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Role of Th1 and Th2 cell-specific polymorphisms and of Regulatory T cells modulated by farm exposure for the determination of childhood allergic diseases

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STATUTORY DECLARATION

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, 30.10.2013

Vera Casaca

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1. Introduction

1.1 ALLERGIC DISEASES

The immune system is a very complex system that protects the body from harmful elements, whether from self or non-self origin. When this system is compromised, infections, cancer, autoimmune diseases or allergic diseases can develop.

Allergic diseases comprise atopic dermatitis (eczema), allergic asthma, allergic rhinitis and conjunctivitis (rhinoconjutivitis or hay fever) and systemic anaphylaxis. These affect both adults and children (Asher, 2004).

Allergic diseases are characterized by an aberrant immune response to generally harmless antigens. The common allergens in westernized countries (antigens that can cause allergic reactions) include house dust mite, grass pollen, *Alternaria alternata*, *Cladosporium herbarum*, cat and dog dander, cow's milk, peanut, hen's egg, soya, cod and wheat (Arshad *et al.* 2001).

Currently, allergic diseases have reached epidemic proportions worldwide, it is estimated that 30-40 % of the world's population is affected (Pawankar, 2011). Therefore, it is of extreme importance to disentangle the origins and mechanisms of allergic diseases for the development of future therapies.

1.1.1 The socio-economic relevance of allergic diseases

The dramatic increase of allergic diseases, about 3 time fold in the last half century (Asher, 2004) lead to a significant financial burden on the healthcare systems (O'Connell, 2004). Predictions estimate that in approximately 10 years there could be 400 million people

suffering from asthma (Pawankar, 2011). The financial costs also impact the families as in medical services and prescription drugs (Suh, 2007).

Likewise there are social and physical costs associated with lowered life quality of the patients. This ranges from missed work and school days, urgent visits to the doctor, inability to practice sports, constant vigilance in food uptake and increased stress levels (O'Connell, 2004; von Mutius, 2000; Klinnert, 2008).

Atopic dermatitis leads for instance to constant itchiness, which is worsened by night time with a direct impact on the sleep patterns and is associated with decreased life quality (Monti *et al.* 1989).

Several studies have shown that there can be severe psychological effects on patients with allergic diseases and their families, which require guidance from the healthcare providers (Klinnert *et al.* 2008). Therefore, patients and closer relatives are not only socio-economically affected by allergic diseases, but also physically and psycologically, becoming a heavy burden on their daily lives.

1.1.2 Clinical manifestations of allergic diseases

1.1.2.1 Atopic dermatitis (eczema)

Atopic dermatitis (AD) is characterized by a chronic and relapsing skin inflammation with pruritus (itching), resulting in dry skin with an impaired function of the healthy epidermal-barrier. The symptoms include eczematous papules and plaques (Bieber, 2008; Simpson, 2010). The location of the lesions varies and can affect several parts of the body, such as the cheeks, scalp, neck and the flexures (elbows, the back of the knees) (Bieber, 2008).

The prevalence of atopic dermatitis is two times higher in children compared to adults (Williams *et al.* 2006). AD usually has a very early-onset, with 60 % starting before the age of 1 year, however, the majority of the children outgrow atopic dermatitis before reaching

adolescence (Novak *et al.* 2003). Histologic analyses show that the plaques are characterized by epidermal oedema and high levels of immune cell infiltrates (such as eosinophils, lymphocytes and dendritic cells) (Bieber *et al.* 2008).

1.1.2.2 Allergic asthma

Asthma is a complex airway disorder that is characterized by four main features: airway obstruction, hyperresponsiveness, spontaneous (or treatment-associated) reversibility and inflammation (Lemanske *et al.* 2010). The manifestation of asthma can be mild, moderate up to extreme cases, life-threatening due to asphyxia (Corry, 2001). The episodes of exacerbation are triggered by exposure to allergens in sensitized subjects (Lemanske *et al.* 2010). This is one of the hallmarks that differentiates allergic from non-allergic asthma, which is generally triggered by physical activity, stress, cold air and viral infections (Romanet-Manent *et al.* 2002). For the majority of the patients, the onset starts during early childhood and is accompanied by a pattern of decreased lung function (Lemanske *et al.* 2010).

Although the onset is usually before the age of 4 years, there are several limitations in assessing the disease. For instance, wheezing can be assessed with or without a stethoscope while airway obstruction can be indirectly measured by detailed lung function testing (which is reliable to perform usually from the age of 5 years on), however there are scarce ways of imaging the airways and lungs (von Mutius, 2009). Furthermore, one of the main features of airway obstruction in asthmatics is its transiency. Although peak flow variability provides valuable information, there are no means to study the airway size and tone, particularly in small children (von Mutius, 2009). Thus this represents a holdup in the study of asthma at very young ages (Frey *et al.* 2009; Depner *et al.* 2013, submitted).

Studies have shown the presence of cellular infiltrates in the lung periphery of inflammatory cells in cases of fatal asthma (Saetta *et al.* 1991; Synek *et al.* 1996). Also the density of these cell infiltrates has been linked to peripheral airway obstruction (Hayley *et al.*1998).

1.1.2.3 Allergic rhinitis

Allergic rhinitis affects an estimate of 1.4 billion people worldwide and following the same pattern of the two other aforementioned allergic diseases, it continues to rise. The symptoms of this disorder comprise itching of the nasal area, inner ear, soft palate, nasal congestion and rhinorrhea in response to airborne allergens. The most common causal allergens are pollen, dust mites and dog or cat dander (Uzzaman *et al.* 2012; Skoner, 2001).

Furthermore, allergic rhinitis is often associated with other conditions such as sinusitis, otitis media, nasal polyps and asthma.

The features of allergic rhinitis result from infiltration of inflammatory cells in the nasal mucosa and mediators modulated by specific Immunoglobulin E (IgE) production in sensitized individuals (Settipane *et al.* 2013).

1.1.2.4 Anaphylaxis

This form of allergic disease is a severe systemic reaction with an acute-onset. The main triggers are food, drugs or insect stings. Although the symptoms can be mild in some cases, it can be life-threatening in a matter of minutes (Simons, 2008).

In the literature anaphylaxis is also referred to as a syndrome and not as a “disease” with varied clinical manifestations such as: hypotension, cardiac arrhythmias, laryngeal oedema, bronchospasm, diffused erythema and pruritus (Kemp *et al.* 2002).

1.2 ROLE OF T CELL LYMPHOCYTES IN ALLERGIC DISEASES

There are different immune cells, such as dendritic cells, mast cells, eosinophils, lymphocytes and other subsets involved in allergies. T lymphocytes (Th1, Th2 and regulatory T cells) are the focus in the present work.

1.2.1 Th1 and Th2 cells

Lymphocytes constitute of different effector T cells. These cells secrete different pattern of cytokines, preferentially express different markers and characteristic transcription factors, respond differently to stimulation and induce different immune responses.

Interferon-gamma (IFN-γ) is the Th1 hallmark cytokine; Th1 cells also produce interleukin (IL)-2 and tumor necrosis factor-beta (TNF-β) which are not produced by Th2 cells. Th1 cytokines are important for elimination of intracellular pathogens (Berger, 2000; Rengarajan *et al.* 2000) but also in the developmental phase of allergies. Th2 but not Th1 cells, produce the characteristic interleukins IL-4, IL-5, IL-13 and IL-10, which are important for immunity against helminthic infections. Both Th1 and Th2 secrete IL-3, IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-alpha, TNF-α (Del Prete, 1992).

Th1 and Th2 can counter-balance each other. Dysregulation of these cell subtypes is known as the Th1/Th2 paradigm (Berger, 2000; Rengarajan *et al.* 2000).

Apart from the classical Th1 and Th2 cells, other effector cell types have been identified: Th17 and Th9 cells, which have characteristic cytokine profiles and transcription factors.

Figure 1 shows the main transcription factors and cytokines secreted by different T cell population.

Th1 and Th2 cells are as well regulated differently and have characteristic transcriptional regulation. T-box expressed in T cells (T-bet, encoded by the *TBX21* gene) is an important transcription factor (TF) of the Th1 lineage. Additionally, it was shown that T-bet interacts closely with another transcription factor, H2.0-like *homeo* box 1, HLX1 (Mullen *et al.* 2002). Together they induce Th1 cell differentiation and suppress the Th2 cell lineage (Mullen *et al.* 2002). In contrast, Th2 cells are strongly regulated by two characteristic transcription factors: signal transducer and activator of transcription 6 (STAT6), which induces the transcription factor GATA-binding protein 3 (GATA3) (Zhu *et al*. 2010).

1.2.2 Regulatory T cells

Although other cell types have recently been shown to have suppressive capacity, regulatory T cells (Treg cells) have the main specialized role in suppressing cellular responses of other immune cells. This is crucial for the maintenance of peripheral homeostasis and for self and foreign tolerance. A large body of mouse and human studies has shown that impairment of these cells is associated with autoimmunity and allergic diseases (Sakaguchi, 2008).

Different Treg subsets have been described based on their phenotype and regulatory mechanisms. Naturally occurring Treg cells (nTreg cells) originate from the thymus and are characterized by the expression of CD4, high levels of CD25 (IL-2 receptor) and expression of Forkhead box protein 3–positive (FOXP3) (Vignali *et al.* 2008). *FOXP3* is known as the master gene regulating the natural Treg cells, encoding for the TF FOXP3.

Furthermore there are induced or adaptive Treg cells (iTreg cells), which acquire suppressive capacity under specific conditions in the periphery. Those include Tr1 cells that secrete the anti-inflammatory cytokine IL-10 and Tr3 cells that produce transforming growth factor beta (TGF-β) (Sakaguchi *et al.* 2010).

Different mechanisms of suppression have been proposed. Besides secretion of the inhibitory cytokines IL-10 and TGF-β, Treg cells can interfere with effector T cells by IL-2 consumption. Moreover they can cause cell apoptosis in a granzyme and perforin-dependent manner.

CTLA4 (Cytotoxic T lymphocyte antigen 4) was shown to be an important inhibitory molecule (Lin *et al.* 1998). Treg cells can also prevent effector T cell activation by inhibiting dendritic cells (DCs) by binding of CTLA-4 to CD80/CD86 (DC activation markers).

Another suppression mechanism is through, LAG3 (Lymphocyte-activation gene 3), selectively expressed on Tregs, binds to MHC class II on DCs leading to DC functional impairment (Vignali *et al.* 2008; Shevach, 2009; Josefowicz, 2012). Mice studies on LAG 3 have shown that antibodies to LAG-3 inhibit suppression by induced Tregs both *in vitro* and *in vivo* (Huang *et al.* 2004).

On the other hand GITR (glucocorticoid-induced tumour-necrosis-factor-receptor-related protein) is constitutively expressed in high levels in Tregs (Ephrem *et al.* 2013). Initially, it was proposed that the main effect of GITR was the dowregulation of Treg-cell activity, however new studies have shown that GITR is important for the stimulation of effector T cells (Shevach *et al.* 2006)

1.2.3 The relevance of Th1, Th2 and regulatory T cells in allergic diseases

In a first, sensitization phase, dendritic cells capture, process and present the allergen to naïve T cells which differentiate to Th2 cells. These allergen-specific Th2 cells then enter clonal expansion and produce pro-inflammatory cytokines which trigger the production of IgE by B cells. Allergen-specific IgE binds to mast cells and basophils. Simultaneously there is the development of antigen memory with the production of allergen-specific memory B and T

cells. The second, effector phase, occurs during repeated exposure to the same allergen. The allergen binds and cross-links the specific IgE displayed on sensitized mast cells and basophils, leading to their activation and secretion of immune mediators (Palomares *et al.* 2010). To counteract the exacerbate immune responses, Treg cells with inhibitory properties are expanded (Palomares *et al.* 2010; Barnes, 2008).

Th2 cells have the ability to produce (and to be activated by) IL-4 and IL-13 which influences the regulation of IgE production. Furthermore IL-5 production by Th2 cells is important for the recruitment and differentiation of eosinophils (Maggi, 1998). Studies have shown more Th2 cells in the airways of asthmatics (Robinson *et al.* 1992; Bentley *et al.* 1992) as well as increased IL-4, IL-5, IL-13 and IL-10 levels in the skin of atopic dermatitis patients and a positive association of IL-5 and IL-13 with IgE levels in those patients (Jeong *et al.* 2003).

