.
- Tost J, Gut IG. DNA methylation analysis by pyrosequencing. *Nat Protoc*. 2007;2:2265-75.
- Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol*. 2009;10:864-71.
- Tsukagoshi H, Sakamoto T, Xu W, Barnes PJ, Chung KF. Effect of interleukin-1 beta on airway hyperresponsiveness and inflammation in sensitized and nonsensitized Brown-Norway rats. *J Allergy Clin Immunol*. 1994;93:464-9.
- Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat Immunol.* Aug 2002;3:715-720.
- Uzzaman A, Story R. Chapter 5: Allergic rhinitis. *Allergy Asthma Proc*. 2012;33 Suppl 1:15-8.
- Van der Velden VH, Laan MP, Baert MR, de Waal Malefyt R, Neijens HJ, Savelkoul HF. Selective development of a strong Th2 cytokine profile in high-risk children who develop atopy: risk factors and regulatory role of IFN-gamma, IL-4 and IL-10. *Clin Exp Allergy*. 2001;31:997-1006.
- Vercelli D. Discovering susceptibility genes for asthma and allergy. *Nat Rev Immunol*. 2008;8:169-82.
- von Mutius E, Radon K. Living on a farm: impact on asthma induction and clinical course. *Immunol Allergy Clin North Am*. 2008;28:631-47, ix-x.
- Wan YY, Flavell RA. TGF-beta and regulatory T cell in immunity and autoimmunity. *J Clin Immunol.* Nov 2008;28:647-659.
- Waterland RA, Lin JR, Smith CA, Jirtle RL. Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus. *Hum Mol Genet*. 2006;15:705-16.
- White GP, Hollams EM, Yerkovich ST, Bosco A, Holt BJ, Bassami MR, et al. CpG methylation patterns in the IFNgamma promoter in naive T cells: variations during Th1 and Th2 differentiation and between atopics and non-atopics. *Pediatr Allergy Immunol*. 2006;17:557-64.
- Wilk JB, Walter RE, Laramie JM, Gottlieb DJ, O'Connor GT. Framingham Heart Study genome-wide association: results for pulmonary function measures. *BMC Med Genet*. 2007;8 Suppl 1:S8.
- Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol*. 2009;9:91-105.
- Wong CK, Ho CY, Li EK, Lam CW. Elevation of pro-inflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. *Lupus*. 2000;9:589-93.
- Wong CK, Ho CY, Ko FW, Chan CH, Ho AS, Hui DS, et al. Pro-inflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin Exp Immunol*. 2001;125:177-83.
- Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell*. 2006;126:375-87.
- Xu H, Zhu J, Smith S, Foldi J, Zhao B, Chung AY, et al. Notch-RBP-J signalling regulates the transcription factor IRF8 to promote inflammatory macrophage polarization. *Nat Immunol*. [10.1038/ni.2304]. 2012;13:642-50.
- Yamaguchi T, Wing JB, Sakaguchi S. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. *Semin Immunol*. 2011;23:424-30.
- Yamamoto Y, Negoro T, Hoshi A, Wakagi A, Shimizu S, Banham AH, et al. Impaired Ca(2)(+) regulation of CD4(+)CD25(+) regulatory T cells from pediatric asthma. *Int Arch Allergy Immunol*. 2011;156:148-58.
- Yan L, Galinsky RE, Bernstein JA, Liggett SB, Weinshilboum RM. Histamine N-methyltransferase pharmacogenetics: association of a common functional polymorphism with asthma. *Pharmacogenetics*. 2000;10:261-6.
- Yang IV, Schwartz DA. Epigenetic mechanisms and the development of asthma. *J Allergy Clin Immunol*. 2012;130:1243-55.

7. REFERENCES

- Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest*. 2006;116:1310-6.
- Yin LM, Jiang GH, Wang Y, Liu YY, Jin WR, Zhang Z, et al. Serial analysis of gene expression in a rat lung model of asthma. *Respirology*. 2008;13:972-82.
- Zhang J, Mendoza M, Guiraldelli MF, Barbu EA, Siraganian RP. Small interfering RNA screen for phosphatases involved in IgE-mediated mast cell degranulation. *J Immunol*. 2010;184:7178-85.
- Zhang H, Xiao Y, Zhu Z, Li B, Greene MI. Immune regulation by histone deacetylases: a focus on the alteration of FOXP3 activity. *Immunol Cell Biol*. 2012;90:95-100.
- Zhao Y, Yang J, Gao YD, Guo W. Th17 immunity in patients with allergic asthma. *Int Arch Allergy Immunol*. 2010;151:297-307.
- Zhu Z, Ma B, Zheng T, Homer RJ, Lee CG, Charo IF, et al. IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodelling. *J Immunol*. 2002;168:2953-62.

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10. ABBREVIATIONS

AA	Allergic asthmatic(s)
AD	Atopic dermatitis
AHR	Airway hyperresponsiveness
BHR	Bronchial hyperresponsiveness
bp	Basepair
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CBMC	Cord blood mononuclear cell
CD	Cluster designation
cDNA	Coding DNA
CLARA	Clinical Asthma Research Association
CT	Cycle threshold
CTLA-4	Cytotoxic T-Lymphocyte antigen 4
Derp1	Dermatophagoides pteronyssinus; house dust mite
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
fwd	Forward
GITR	Glucocorticoid-induced TNF receptor
GMC-SF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HC	Healthy control(s)
HWE	Hardy-Weinberg equilibrium
IFN-γ	Interferon-gamma
IgE	Immunglobulin E
IL	Interleukin
IQR	Interquartile range
LAG3	Lymphocyte activation gene-3
LD	Linkage disequilibrium
LpA	Lipid A
M	Media, unstimulated
MAF	Minor allele frequency
MALDI-TOF MS	Matrix assisted laser desorption-time of flight mass spectrometry
mRNA	Messenger RNA
NA	Non-allergic asthmatic(s)
OR	Odds ratio
p	p-value
PI	PMA/Ionomycin
PAULCHEN	Prospective Cord Blood Study in Rural Southern Germany
PAULINA	Paediatric Alliance for Unselected Longitudinal Investigation of Neonates for Allergies
PBMC	Peripheral blood monocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate

10. ABBREVIATIONS

Ppg	Peptidoglycan
Real-time RT-PCR	Real-time reverse-transcriptase-PCR
rev	Reverse
RA	Retinoic acid
RAST	Radioallergosorbent test
RNA	Ribonucleic acid
RORC	Retinoic acid receptor-related orphan receptor C
rs	Reference SNP
S100A9	S100 calcium binding protein A9
SD	Standard deviation
SNP	Single nucleotide polymorphism
TCR	T cell receptor
Th cells	T helper cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha
Treg	Regulatory T cell
TREM1	Triggering receptor expressed on myeloid cells 1
TSDR	Treg-specific demethylated region
t-test	Statistical test: Student's t distribution
UTR	Untranslated region
WT	Wildtype allele

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