Due to their importance in inflammatory processes, Th2 imbalance promoted by genetic alterations was shown to be a relevant element for the study of allergic diseases. Two transcription factors are important in Th2 and IgE regulation, GATA3 and STAT6 (Figure 1). In a mouse model, *STAT6* knock-out was shown to result in reduced IL-4 mediated functions, such as Th2-differentiation and IgE class switching (Takeda *et al.* 1996; Shimoda *et al.* 1996). Human studies revealed that genetic changes like single nucleotide polymorphisms (SNPs) in the *STAT6* gene were associated with IgE levels in different populations of children and adults (Schedel *et al.* 2004, Duetsch *et al.* 2002; Weidinger *et al.* 2004; Weidinger *et al.* 2008).

Th1 cells also play an important role in allergic diseases as IFN-γ suppresses Th2 cells. Decreased IFN-γ in neonates was associated with a higher risk of developing atopic dermatitis during the first 2 years of life (Herberth *et al.* 2010).

TBX21 and HLX1 play a major role in controlling Th1 lineage (Figure 1) and polymorphisms in these transcription factors were reported to affect asthma risk in children (Suttner *et al.* 2009; Suttner *et al.* 2009).

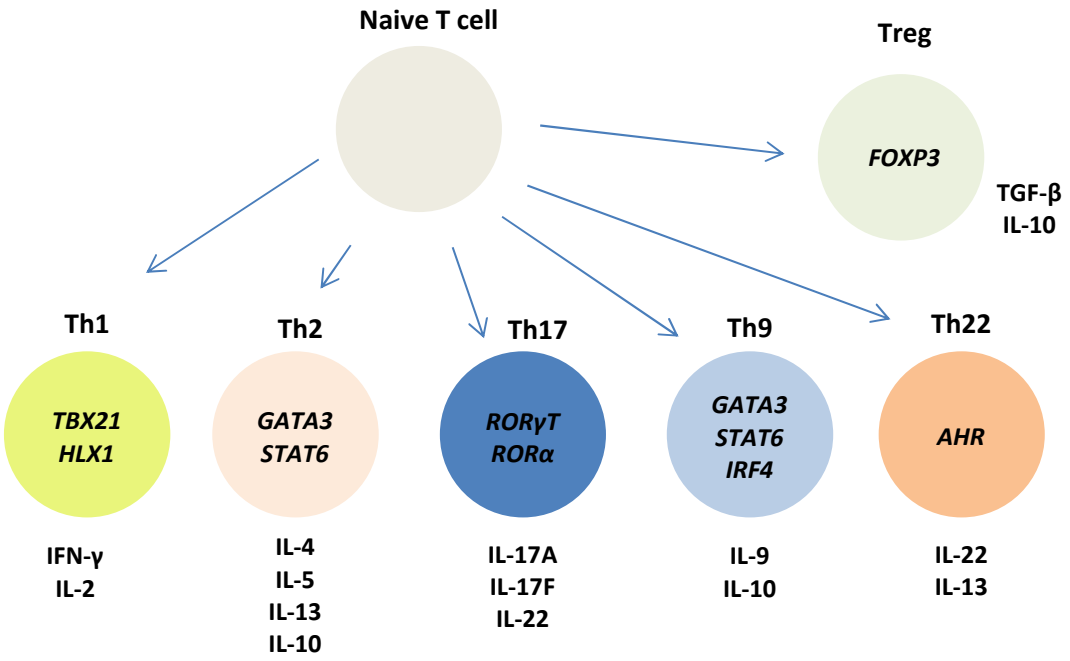


Figure 1. T cells are characterized by their transcription factors as well as different cytokine secretion patterns.

Due to their ability to control effector T cell responses, Treg cells play a major role in the development and maintenance of autoimmune and allergic diseases. Their importance has been confirmed by mouse studies, reporting that depletion of Treg cells during the priming phase of an active immune response led to a dramatic exacerbation of allergic airway inflammation (Baru *et al.* 2010) and worsened allergic airway hyperreactivity (Suto *et al.* 2001).

Treg cells have been shown to be impaired in pediatric asthma (Hartl *et al.* 2007) and in children with egg allergy (Smith *et al.* 2008). Furthermore, neonates from atopic mothers (a risk factor for atopic diseases) showed decreased Treg cell numbers and function at birth (Schaub *et al.* 2008).

1.3 THE ROLE OF ENVIRONMENTAL AND GENETIC FACTORS IN ALLERGIC DISEASES

1.3.1 Impact of the environment on allergic diseases

In the last decades, in parallel to the increasing number of people suffering from allergic diseases, major changes have been observed in lifestyle, particularly in industrialized countries (Masoli *et al.* 2004; von Mutius, 2000).

The relationship between a "traditional" lifestyle and lower prevalence of allergies and asthma has been consistently demonstrated in several epidemiological studies in different countries and both in children and adults (von Mutius, 2008).

Children from farming households in areas of Austria, Germany and Switzerland were shown to have lower prevalence of hay fever, atopic sensitization and atopic asthma (Braun-Fahrländer *et al.* 2002). In accordance, it was shown in Australia that children who have lived on a farm were less prone to develop atopy (Downs *et al.* 2001).

Identification of specific exposures such as endotoxin levels (Braun-Fahrländer *et al.* 2002), contact to different animal species (Remes *et al*. 2003, Ege *et al.* 2006), consumption of farm milk (Perkin, 2006; Riedler *et al.* 2001; Loss *et al.* 2011; Waser *et al.* 2007) and microbial diversity (Ege *et al.* 2011) have been shown to be particularly important for the allergy protective effect (Lluis, Schaub, 2012).

Involvement of innate receptors, CD14 and TLR2 (Launer, 2002) and of regulatory T cells (Schaub *et al.* 2009) have been demonstrated. However the exact mechanisms of how the environment changes allergic disease susceptibility are still unknown.

Furthermore, it seems that the modulation of the immune system through external exposures may start already *in utero* (Douwes *et al.* 2008; Schaub *et al*. 2009, Ege *et al.* 2006).

This concept of rural and farm environments providing protection against allergic disease development is in sync with the theory of the "hygiene hypothesis". This concept was proposed in 1989 by Strachan (Strachan, 1989) and described that family size was inversely related to allergic diseases. This finding led to the speculation that lower allergy prevalence could be related to a higher rate of infections transmitted by the older siblings.

The idea then extended to environmental factors like higher intake of antibiotics and vaccination, better hygiene, sew system, clean water (Bloomfield *et al.* 2006; Weiss, 2002)

1.3.2 The influence of genetics for disease predisposition

Despite the understanding of the environmental protective factors for the development of allergic diseases, it is important to consider that not all children living under a "risk" or "protective" environmental conditions will develop or not develop allergic diseases. Allergic diseases are a complex result from the interaction of individual genetic susceptibility and environmental exposures.

Large genetic studies such as Genome-wide association study (GWAS) have been extremely valuable in identifying susceptibility *loci* for allergic diseases (Tamari *et al.* 2013). In this context, more functional assays and pathway studies are still needed to understand the underlying pathophysiological mechanisms (Tamari *et al.* 2013, Vercelli, 2008; Holloway *et al.* 2010).

According to Vercelli, there are four main groups of genes associated with asthma, the ones associated with (Vercelli, 2008):

- Innate immunity and immunoregulation
- Th2 differentiation and effector functions
- Epithelial biology and mucosal immunity
- Lung function, airway remodeling and disease severity

In regards to Th2 differentiation, polymorphisms in *GATA3*, *TBX21*, *IL-4*, *IL4RA35*, *STAT6* and *IL12B* have been associated with asthma and allergy (Vercelli, 2008). It is important to note that Th2-related polymorphisms also influence further pathways such as IgE amplification, dependent on Th2 cytokines expressed by basophils and mast cells (Vercelli, 2008).

A focus on gene-environment interactions is also necessary, using both *in vitro* and *in vivo* models and epidemiological studies.

1.4 AIM OF THE STUDY

Early life immune modulation is an extremely relevant window period for the development of allergic diseases. Important Th1 and Th2-associated genetic alterations have been associated with disease development in German school-age (9-11 years) children (Schedel *et al.* 2004, Suttner *et al.* 2009, Suttner *et al.* 2009). Nevertheless, up to date, no studies were conducted to examine whether those genetic changes can already modulate the immune system at birth, and how that relates to early allergic disease development during the first years of life. Moreover it is known that farm exposures influence regulatory T cells at birth (Schaub *et al*. 2009) and early childhood (4.5 years) (Lluis, Depner *et al.* 2013), still no data are available in regards to the effect of farm on regulatory T cells with continuous immune maturation.

This work aimed to investigate whether:

- Th1 and Th2 polymorphisms which were previously associated with the development of allergic diseases in German school-age children, are associated with changes with immune responses already at birth – (cord blood study)
- Neonatal immune responses modulated by polymorphisms are associated with allergic and respiratory disease development during the first 3 years of life
- Regulatory T cells are modulated (in number and suppressive capacity) by farm-associated exposures such as contact to hay, to stables, farm milk consumption in children at age of 6 years
- Regulatory T cells participate in the protective farm effect against the development of allergic diseases at age 6 years

2. Materials and Methods

2.1 MATERIALS

Reagents and chemicals

100bp ladder (500µg/mL)	New England BioLabs, Ipswich, USA
ACK Lysis Buffer	Cambrex, East Rutherford, USA
Anti-human CD3-PE antibody	Beckmann Coulter, Fullerton, USA
Anti-human CD4-FITC antibody	BD Pharmingen, Franklin Lakes, USA
Anti-human CD4-FITC antibody	Beckmann Coulter, Fullerton, USA
Anti-human CD25-APC antibody	BD Pharmingen, Franklin Lakes, USA
Anti-human CD25-PC5 antibody	Beckmann Coulter, Fullerton, USA
Anti-human CD127-PE antibody	eBioscience, San Diego, USA
Anti-human FOXP3-PE antibody	BD Pharmingen, Franklin Lakes, USA
Anti-human FOXP3-PE antibody	eBioscience, San Diego, USA
Anti-human IgG1-FITC antibody	Dako Cytomation, Glostrup, Denmark
Anti-human IgG1-PE antibody	BD Pharmingen, Franklin Lakes, USA
Anti-human IgG1-PE antibody	Dako Cytomation, Glostrup, Denmark
Anti-human IgG2a-PC5 antibody	Beckmann Coulter, Fullerton, USA
Anti-human IgG2a-PE antibody	eBioscience, San Diego, USA
Bioplex Sheath Fluid	Biorad, Hercules, USA
Boric acid	Sigma-Aldrich, Steinheim, Germany
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Steinheim, Germany
Bromophenol blue	Roth, Karlsruhe, Germany
D. pteronyssinus allergen 1 (Derp1)	Indoor Biotechnologies, Charlottesville, USA
Ethanol 100 %	Merck, Darmstadt, Germany
Ethidiumbromide (10mg/mL)	Biorad, Hercules, USA
Ethylene diamine tetraacetic acid EDTA (0.5M)	Sigma-Aldrich, Steinheim, Germany
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	eBioscience, San Diego, USA
Fixation/Permeabilization Diluent	eBioscience, San Diego, USA
Fluorescein Calibration Dye	Biorad, Hercules, USA
Fetal Bovine Serum Gold (FCS)	PAA Laboratories GmbH, Pasching, Austria
Glycerol	Sigma-Aldrich, Steinheim, Germany
H_2O bidest	H. Kerndl GmbH, Weißenfeld, Germany
Human serum	Sigma-Aldrich, Steinheim, Germany
Ionomycin	Sigma-Aldrich, Steinheim, Germany
Isopropanol 100 %	Merck, Darmstadt, Germany
LiChrosolv H_2O (HPLC)	Merck, Darmstadt, Germany
Liquemin N 7500 (Na-Hep)	Roche Diagnostics, Mannheim, Germany
Lipid A	Sigma-Aldrich, Steinheim, Germany
Lipopolysaccharide	Sigma-Aldrich, Steinheim, Germany
Nuclease-free water	Ambion, Austin, USA
Paraformaldehyde	Sigma-Aldrich, Steinheim, Germany
PBS with EDTA (2mM)	Apotheke Innenstadt Uni München, Munich, Germany
Penicillin/Streptomycin	Gibco, Carlsbad, USA
Peptidoglycan	Sigma-Aldrich, Steinheim, Germany
Permeabilization Buffer (10X)	eBioscience, San Diego, USA
Phosphate-Buffered Saline (PBS)	Gibco, Carlsbad, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Steinheim, Germany
Phytohemagglutinin	Sigma-Aldrich, Steinheim, Germany

Primers	Invitrogen, Carlsbad, USA
Rat serum	eBioscience, San Diego, USA
RPMI 1640 + GlutaMax	Gibco, Carlsbad, USA
Sodium hydroxide (NaOH)	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
Water, Mol Bio grade	5 Prime, Gaithersburg, USA

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
5.5 % BSA	5.5g Bovine Serum Albumin 100mL PBS with EDTA (2mM)
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 ladder 80µl 0.5x TBE-Buffer 10µl Loading Dye
Ethidiumbromide (500µg/mL)	100µl Ethidiumbromide 1900µl dH_2O
FACS buffer	25mL 10X PBS Ad 250mL LiChrosolv H_2O 12.5mL FCS (5 %) 1.25mL Tween 20 (0.5 %)
Isolation buffer (0.55% BSA)	40mL PBS with EDTA (2mM) 4mL 5.5 % BSA
Loading dye diluted solution	5mL Loading dye stock solution 13.5mL glycerol 31.5mL dH_2O
Loading dye stock solution	0.25g bromophenol blue 0.25g xylene cyanol 30 % glycerol 70mL dH_2O
Medium 10 % human serum	440mL RPMI 1640 + GlutaMAX 10mL Penicillin/Streptomycin 50mL inactivated human serum

Reagent systems (Kits)

Allergy Screen test panel for atopy	Mediwiss Analytic, Moers, Germany
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
Human FoxP3 Buffer Set	BD Pharmingen, Franklin Lakes, USA
Roche LightCycler® 480 Probes Master	Roche Diagnostics, Mannheim, Germany
SYBR Green PCR Master Mix	Applied Biosystems, Foster City, USA

Consumables

Filter Mat Glass Fiber Paper	Skatron Instruments, Lier, Norway
iQ™ 96-Well PCR Plates	Biorad, Hercules, USA
Biosphere® filter tips 10µL M 40mm type D	Sarstedt, Nümbrecht, Germany
Biosphere® filter tips 100µL	Sarstedt, Nümbrecht, Germany
LD columns	Miltenyi Biotec, Bergisch Gladbach, Germany
LS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Microtest™ 96 well plates	BD Biosciences, Heidelberg, Germany
MS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Multiwell™ 6 well plates	BD Biosciences, Heidelberg, Germany
SafeGuard Filter tips 100-1000µL	Peqlab, Erlangen, Germany
Serum-Gel S-Monovette	Sarstedt Nümbrecht, Germany

Additional laboratory equipment

Centrifuge 5810 R / 5417 R / 5415 R	Eppendorf, Hamburg, Germany
Centrifuge Rotanta 460R / S	Hettich, Tuttlingen, Germany
Combi cell harvester	Skatron Instruments, Lier, Norway
Electrophoresis Power Supply	VWR International, Radnor, USA
FACS MoFlo XDP	Beckman Coulter, Fullerton, USA
FACSCalibur	Becton-Dickinson, Heidelberg, Germany
FACSCanto II	Becton-Dickinson, Heidelberg, Germany
Gel iX Imager	Intas Science Images Instruments, Göttingen, Germany
iCycler iQ™ Real Time PCR Detection System	Biorad, Hercules, USA
Incubator Hera Cell 240	Hereus, 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
Microplate shaker Type Rotamax 120	Heidolph Instruments, Schwabach, Germany
Microscope Axiovert 40C	Zeiss, Göttingen, Germany
Microwave	Siemens, Munich, Germany
MidiMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach, Germany

MiniMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Neubauer cell counter	Karl Hecht KG Assistent, Sondheim, Germany
Owl D3-14 Wide Gel System	Thermo Scientific, Waltham, USA
Roche LightCycler 480 System	Roche Diagnostics, Mannheim, Germany
Sterile Hood LaminAir HBB2472	Heraeus, Hanau, Germany
Thermocycler Eppendorf Mastercycler	Eppendorf, Hamburg, Germany
Vacuum regulator	Biorad, Hercules, USA
Vortex Genie 2	Scientific Industries, Bohemia, USA
Waterbath Köttermann	Köttermann GmbH, Uelze, Germany

Software

Adobe Photoshop	Adobe Systems, Edinburgh, UK
Bio-plex Manager Software 4.1	Biorad, Hercules, USA
Cell Quest Software	Becton-Dickinson, Heidelberg, Germany
Cell Quest Pro Software	Becton-Dickinson, Heidelberg, Germany
EndNote X3/X4	ISI ResearchSoft, Berkeley, USA
Ensembl Genome Browser	http://www.ensembl.org/
FACSDiva software	Becton-Dickinson, Heidelberg, Germany
FCS express v3/v4	De Novo Software, Los Angeles, USA
Haploview	http://www.broad.mit.edu/mpg/haploview/
iCycler iQ Optical System Software v3.1	Biorad, Hercules, USA
Microsoft Office	Microsoft, Redmont, USA
National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
Sigmastat version 1.0	Systat Software Inc., Chicago, USA
SPSS version 20	SPSS IBM Inc., Armong, USA
WinMDI 2.8	The Scripps Research Institute, La Jolla, USA

2.2 METHODS

2.2.1 Cord blood studies

2.2.1.1 Study Population

Between July 2005 to September 2007 pregnant women were approached for the study's recruitment during their last trimester of pregnancy. The recruitment took place in the Munich metropolitan area, Germany.

Participants were excluded in case of preterm deliveries, multiple gestations, perinatal infections, fever around birth, maternal intake of medication and maternal chronic diseases (Schaub *et al.* 2009; Schaub *et al.* 2008; Casaca *et al*. 2012, Casaca *et al*. 2013). A total of 200 neonates took part in the study.

All mothers answered comprehensive questionnaires which included assessment of potential covariates such as sex, smoking, birth characteristics, race/ethnicity, siblings, education, previous cesarean section and miscarriage. Written informed consent was obtained from all mothers. Ethical approval for the study was obtained from the local human research committee of the Bavarian Ethical Board, LMU Munich, Germany (Casaca *et al.* 2013).

2.2.1.2 Definition of clinical phenotypes

Doctor's diagnosis of asthma and/or eczema and/or hay fever was documented by personal interview and defined the maternal atopic status. Furthermore, maternal sera were used to measure total and specific maternal IgE levels by RAST (radioallergosorbent test). A positive specific IgE was defined as 1 or more positive reactions $\geq$ 0.35 IU/ml to a panel of common allergens: aeroallergens (mites, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, birch pollen, hazelnut pollen, timothy grass, mugwort, plantain), animals (cat, horse,

dog), *Alternaria*, and food including egg white, milk protein, peanut, hazelnut, carrot, wheat flour and soy as previously described by Schaub *et al*. (Schaub *et al.* 2008).

From the mothers defined as atopic, 82.6 % were confirmed to have a positive RAST test, while non-atopic mothers had no atopic disease and sensitization levels comparable to previous epidemiological studies among non-diseased subjects (Heinrich *et al.* 2002).

When the children turned 3 years, parents completed detailed questionnaires assessing allergic and respiratory diseases: obstructive bronchitis was defined as a doctor's diagnosis of asthma ever or a repeated diagnosis of obstructive bronchitis; food allergy as clinical symptoms or as doctor's diagnosis of food allergy; atopic dermatitis (AD) as doctor's diagnosis of AD and wheeze was defined by airway obstructive symptoms from birth until the age of 3 years.

2.2.1.3 Cell isolation and culture

Umbilical blood was collected immediately after delivery. Blood was collected into sodium heparin or EDTA tubes. Within 24 h after delivery, EDTA tubes were frozen until DNA extraction. DNA was extracted using the FlexiGene DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Sodium heparin tubes were processed within 24 h for isolation of cord blood mononuclear cells (CBMCs). CBMCs were isolated by density gradient Ficoll-Hypaque^{TM} PLUS (GE Healthcare, Piscatay, USA). Cells were stimulated for 3 days with Lipid A (LpA, 0.1μg/ml), Peptidoglycan (Ppg, 10μg/ml), the allergen house dust mite (Derp1, 30μg/ml), a combination of Derp1 and LpA (D+L) or cultured without stimulation. After 3 days cells were collected for mRNA extraction, cytokine measurement and flow cytometry.

2.2.1.4 Cytokine secretion

Supernatants were used for measurement of cytokines including: IFN-γ, IL-5, IL-6, IL-13, GM-CSF and TNF-α. Cytokines were measured by Human Cytokine-Multiplex-Assay-Kit

(Bio-Rad, Munich, Germany) according to the manufacturer's instructions. The limits of detection (pg/ml) were as follows: 1.3 (IFN-γ), 1.8 (IL-5), 0.5 (IL-6), 2.1 (IL-13), 1.0 (GM-CSF) and 3.0 (TNF-α).

2.2.1.5 Flow cytometry

For the assessment of activated T cells ($CD4^+CD25^+$) a 3-color flow cytometer (FACScan; BD Biosciences, Heidelberg, Germany) was used. Cells were incubated with 2 µl of anti-human CD4–fluorescein isothiocyanate (FITC) and 1 µl of CD25–RPE-Cy5 (Dako Cytomation, Glostrup, Denmark). For isotype control 1 µl of IgG1-FITC (Dako Cytomation) and 0.5 µl of IgG2a RPE-Cy5 (BD Biosciences) were used (Schaub *et al.* 2009). The flow cytometry data were analyzed using CellQuest software (BD Biosciences) and post-acquisition analysis was performed with WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA).

2.2.1.6 Polymorphisms' selection and genotyping

The Th1 and Th2 cell lineage polymorphisms selected for this study were based on previous reports that showed their putative functional importance and an association with disease development or IgE levels in German children (9-11 years old) and other study populations (Suttner *et al.* 2009; Suttner *et al.* 2009; Schedel *et al.* 2004; Schedel *et al.* 2009; Weidinger *et al.* 2004).

The following polymorphisms were chosen: Th2 *STAT6* rs1059513 and rs324011 and Th1 *TBX21* rs17250932 and rs11079788 and *HLX1* rs2738751, rs3806325 and rs12141189.

The genotypes of *STAT6*, *TBX21* and *HLX1* were determined by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Sequenom Inc.,

San Diego, CA, USA). SpectroDESIGNER software (Sequenom Inc.) was used to design polymerase chain reaction assays and associated extension reactions. Amplification and extension reaction conditions were previously described (Schedel *et al.* 2004; Casaca *et al.* 2013).

2.2.1.7 Quantitative Real-Time-PCR

Total RNA was isolated with TRI reagent from CBMCs after 72 h of incubation with LpA, Ppg, Derp1, D+L or from unstimulated cells. RNA was reverse transcribed into cDNA following manufacturer's instructions (Invitrogen, Karlsruhe, Germany).

Forward and reverse primers for the housekeeping gene *18S*rRNA, Treg-related *FOXP3*, *LAG3*, *GITR*, Th2-related *STAT6*, *STAT6d*, *STAT6e*, *GATA3*, Th1-related *TBX21*, *HLX1*, *IRF1*, Th17-related *IL-22* and Th9-related *IL-9* genes were designed with Vector NTI advance 10 (Invitrogen, Karlsruhe, Germany). Gene expression was measured by the increase in fluorescence caused by the binding of SYBR Green to double stranded DNA. Normalization of the values was done by subtracting the corresponding *18S* RNA threshold cycle (Ct) value from the Ct of the gene of interest: ΔCt = Ct (gene of interest) - Ct (housekeeping gene). Higher ΔCt represents a lower gene expression and vice-versa (Casaca *et al.* 2012; Casaca *et al.* 2013). The housekeeping gene was previously by lab work as it was shown to be have a stable profile independent of stimulation. All primers were tested for specificity.

2.2.1.8 Statistical analyses

Data were generally reported in 3 group comparisons including the health outcomes in the 3 year follow-up. The non-parametric Kruskal-Wallis test was applied to analyze cytokine secretion as data were generally not normally distributed and could not be transformed to normality. The value of 0.01 was assigned to undetectable cytokine concentrations so that the

undetectable values could be included in the analysis. Gene expression data were also analyzed with Kruskal-Wallis test. Immunological data were shown as medians, first and third quartiles.

Results were further analyzed using genetic models, recessive or dominant models, and differences were tested with Wilcoxon test. Dominant model compares wildtype *vs* heterozygous and SNP homozygous and the recessive model compares SNP homozygous *vs* heterozygous and wildtype.

Correlation of gene expression and cytokine secretion were assessed by Spearman correlation coefficients. Differences were considered significant with $p \leq 0.05$ or as borderline significant with $p \leq 0.1$.

As expression of the Treg-related gene markers *FOXP3*, *GITR* and *LAG3* was correlated with each other and also pro-inflammatory and Th2 cytokines were correlated, data were not adjusted for multiple testing.

Mantel–Haenszel test was used to test differences in the health outcomes between the groups.

In total 200 children participated in the study; however the number for single analyses varied due to sample availability or non-participation in the follow up at age 3 years.

Statistical analyses were performed by SAS (version 9.2, SAS Institute, Cary, NC, USA).

(Casaca *et al.* 2013).

Of note: The cord blood samples were collected between 2005 and 2007 and processed freshly. During my thesis the samples had been measured and my worked was focused in gathering the data and analysis. However for the reader's understanding, the lab techniques were described in the Methods.

2.2.2 PASTURE/EFRAIM study

2.2.2.1 Study population

The PASTURE (Protection against allergy: study in rural environments) study and the follow-up study EFRAIM (Mechanisms of Early Protective Exposures on Allergy Development) is a large international birth cohort study that includes children from 5 European countries: Austria, Finland, France, Germany and Switzerland (von Mutius *et al.* 2006) to investigate the protective factors against the development of allergies in early life.

The PASTURE/EFRAIM cohort comprises more than 1000 children. For the investigation of this thesis a subgroup of 143 German children with available Treg cell data were followed until age 6 years.

Initially, women who lived in rural areas were recruited: women who lived on family-run livestock farms were considered as farming mothers and women not living on a farm, but from the same rural areas as non-farming mothers. The study was approved by the local research ethics committee and informed consent was obtained from all parents (von Mutius *et al.* 2006).

2.2.2.2 Assessment of farm exposures

Parents completed several questionnaires overtime: when the children were 2, 12, 18, 24, 36, 48 months, 4.5 and 6 years.

The questions were based on previous studies: the ALEX (Allergy and Endotoxin Study) (Riedler *et al.* 2001), the AMICS (Asthma Multicenter Infants Cohort Study) (Basagana *et al.* 2002), the PARSIFAL study (Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle) (Alfven *et al.* 2006) and questions derived from the ATS (American Thoracic Society) questionnaire (Ferris, 1978). The questionnaires

inquired about the general health of the child and family and detailed farm exposures. A child was defined as farm child if he/she lived on a farm.

Farm milk consumption was defined if the child drank any farm milk within the last 12 months prior to the questionnaire or whether the mother drank farm milk during pregnancy. Contact to hay was defined by children who had regular contact to hay, at least once a week. Stable exposure defined children which regularly spent time in the stable of their own or another farm. Both exposures were assessed in regards to the last 12 months. A single contact to the stable was enough to classify them as children with stable contact (Lluis, Depner, *et al.* 2013).

2.2.2.3 Clinical phenotypes

The asthmatic group at age 6 years included children that had a life time prevalence of a doctor's diagnosis of asthma ever and/or a repeated diagnosis of obstructive bronchitis and/or children which are intermediate, late onset and persistent wheezers after the first 6 years and/or children with wheeze without a cold and symptoms between wheeze from age 18 months to age 6 years. Current asthma defines children with a doctor diagnosis of asthma ever and any wheeze episodes in the last year.

Specific IgE was measured in sera at age 6 years of the children to assess allergic sensitization. Allergens tested included: food allergens, hen's egg, cow's milk, peanut, hazelnut, carrot and wheat flour; and inhalant allergens, *D. pteronyssius*, *D. farinae*, cat, horse, dog, *Alternaria*, mugwort, plantain, alder, birch pollen, hazel pollen, rye pollen, and a grass pollen mix.

Sensitization against food or inhalant IgE were assessed using three RAST class cut-offs:

Class I $\geq$ 0.35 IU/ml; Class II $\geq$ 0.7 IU/ml and Class III $\geq$ 3.5 IU/ml.

2.2.2.4 Isolation of peripheral blood mononuclear cells

Peripheral blood was collected in sodium heparin tubes and peripheral blood mononuclear cells (PBMCs) were isolated within 24 hours using density gradient centrifugation (Ficoll-Paque™ PLUS). Cells were washed with RPMI 1640 + GlutaMax and counted.

2.2.2.5 Culture for Treg cell frequency assessment

PBMCs were set to the final concentration of $5x10^6$cells/ml in RPMI with 10 % human serum and incubated for 24 h, at 37°C with 5 % CO_2 with no addition of stimulus (control - media) or with PMA (5 ng/ml) and Ionomycin (1 µg/ml) or lipopolysaccharide (Lps, 0.1 µg/ml). After 24 h cells were washed and stained for flow cytometry

2.2.2.6 Treg frequency assessment

For Treg cell frequency assessment, cells were harvested after 24 h and diluted in 5 ml RPMI for counting. Cells were resuspended at final concentration of $1x10^7$ cells/ml in RPMI and incubated with 8 µl of CD4-FITC, 4 µl of CD25-PE for surface staining markers. The cells were then fixed and permeabilized (Human FoxP3 Buffer Set, BD Pharmingen, Franklin Lakes, USA) before addition of 5 µl of FOXP3-PE. The isotypes used were: IgG1 FITC, IgG2a PC5 and IgG2a-PE. Treg cells were defined as the CD4 positive cell expressing high levels of CD25 (upper top 20 %) and FOXP3 (figure 5).

2.2.2.7 Cell isolation for Treg functional assay

To isolate the different cell subtypes (antigens presenting cells, effector T cells and Treg cells) a magnetic bead system was used. PBMCs were resuspended in autoMACS® Running Buffer. Approximately $2x10^6$ cells were taken for CD3-marker isolation and the remaining was used for CD4 isolation. For CD3 isolation CD3 microbeads were added and incubated for 15 min at 4º C before separation in the autoMACS Pro Separator. $CD3^-$ cells (APCs) were

resuspended at the final concentration of $8x10^5$ cells/ml in RPMI with Human serum 10 % and irradiated at 30Gy (3000rad) for 10 min. $CD3^+$ fraction was used for isotype and compensation controls. For $CD4^+$ cell isolation 10 µL per $10x10^7$ cells of Biotin-Antibody Cocktail was added and mixed. 20 µL of Anti-Biotin MicroBeads were added following additional 15 min of incubation at 4 °C. $CD4^+$ cells were resuspended in 90 µL of buffer and used for effector and Treg cell isolation. 10 µL of CD25 MicroBeads were added and cells were incubated for 15 min at 4°C.

$CD4^+CD25^{+(high)}$ (Treg cells) were washed and resuspended at $4x10^5$ cells/ml in RPMI with Human serum 10 % and $CD4^+CD25^-$ (effector cells) were labelled with CD25 microbeads for a second round of isolation to increase the purity. The second $CD4^+CD25^-$ fraction was washed and resuspended in 1 ml of PBS/10 % FCS for CFSE staining.

After incubation with the antibodies, cells were washed and resuspended in FACS buffer and cell purity was measured by flow cytomery in FACSCanto II.

2.2.2.8 CFSE staining of $CD4^+CD25^-$ cells

For preparation of 5 mM CellTrace™ CFSE stock solution the content of component A (CellTrace™ CFSE) was dissolved in 18 µL of the DMSO provided (Component B). 5 µl of A+B were added to 5 ml of PBS/10 % FCS and 1 ml was taken and added to the cells. Cells were incubated for 5 min in the dark and then washed 3x (10 min) with PBS/10 % FCS. Cells were then resuspended in 2 ml of RPMI with 10 % Human serum for 1-2 h at 37 °C. Afterwards, cells were resuspended at $4x10^5$cells/ml in RPMI with Human serum 10 %.

2.2.2.9 Cell culture for Treg functional assay

For the determination of Treg suppressive capacity co-cultures were performed as follows: CFSE-treated $CD4^+CD25^-$ cells ($2x10^4$ cells/well) were incubated with irradiated $CD3^-$ cells ($4x10^4$ cells/well), with or without $CD4^+CD25^{high}$ T cells at the ratios $CD4^+CD25^-$/

$CD4^{+}CD25^{high}$, 1:0.5 or 1:0.25. For stimulation, PHA 0.8 µg/ml was used. Cells were incubated in a 96-round bottom well plate for 3 days at 37 °C with 5 % CO2. After culture cells were harvested and the supernatants collected for cytokine measurement.

2.2.2.10 Treg functional assay assessment

For Treg suppressive capacity, cells were harvested after 72 hours, counted and resuspended in 50 µl of FACS buffer. 2 µl of CD4-APC-H7 and 2 µl CD25-PE were added for 10 minutes. Cells were then washed and resuspended in FACS buffer for purity measurement in FACSCanto II. Immediately before measurement, 2 µl of PI were added for dead cell exclusion. Suppressive capacity was assessed by the division of CFSE-labelled effector cells (figure 6).

2.2.2.11 Cytokine concentrations

Cytokines were collected from the suppression assays cell cultures. The cytokines, IL-2, IL-5, IL-9, IL-10, IL-13 and IFN-γ were measured with 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: 4.25 (IL-2), 0.24 (IL-5), 0.21 (IL-9), 0.14 (IL-10), 0.17 (IL-13), 2.26 (IFN-γ) pg/ml.

2.2.2.12 Statistical analyses

Percentages of Treg cells were in relation to the lymphocyte gate defined through flow cytometry. Association of Treg cells and farm exposures and clinical outcomes was analysed with the nonparametric Wilcoxon test.

For Treg cell suppression data, before conducting statistical analysis, the data were log-transformed to achieve normality. Data were close to normality to allow analysis with parametric test. Cytokine data distribution below the respective cytokine detection limit was

imputed according to Lubin (Lubin *et al.* 2004). Subsequently, a linear mixed model with varying intercept and slope was applied. Statistical significance was defined by a p-value $\leq$ 0.05.

Analyses were performed using SAS 9.2 (SAS Institute, Cary, USA) and R (R Core Team, 2012).

3. Results

3.1 IMPACT OF T CELL POLYMORPHISMS ON CORD BLOOD IMMUNE MODULATION AND ASSOCIATION WITH EARLY DISEASE DEVELOPMENT

Understanding the modulation of immune responses at birth is crucial as this is an important period in shaping the immune system. In 200 neonates, polymorphisms in Th1 and Th2 transcription factors were investigated to assess their influence on neonatal immune responses and subsequent development of allergic and respiratory diseases during the first 3 years of life. Table I displays the study population characteristics.

Table I. Cord blood study: population characteristics.

Parameters	n	N
Female sex, n (%)	96 (48.0%)	200
Gestational age, weeks, median (Q1;Q3)	40.0 (39.1;40.7)	200
Maternal atopic diseases, n (%)	71 (35.5%)	200
Asthma, n (%)	14 (7.0%)	200
Hay fever, n (%)	52 (26.0%)	200
Atopic eczema, n (%)	16 (8.0%)	200
Maternal serum total IgE (IU/mL), median (Q1;Q3)	33.5 (13.1;87.3)	196
Maternal specific IgE >0.35 IU/mL, n (%)	83 (70.9%)	117
Vaginal delivery, n (%)	177 (88.5%)	200
Siblings ≥1, n (%)	89 (44.5%)	200
Clinical outcome up to age 3 years, n (%)		
Doctor's diagnosis atopic dermatitis	26 (13.0%)	200
Doctor's diagnosis food allergy	5 (2.5%)	200
Symptoms of food allergy	24 (12.0%)	200
Asthma/obstructive bronchitis	23 (11.5%)	200
Wheeze	75 (37.5%)	200

Q1 = 1st quartile; Q3 = 3rd quartile. Total N = 200, total IgE measurements were available in 196 mothers and specific IgE in 117 mothers (Casaca *et al.* 2013).

As shown in Table II, a total of 7 polymorphisms were investigated: two in the *STAT6* gene, two in *TBX21* and three in *HLX1*. The minor allele frequencies (MAF) were generally

comparable to the MAF of the ISAAC study (Schedel *et al.* 2004, Suttner *et al.* 2009, Suttner *et al.* 2009). None of the polymorphisms significantly deviated from the Hardy-Weinberg equilibrium (HWE).

Table II. Characteristics of the studied polymorphisms.

Gene	Rs number*	Position in relation to 1.ATG	Position in the gene structure	MAF (ISAAC†)	MAF (our cohort)	P value HWE	Call rate
STAT6	rs1059513	T12888C	3' UTR	0.12	0.10	0.46	98.56
STAT6	rs324011	C2892T	Intron 2	0.37	0.38	0.93	98.85
TBX21	rs17250932	T1514C	Promoter	0.16	0.22	0.60	97.14
TBX21	rs11079788	C9902T	Intron 3	0.22	0.30	0.83	97.14
HLX1	rs2738751	C1486G	Promoter	0.14	0.15	0.88	97.14
HLX1	rs3806325	C1407T	Promoter	0.19	0.18	0.47	97.62
HLX1	rs12141189	T346C	Exon 1	0.25	0.25	0.40	97.62

*SNP number according to database SNP (http://www.ncbi.nlm.nih.gov/snp).
†ISAAC, International Study of Asthma and Allergy in Childhood phase II (school-age German children).
MAF = Minor allele frequency, HWE = Hardy-Weinberg equilibrium.
(Casaca *et al*. 2013).

3.1.1 Modulation of neonatal immune responses by Th2 polymorphisms: *STAT6*

3.1.1.1 Downregulation of Treg cell-related genes at birth is associated in carriers of *STAT6* rs324011 polymorphism

Carriers of *STAT6* rs324011 had significantly decreased *LAG3* mRNA expression upon Derp1-stimulation (p = 0.053, Fig. 2C). This pattern of downregulation was similar for *FOXP3* and *GITR* mRNA-expression, although not significant: *FOXP3* (Ppg and Derp1-stimulated, p = 0.063/ 0.063, Fig. 2A), *GITR* (Ppg, p = 0.095, Fig. 1B). *LAG3* also showed a trend to lower expression in carriers of rs324011 (unstimulated, p = 0.095 and LpA p=0.077, Fig. 1C) (Casaca *et al.* 2013).

As shown in Table III, applying the recessive model, the differences of lower *FOXP3* (unstimulated, Ppg, Derp1), *GITR* (Ppg) and *LAG3*-expression (unstimulated, LpA, Derp1) between the homozygous neonates and the heterozygous and wildtype became significant ($p \leq 0.05$) in comparison to the 3 group comparison analyses (Figure 2).

No association was found for *STAT6* rs1059513 polymorphism with expression of Treg cell-related genes birth.

STAT6 rs324011 and rs1059513 SNPs were further studied to assess whether they affected the expression of other Th-related genes in cord blood, including *TBX21* and *HLX1* (Th1), *STAT6* and two *STAT6* isoforms (*STAT6d, STAT6e)* and *GATA3* (Th2), *IL-22* (Th22, Th17) and *IL-9*-expression (Th9), however no significant differences were found (Casaca *et al.* 2013).

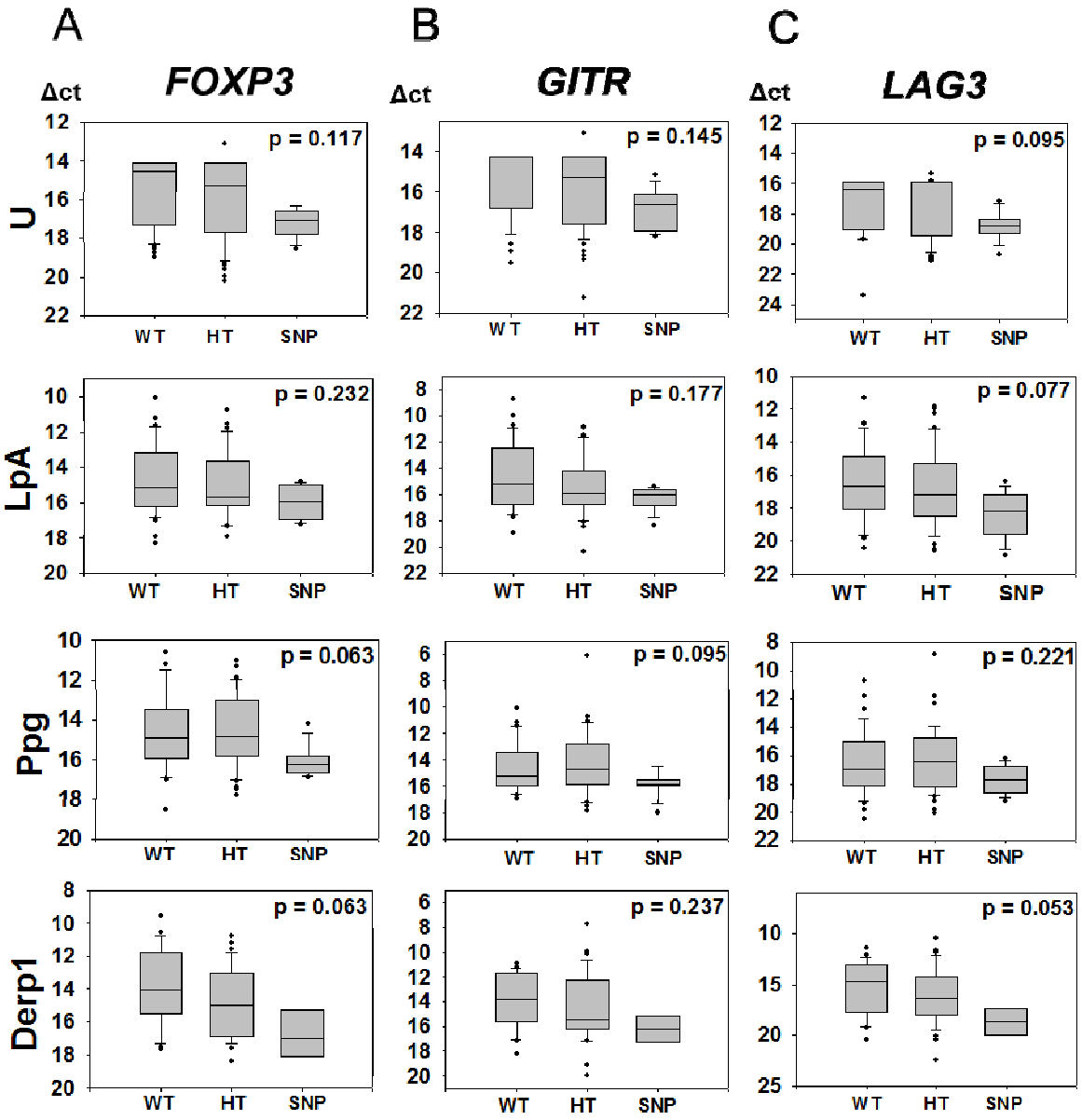


Figure 2. *STAT6* rs324011 allele associated with downregulation gene expression of Treg cell-related markers.

A) *FOXP3*; B) *GITR*; C) *LAG3*. Corresponding box plots represent mRNA expression in ΔCt (normalized with *18S*); higher Δct represents lower mRNA expression and *vice-versa*. Data were shown as medians, first and third quartile. WT = wildtype, HT = heterozygous, SNP = SNP homozygous, U = Unstimulated, LpA = Lipid A, Ppg = Peptidoglycan, Derp1 = *Dermatophagoides pteronyssinus*. Data were analyzed with Kruskal-Wallis test. Maximum number for gene expression analysis: *STAT6* rs324011 n (WT) = 43; n (HT) = 55; n (SNP) = 10. (Casaca *et al.* 2013).

Table III. Dominant and recessive models comparing results of expression of Treg-related genes depending on *STAT6* rs324011 genotypes.

Parameter Δct	**Stim.**	**WT**	**HT+SNP**	**P value* Dominant†**
Foxp3	U	14.52 (14.09-17.30)	16.30 (14.09-17.75)	0.245
	LpA	15.15 (13.45-16.20)	15.70 (14.60-16.30)	0.250
	Ppg	14.90 (13.45-15.90)	15.00 (13.20-16.20)	0.596
	Derp1	14.05 (11.80-15.50)	15.40 (13.10-16.90)	0.095
GITR	U	14.28 (14.28-16.85)	16.05 (14.28-17.63)	0.311
	LpA	15.18 (12.50-16.80)	16.00 (14.90-16.85)	0.121
	Ppg	15.25 (13.48-15.98)	15.05 (13.95-16.00)	0.877
	Derp1	13.80 (11.75-15.50)	15.50 (12.40-16.20)	0.227
LAG3	U	16.36 (15.93-19.05)	17.50 (15.93-19.40)	0.587
	LpA	16.70 (14.90-18.05)	17.65 (15.85-19.05)	0.233
	Ppg	16.92 (15.03-18.15)	16.80 (14.95-18.28)	0.960
	Derp1	14.75 (13.00-17.75)	16.90 (14.43-18.15)	0.100
Parameter Δct	**Stim.**	**WT+HT**	**SNP**	**P value* Recessive†**
Foxp3	U	14.95 (14.09-17.40)	17.08 (16.58-17.80)	**0.054**
	LpA	15.40 (13.43-16.15)	15.98 (15.00-16.93)	0.129
	Ppg	14.88 (13.10-15.83)	16.20 (15.80-16.63)	**0.018**
	Derp1	14.60 (13.00-16.60)	17.00 (15.30-18.10)	**0.050**
GITR	U	14.85 (14.28-17.50)	16.63 (16.10-17.90)	0.059
	LpA	15.50 (13.70-16.80)	16.00 (15.75-16.85)	0.156
	Ppg	15.05 (12.95-15.95)	15.88 (15.50-16.00)	**0.035**
	Derp1	15.00 (12.20-16.20)	16.25 (15.15-17.25)	0.163
LAG3	U	15.93 (15.93-19.13)	18.85 (18.35-19.30)	**0.030**
	LpA	16.90 (15.08-18.48)	18.20 (17.20-19.50)	**0.030**
	Ppg	16.75 (14.75-18.20)	17.73 (16.80-18.60)	0.095
	Derp1	15.60 (13.70-17.90)	18.65 (17.40-20.00)	**0.041**

Data presented as medians (first/third quartile). *P values calculated with non-parametric Wilcoxon test for group comparison of the medians. †Dominant model = wildtype *vs* heterozygous and SNP homozygous. Recessive model = SNP homozygous *vs* heterozygous and wildtype. WT = wildtype, HT = heterozygous, SNP

= SNP homozygous, U = unstimulated, LpA = Lipid A, Ppg = Peptidoglycan, Derp1 = *Dermatophagoides pteronyssinus*. ↑ = upregulation or ↓ = downregulation in the presence of the polymorphic allele. Maximum number for gene expression analysis: *STAT6* rs324011 n (WT) = 43; n (HT) = 55; n (SNP) =10. Significant values are marked in bold. (Casaca *et al.* 2013).

3.1.1.2 Changes in cytokine secretion are associated with *STAT6* polymorphisms

Next cytokine concentrations were assessed in relation to the different *STAT6* genotypes. As shown in Table IV, following LpA- and Derp1-stimulation, homozygous carries of *STAT6* rs1059513 had increased protein levels of TNF-α (p = 0.038/0.052) and of GM-CSF (p = 0.023/0.033).

Furthermore, neonates carrying the minor polymorphic allele of *STAT6* rs1059513showed increased IFN-γ secretion in unstimulated cells (p = 0.040) (although the expression was rather low) and after Ppg-stimulation (p = 0.081).

Lower TNF-α secretion in carriers of *STAT6* rs324011 after Derp1-stimulation (p = 0.044) and after LpA-stimulation (trend, p = 0.085) (Table IV) was found. No changes in GM-CSF expression were observed. Changes in Th2 cytokines were also detected: trend to lower IL-13 secretion after Derp1-stimulation (p = 0.063) and IL-5 after innate (LpA, p = 0.061) and allergen-stimulation (Derp1, p = 0.059), yet not significant.

On the other hand, IFN-γ (Th1) showed an upregulation in homozygous and heterozygous carriers of *STAT6* rs324011 polymorphism and after stimulation with LpA (p = 0.020), Ppg (p = 0.033) and Derp1 (p = 0.004) (Casaca *et al.* 2013).

Table IV. Cytokine secretion in cord blood in relation to *STAT6* rs1059513 and rs324011 polymorphisms.

Cytokine pg/ml	Stimuli	WT	HT	SNP	P value*
rs1059513					
TNF-α	U ↑	0.57 (0.01-1.16)	0.55 (0.01-1.35)	0.91 (0.01-9.13)	0.870
	LpA ↑	943.73 (357.01-1757.48)	1398.86 (423.04-2046.32)	4308.41 (2096.94-7875.38)	**0.038**
	Ppg ↑	2254.23 (1302.73-3422.19)	2072.61 (1313.43-3135.92)	6546.62 (2720.25-13075.70)	0.111
	Derp1 ↑	1024.73 (435.67-1896.18)	1100.52 (547.41-3060.25)	7028.02 (1729.37-13401.76)	**0.052**
GM-CSF	U ↑	0.01 (0.01-0.21)	0.01 (0.01-6.36)	2.78 (0.01-14.28)	**0.041**
	LpA ↑	2.19 (0.01-14.64)	6.42 (0.01-55.67)	295.54 (13.35-301.23)	**0.023**
	Ppg ↑	84.43 (6.07-364.82)	9.72 (0.01-194.80)	112.70 (18.04-781.61)	0.178
	Derp1 ↑	13.14 (0.01-105.23)	37.24 (1.42-275.90)	623.82 (85.25-649.46)	**0.033**
IL-13	U ↓	0.43 (0.24-0.78)	0.37 (0.06-0.84)	0.01 (0.01-0.15)	**0.045**
	LpA ↑	4.68 (1.87-12.34)	6.23 2.01-15.25	7.75 (7.70-7.92)	0.591
	Ppg ↑	26.80 (10.48-61.24)	25.39 (9.16-53.74)	27.36 (18.77-34.65)	0.720
	Derp1 ↑	10.12 (3.40-19.84)	10.22 (3.42-27.26)	18.00 (13.42-20.59)	0.498
IL-5	U ↑ ↓	0.10 (0.01-0.17)	0.10 (0.01-0.16)	0.01 (0.01-0.10)	0.441
	LpA ↑	6.31 (2.64-17.20)	8.23 (3.22-14.60)	9.47 (1.49-47.03)	0.675
	Ppg ↑	28.94 (11.82-67.65)	25.11 (5.28-44.41)	30.87 (1.79-74.45)	0.514
	Derp1 ↑	10.73 (4.71-22.59)	9.29 (6.29-22.43)	15.26 (2.70-106.90)	0.833
IFN-γ	U ↑	0.01 (0.01-1.01)	0.13 (0.01-2.49)	2.53 (0.01-3.18)	**0.040**
	LpA ↓	43.84 (6.18-98.95)	24.99 (3.72-76.65)	17.29 (13.78-45.23)	0.514
	Ppg ↓	49.64 (15.98-130.31)	23.80 (4.96-100.08)	32.95 (25.26-61.60)	0.081
	Derp1 ↓	47.41 (9.71-121.80)	36.62 (5.63-118.62)	30.56 (13.57-67.71)	0.737
rs324011					
TNF-α	U ↓	0.77 (0.01-1.24)	0.58 (0.01-1.15)	0.11 0.01-0.97	0.161
	LpA ↓	1124.07 (530.60-1970.03)	938.18 (350.42-1757.48)	787.74 (207.11-1502.44)	0.085
	Ppg ↓	2589.49 (1403.13-3560.77)	1997.07 (1345.51-3316.99)	1670.61 (1172.47-3144.03)	0.214
	Derp1 ↓	1121.41 (648.25-2884.49)	1095.66 (395.68-1741.80)	738.75 (346.33-1538.55)	**0.044**
GM-CSF	U -	0.01 (0.01-2.78)	0.01 (0.01-0.16)	0.01 (0.01-1.02)	0.470

	LpA ↓	6.07 (0.01-25.20)	1.47 (0.01-20.12)	0.78 (0.01-10.14)	0.484
	Ppg ↑	61.45 (5.84-219.57)	106.04 (3.64-348.92)	84.94 (9.26-399.79)	0.698
	Derp1 ↓	34.22 (0.92-208.03)	10.48 (0.01-104.79)	7.60 (0.01-49.87)	0.204
IL-13	U ↑	0.37 (0.15-0.74)	0.47 (0.27-0.79)	0.41 (0.18-0.79)	0.258
	LpA ↑ ↓	4.33 (2.24-14.72)	6.03 (1.93-12.99)	3.11 (1.19-7.80)	0.222
	Ppg ↑↓	24.39 (11.16-60.86)	28.01 (10.53-65.84)	19.16 (9.32-44.73)	0.528
	Derp1 ↓	10.46 (5.98-20.59)	10.50 (4.09-28.85)	5.14 (1.99-12.85)	0.063
IL-5	U ↓	0.13 (0.01-0.19)	0.02 (0.01-0.13)	0.04 (0.01-0.16)	0.069
	LpA ↓	8.47 (3.66-21.82)	6.25 (2.45-15.35)	4.69 (1.24-8.49)	0.061
	Ppg ↓	29.13 (10.80-72.92)	32.40 (10-78-68.69)	21.62 (11.82-48.29)	0.567
	Derp1 ↓	10.97 (6.90-22.59)	11.00 (3.96-26.54)	5.95 (2.94-12.71)	0.059
IFN-γ	U -	0.01 (0.01-1.02)	0.01 (0.01-1.03)	0.01 (0.01-1.02)	0.765
	LpA ↑	20.90 (3.70-85.07)	48.82 (15.19-105.78)	27.85 (3.86-51.04)	**0.020**
	Ppg ↑	27.88 (8.74-117.59)	58.12 (19.30-134.00)	42.51 (7.14-59.11)	**0.033**
	Derp1 ↑	23.53 (5.99-119.48)	72.37 (21.79-130.23)	31.87 (5.88-51.69)	**0.004**

Data presented as medians (first/third quartile). WT = wildtype, HT = heterozygous, SNP = SNP homozygous. U = Unstimulated, LpA = Lipid A, Ppg = Peptidoglycan, Derp1 = *Dermatophagoides pteronyssinus*. *Kruskal-Wallis test. Significant results are marked in bold. ↑ = upregulation or ↓ = downregulation in the presence of the polymorphic allele. Maximum number for cytokine analysis: *STAT6* rs1059513 n (WT) =152; n (HT) = 32; n (SNP) = 3 and *STAT6* rs324011 n (WT) = 73; n (HT) = 90; n (SNP) = 24. (Casaca *et al.* 2013).

When the genetic models (dominant and recessive) were applied most of the cytokine data retained their statistical significance as in the 3 group comparisons, for both *STAT6* polymorphisms (Table V).

Table V. Dominant and recessive models comparing results of cytokine secretion depending on *STAT6* polymorphisms.

		Dominant† Model					
		rs1059513			**rs324011**		
Cytok. pg/ml	**Stim.**	WT	HT+SNP	**P * value**	WT	HT+SNP	**P * value**
TNF-α	U	0.57 (0.01-1.16)	0.55 (0.01-1.47)	0.972	0.77 (0.01-1.24)	0.50 (0.01-1.15)	0.238
	LpA	943.73 (357.01-1757.48)	1555.43 (433.90-2345.46)	0.094	1124.07 (530.60-1970.03)	875.64 (307.17-1721.07)	0.056
	Ppg	2254.23 (1302.73-3422.19)	2112.51 (1330.50-3253.58)	0.975	2589.49 (1403.13-3560.77)	1958.18 (1250.78-3316.99)	0.101
	Derp 1	1024.73 (435.67-1896.18)	1291.52 (688.71-3105.12)	0.114	1121.41 (648.25-2884.49)	976.49 (390.06-1703.11)	**0.038**
GM-CSF	U	0.01 (0.01-0.21)	0.01 (0.01-6.61)	**0.017**	0.01 (0.01-2.78)	0.01 (0.01-0.26)	0.545
	LpA	2.19 (0.01-14.64)	8.09 (0.01-162.20)	**0.054**	6.07 (0.01-25.20)	0.78 (0.01-16.90)	0.243
	Ppg	84.43 (6.07-364.82)	29.03 (1.76-194.80)	0.138	61.45 (5.84-219.57)	98.13 (4.31-391.45)	0.480
	Derp 1	13.14 (0.01-105.23)	41.53 (4.25-276.40)	**0.055**	34.22 (0.92-208.03)	10.28 (0.01-103.27)	0.083
IL-13	U	0.43 (0.24-0.78)	0.37 (0.01-0.77)	0.183	0.37 (0.15-0.74)	0.47 (0.25-0.79)	0.175
	LpA	4.68 (1.87-12.34)	7.70 (2.24-14.72)	0.348	4.33 (2.24-14.72)	5.21 (1.61-11.62)	0.442
	Ppg	26.80 (10.48-61.24)	25.44 (9.51-50.36)	0.439	24.39 (11.16-60.86)	27.66 (10.42-59.34)	0.714
	Derp 1	10.12 (3.40-19.84)	15.09 (4.09-24.84)	0.382	10.46 (5.98-20.59)	10.17 (3.23-20.92)	0.488
IL-5	U	0.10 (0.01-0.17)	0.10 (0.01-0.16)	0.960	0.13 (0.01-0.19)	0.02 (0.01-0.15)	**0.023**
	LpA	6.31 (2.64-17.20)	8.24 (3.05-15.37)	0.389	8.47 (3.66-21.82)	5.45 (2.43-14.15)	**0.044**
	Ppg	28.94 (11.82-67.65)	27.99 (5.28-44.71)	0.250	29.13 (10.80-72.92)	27.69 (11.38-60.34)	0.655
	Derp 1	10.73 (4.71-22.59)	10.13 (6.29-22.43)	0.627	10.97 (6.90-22.59)	9.12 (3.81-22.43)	0.080
IFN-γ	U	0.01 (0.01-1.01)	0.24 (0.01-2.53)	**0.017**	0.01 (0.01-1.02)	0.01 (0.01-1.03)	0.466
	LpA	43.84 (6.18-98.95)	22.69 (3.74-72.90)	0.253	20.90 (3.70-85.07)	44.23 (9.24-98.31)	0.105
	Ppg	49.64 (15.98-130.31)	24.93 (5.74-61.60)	**0.028**	27.88 (8.74-117.59)	50.88 (17.40-123.26)	0.073
	Derp 1	47.41 (9.71-121.80)	33.59 (6.89-99.12)	0.440	23.53 (5.99-119.48)	52.53 (13.77-118.44)	0.120

Table V. (Continued).

	Recessive† model						
	rs1059513					rs324011	
Cytok. pg/ml	**Stim.**	WT+HT	SNP	**P value ***	WT+HT	SNP	**P value ***
TNF-α	U	0.55 (0.01-1.17)	0.91 (0.01-9.13)	0.623	0.65 (0.01-1.17)	0.11 0.01-0.97	0.073
	LpA	974.66 (369.02-1857.59)	4308.41 (2096.94-7875.38)	**0.021**	1045.80 (391.87-1934.48)	787.74 (207.11-1502.44)	0.097
	Ppg	2171.48 (1313.43-3313.31)	6546.62 (2720.25-13075.70)	**0.045**	2270.20 (1359.88-3328.75)	1670.61 (1172.47-3144.03)	0.275
	Derp1	1035.23 (451.42-1977.79)	7028.02 (1729.37-13401.76)	**0.027**	1103.84 (495.75-2223.45)	738.75 (346.33-1538.55)	**0.052**
GM-CSF	U	0.01 (0.01-0.87)	2.78 (0.01-14.28)	0.152	0.01 (0.01-0.87)	0.01 (0.01-1.02)	0.408
	LpA	2.62 (0.01-20.12)	295.54 (13.35-301.23)	**0.017**	4.03 (0.01-20.61)	0.78 (0.01-10.14)	0.527
	Ppg	78.85 (4.86-338.54)	112.70 (18.04-781.61)	0.506	78.85 (4.13-332.10)	84.94 (9.26-399.79)	0.511
	Derp1	15.81 (0.01-107.72)	623.82 (85.25-649.46)	**0.025**	16.68 (0.01-122.80)	7.60 (0.01-49.87)	0.368
IL-13	U	0.41 (0.21-0.78)	0.01 (0.01-0.15)	**0.017**	0.40 (0.21-0.77)	0.41 (0.18-0.79)	0.639
	LpA	4.89 (1.87-13.19)	7.75 (7.70-7.92)	0.524	5.21 (1.93-13.29)	3.11 (1.19-7.80)	0.087
	Ppg	25.71 (9.95-60.42)	27.36 (18.77-34.65)	0.991	25.71 (10.53-62.05)	19.16 (9.32-44.73)	0.365
	Derp1	10.12 (3.42-21.83)	18.00 (13.42-20.59)	0.314	10.48 (4.30-24.84)	5.14 (1.99-12.85)	**0.019**
IL-5	U	0.10 (0.01-0.16)	0.01 (0.01-0.10)	0.225	0.10 (0.01-0.16)	0.04 (0.01-0.16)	0.748
	LpA	6.42 (2.70-15.37)	9.47 (1.49-47.03)	0.679	7.61 (2.89-18.15)	4.69 (1.24-8.49)	0.075
	Ppg	27.69 (11.78-65.77)	30.87 (1.79-74.45)	0.767	31.37 (10.78-71.01)	21.62 (11.82-48.29)	0.292
	Derp1	10.45 (4.84-22.51)	15.26 (2.70-106.90)	0.634	10.99 (5.14-23.12)	5.95 (2.94-12.71)	**0.041**
IFN-γ	U	0.01 (0.01-1.103)	2.53 (0.01-3.18)	0.149	0.01 (0.01-1.03)	0.01 (0.01-1.02)	0.780
	LpA	40.13 (5.74-96.96)	17.29 (13.78-45.23)	0.662	43.20 (6.40-101.16)	27.85 (3.86-51.04)	0.092
	Ppg	45.49 (12.38-120.71)	32.95 (25.26-61.60)	0.877	46.27 (14.07-127.84)	42.51 (7.14-59.11)	0.209
	Derp1	47.13 (7.95-120.33)	30.56 (13.57-67.71)	0.749	51.00 (10.60-129.22)	31.87 (5.88-51.69)	**0.017**

Data presented as medians (first/third quartile).
*P values calculated with non-parametric Wilcoxon test for group comparison of the medians. †Dominant model = wildtype *vs* heterozygous and SNP homozygous. Recessive model = SNP homozygous *vs* heterozygous and wildtype. *STAT6* rs1059513 n (WT) = 152; n (HT) = 32; n (SNP) = 3 and *STAT6* rs324011 n (WT) = 73; n (HT) = 90; n (SNP) = 24. WT = wildtype, HT = heterozygous, SNP = SNP homozygous**.** U = unstimulated, LpA

= Lipid A, Ppg = Peptidoglycan, Derp1 = *Dermatophagoides pteronyssinus*. ↑ = upregulation or ↓ = downregulation in the presence of the polymorphic allele. Significant values are marked in bold.
(Casaca *et al.* 2013).

To further understand possible immune regulation dependent on the *STAT6* rs324011 genotype, potential correlations of *FOXP3* (Table VI) with changes at protein level of IFN-γ (Th1), IL-5, IL-13 (Th2) and TNF-α (pro-inflammatory) secretion were assessed without and following stimulation.

Wildtype and heterozygous carriers showed a moderate positive correlation between *FOXP3* expression and IFN-γ ($r = 0.4$, $p = 0.01$; $r = 0.3$, $p = 0.039$, respectively) at baseline (media, U). On the other hand, the homozygous carriers of the SNP showed a trendwise strong negative correlation ($r = -0.7$, $p = 0.11$) of *FOXP3* and IFN-γ. Regarding correlations with Th2 and pro-inflammatory cytokine secretion, significant negative associations between *FOXP3* expression and IL-5 ($r = -0.7$, $p < 0.0001$), IL-13 ($r = -0.4$, $p = 0.006$) and TNF-α ($r = -0.5$, $p = 0.0017$) were found in neonates carrying the wildtype allele of *STAT6* rs324011. Further negative correlations were observed in heterozygous carriers of *STAT6* rs324011, for *FOXP3* expression and IL-5 ($r = -0.4$, $p = 0.001$) and TNF-α ($r = -0.4$, $p=0.0008$). The overall population showed a comparable pattern which was not observed in the homozygous carriers of *STAT6* rs324011 Similarly to *FOXP3*, gene expression of *LAG3* was also highly inversely correlated with IFN-γ secretion ($r = -0.8$, $p = 0.011$, LpA) in homozygous carriers of rs324011. Homozygous carriers of the SNP also showed a positive correlation of *GITR* gene expression and TNF-α ($r = 0.7$, $p=0.026$) and borderline significant with IL-5 ($r = 0.6$, $p = 0.073$). On the other hand, heterozygous and wildtype carriers showed a significant negative correlation of *GITR* with IL-5 and TNF-α (Casaca *et al.* 2013). Overall, correlation of *FOXP3* gene expression with Th1 IFN-γ and with Th2 cytokines IL-5 and IL-3 was different in homozygous carriers of *STAT6* rs324011 and heterozygous/wildtype neonates.

For the second *STAT6* SNP, rs1059513, wildtype neonates showed a significant correlation of *FOXP3* with IFN-γ (r = 0.4, p = 0.0001), IL-5 (r = -0.5, p < 0.0001), TNF-α (r = -0.5, p ≤ 0.0001) and almost significant with IL-13 (*r* = -0.2, p = 0.060, unstimulated). The homozygous carriers of *STAT6* rs1059513 had a similar pattern of correlation between *FOXP3* and IL-5 and between *FOXP3* and IL-13 (p ≤ 0.05). Gene expression of the other two Treg-associated markers, *GITR* and *LAG3* showed an overall pattern of correlation with cytokines (protein level) as *FOXP3* (mRNA level) (Casaca *et al*. 2013). In summary wildtype and carriers of *STAT6* rs1059513 showed similar correlation of Treg-related FOXP3 gene expression and Th2 cytokines.

Table VI. Correlations of *FOXP3* gene expression with cytokine secretion in CBMCs depending on *STAT6* polymorphisms.

Treg marker	Stimuli	Genotype		IFN-γ (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	TNF-α (pg/ml)
				rs1059513			
FOXP3 (ΔCt)	U	WT	r	0.4	-0.5	-0.2	-0.5
			p	0.0001	< 0.0001	0.060	< 0.0001
			n	83	84	84	84
		HT + SNP	r	-0.1	-0.5	-0.5	-0.3
			p	0.820	0.022	0.054	0.275
			n	18	18	18	18
	LpA	WT	r	0.1	-0.3	-0.3	-0.1
			p	0.526	0.018	0.011	0.287
			n	72	70	71	72
		HT + SNP	r	0.1	0.2	-0.0	0.1
			p	0.858	0.558	0.979	0.628
			n	13	13	13	13
	Ppg	WT	r	-0.06	0.0	-0.11	-0.2
			p	0.591	0.731	0.365	0.072
			n	66	65	66	66
		HT + SNP	r	-0.3	0.2	0.01	-0.2
			p	0.325	0.415	0.957	0.494
			n	13	13	13	13
	Derp1	WT	r	0.1	-0.3	-0.2	-0.3
			p	0.611	0.061	0.187	0.075
			n	47	46	46	46
		HT + SNP	r	0.4	-0.4	-0.5	-0.7
			p	0.385	0.319	0.233	0.047
			n	8	8	8	8

				rs324011			
FOXP3 (ΔCt)	U	WT	r	0.4	-0.7	-0.4	-0.5
			p	0.010	< 0.0001	0.006	0.0017
			n	41	41	41	41
		HT	r	0.3	-0.4	-0.18	-0.4
			p	0.039	0.001	0.196	0.0008
			n	54	55	55	55
		SNP	r	-0.7	0.3	-0.4	0.4
			p	0.111	0.538	0.351	0.346
			n	7	7	7	7
	LpA	WT	r	-0.1	-0.1	-0.3	-0.2
			p	0.649	0.524	0.097	0.231
			n	36	36	36	36
		HT	r	0.3	-0.24	-0.3	0.0
			p	0.081	0.131	0.047	0.813
			n	43	42	42	43
		SNP	r	0.1	-0.0	-0.3	0.0
			p	0.969	0.957	0.478	0.939
			n	7	6	7	7
	Ppg	WT	r	-0.1	0.3	0.1	-0.0
			p	0.475	0.095	0.471	0.960
			n	32	32	32	32
		HT	r	0.0	-0.1	-0.2	-0.2
			p	0.924	0.642	0.234	0.231
			n	40	40	40	40
		SNP	r	0.14	-0.6	-0.7	-0.3
			p	0.760	0.208	0.071	0.482
			n	6	6	7	7
	Derp1	WT	r	0.3	-0.3	-0.31	-0.3
			p	0.162	0.141	0.164	0.164
			n	21	21	21	21
		HT	r	0.0	-0.1	-0.0	-0.1
			p	0.963	0.585	0.893	0.711
			n	30	29	29	29
		SNP	r	-0.8	-0.4	-0.4	0.0
			p	0.200	0.600	0.600	1.000
			n	4	4	4	4

r = Spearman correlation coefficients. P ≤ 0.01 is significant. WT = wildtype, HT = heterozygous, SNP = SNP homozygous. U = Unstimulated, LpA = Lipid A, Ppg = Peptidoglycan, Derp1 = *Dermatophagoides pteronyssinus*. Heterozygotes and minor allele homozygotes of rs1059513 were combined to increase statistical power. (Casaca *et al.* 2013).

In summary, *STAT6* rs324011 polymorphism was associated with downregulation of Treg-associated genes. Furthermore, neonates with this polymorphic allele showed reduced production of IFN-γ. On the other hand *STAT6* rs1059513 polymorphism was associated with increased concentrations of GM-CSF and TNF-α.

3.1.2 Modulation of neonatal immune responses by Th1 polymorphisms: *TBX21* and *HLX1*

3.1.2.1 Changes in cytokine secretion are associated with *TBX21* and *HLX1* polymorphisms

Neonates carrying *TBX21* rs17250932 and *HLX1* rs2738751 showed a decrease (or trend) for IL-5 (p = 0.03; 0.05) and IL-13 (p = 0.06, 0.05) secretion upon LpA-stimulation. Also carriers of *HLX1* rs2738751 showed lower IL-13 after Ppg-stimulation compared to wildtype and heterozygous. These two polymorphic genotypes (*TBX21* rs17250932 and *HLX1* rs2738751) were also associated with low secretion of TNF-α (Figure 3).

Investigating the influence of *HLX1* SNP rs12141189 showed that homozygous carriers had significant (or trend to) higher IL-5 (p = 0.007/0.1), IL-13 (p = 0.1) and GM-CSF (p = 0.03/0.05) secretion upon LpA and Ppg-stimulation. IFN- γ Th1-associated cytokine secretion was downregulated (trend, p = 0.1) in the homozygous carriers (upon D+L-stimulation) (Figure 4).

The presence of *HLX1* SNP rs3806325 was associated with higher secretion of IL-13 and IL-6 at baseline (Table VII). No associations were found between *TBX21* SNP rs11079788 and cytokine modulation, however the SNP carriers presented increased activation of effector T cells ($CD4^+CD25^+$).

Application of the recessive model generally led to increased statistical significance of the findings (Table VII) (Casaca *et al.* 2012).

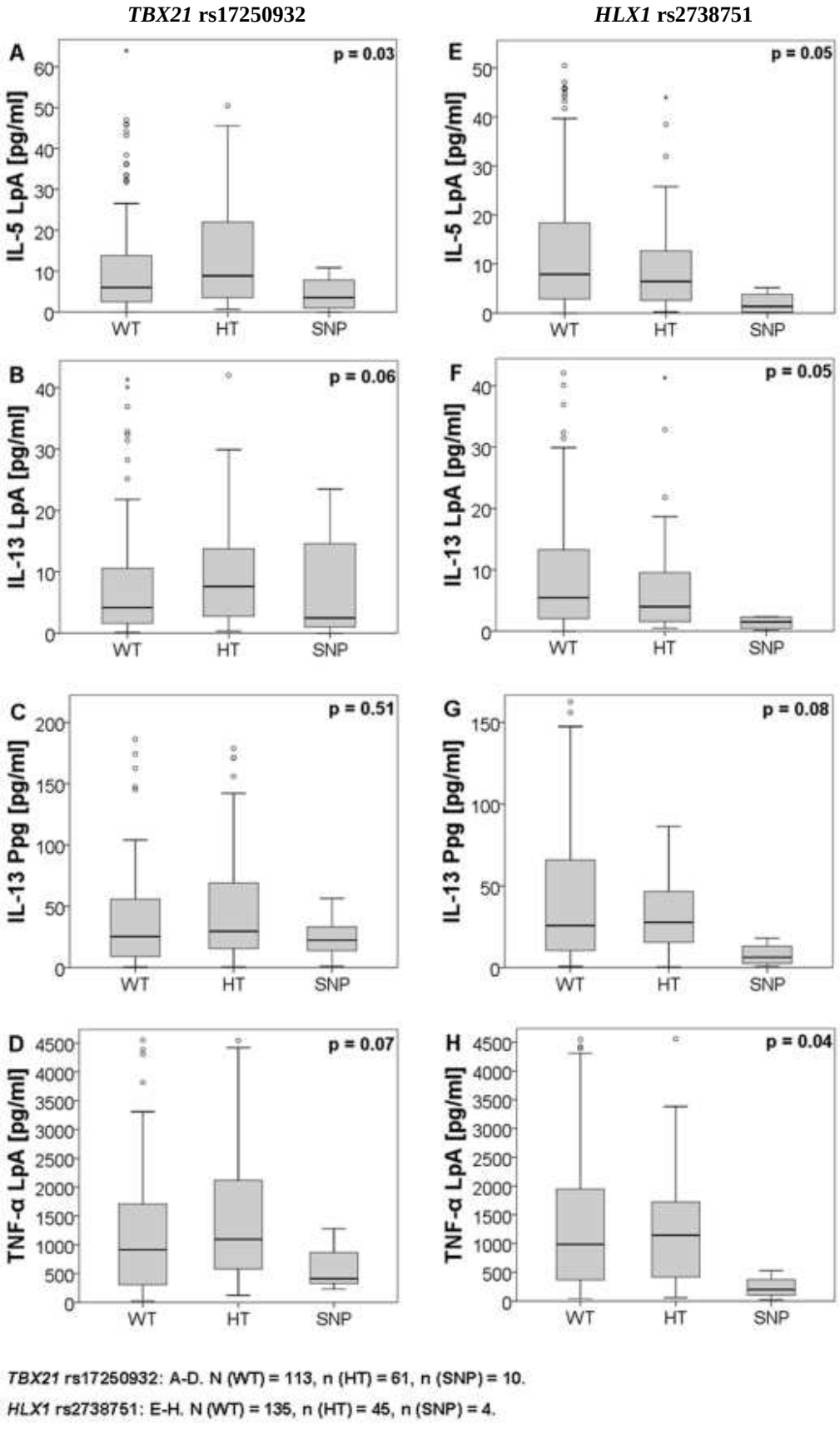
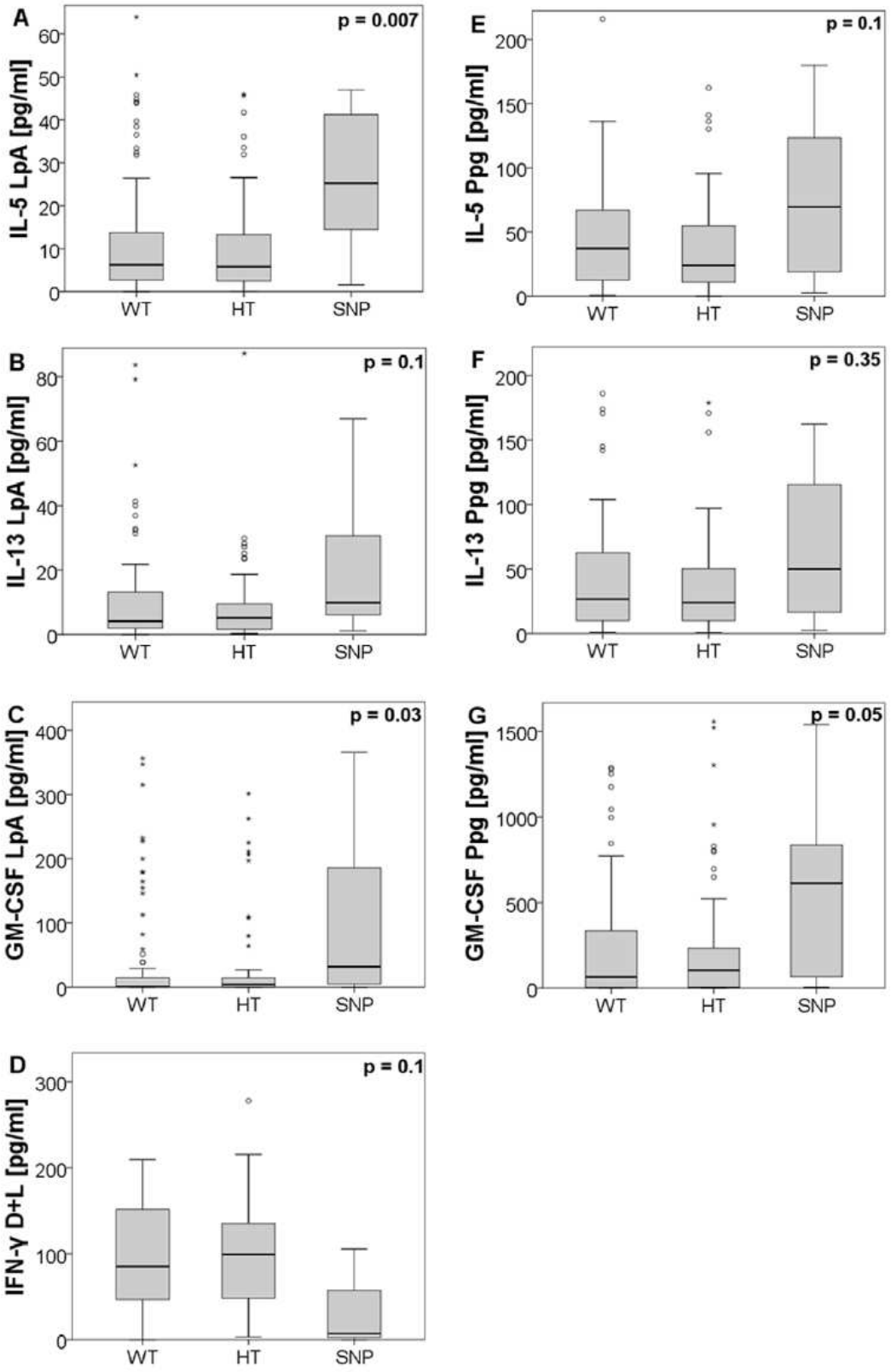


Figure 3. Cytokine secretion of wildtype, heterozygous and homozygous SNP carriers of *TBX21* rs17250932 and *HLX1* rs2738751. Data were shown in boxplots (first, third quartile, median), the whiskers indicate the maximum and minimum values, dots indicate outliers, analyzed by Kruskal-Wallis-test. Values were shown in pg/ml. *TBX21* rs17250932 n (WT) = 113, n (HT) = 61, n (SNP) = 10, and *HLX1* rs2738751 n (WT) = 135, n (HT) = 45, n (SNP) = 4 (Casaca *et al.* 2012).

***HLX1* rs12141189**



HLX1 rs12141189: A-G. N (WT) = 104, n (HT) = 68, n (SNP) = 12.

Figure 4. Cytokine secretion of wildtype, heterozygous and homozygous SNP carriers of *HLX1* rs12141189. Data were shown in boxplots (first, third quartile, median), the whiskers indicate the maximum and minimum values, dots indicate outliers, analyzed by Kruskal-Wallis-test. Values were shown in pg/ml. n (WT) = 104, n (HT) = 68, n (SNP) = 12 (Casaca *et al.* 2012).

3.1.2.2 Gene expression of Th1 and Th2-related markers is associated with *TBX21* and *HLX1* polymorphisms

Next, gene expression at mRNA level was assessed to investigate influence of the polymorphisms on the transcription of Th1 and Th2-related genes. We investigated: *TBX21*, *HLX1*, *IRF1*, *GATA3*, *STAT6* and *STAT6e*. Table VII shows the gene expression (mRNA) related to the findings at protein level. *TBX21* rs17250932 was associated with decreased *STAT6e* expression in parallel with lower Th2 and pro-inflammatory cytokines. *TBX21* rs11079788 polymorphism was associated with higher *GATA3*, *HLX1* and *IRF1* at baseline in parallel with high activated T cells.

Also, *HLX1* rs3806325 was associated with higher expression of *GATA3* and *STAT6e* upon LpA-stimulation, in parallel with increased IL-13 and IL-6 cytokine secretion at baseline (Table VII).

*G*ene expression of the Th1 transcription factor *TBX21* was lower in *HLX1* rs12141189 SNP carriers upon D+L-stimulation. Of note, these carriers presented decrease IFN-γ production. Significant changes at gene levels were not observed in relation to *HLX1* rs2738751 SNP polymorphism (Casaca *et al.* 2012).

In summary, the studied Th1 *TBX21* and *HLX1* polymorphisms showed an association with regulation mainly of Th2 cytokines IL-5 and IL-13 upon innate stimulation with LpA.

Table VII. Effects of *TBX21* and *HLX1* polymorphisms on cytokine secretion, T cells and mRNA regulation.

Gene/ rs number	Cytokine secretion/ T cell regulation	P Overall	P recessive model	mRNA regulation	P Overall
TBX21 rs17250932	IL-5 (LpA)↓ IL-13 (LpA)↓ TNF-α (LpA)↓	**0.03** 0.06 0.07	**0.03** 0.28 0.16	No changes with LpA *STAT6e* (U)↓	- **0.01**
TBX21 rs11079788	$CD4^+CD25^+$ (U)↑	**0.04**	0.23	*GATA3* (U) ↑ *HLX1* (U) ↑ *IRF1* (U) ↑	0.08 **0.02** **0.01**
HLX1 rs2738751	IL-5 (LpA) ↓ IL-13 (LpA) ↓ IL-13 (Ppg)↓ TNF-α (LpA) ↓	**0.05** **0.05** 0.08 **0.04**	**0.02** **0.02** **0.03** **0.01**	No changes with LpA or Ppg	-
HLX1 rs3806325	IL-13 (U) ↑ IL-6 (U) ↑	**0.005** **0.03**	**0.003** **0.05**	*GATA3* (LpA) ↑ *STAT6e* (LpA) ↑	**0.003** **0.007**
HLX1 rs12141189	IL-5 LpA ↑ IL-13 LpA ↑ GM-CSF LpA ↑ IL-5 (Ppg) ↑ GM-CSF (Ppg) ↑ IFN- γ (D+L) ↓	**0.007** 0.1 **0.03** 0.1 **0.05** 0.1	**0.002** **0.04** **0.009** 0.09 **0.02** **0.03**	*TBX21* (D+L) ↓	**0.02**

LpA=Lipid A; Ppg=Peptidoglycan and U=unstimulated, D+L=*Dermatophagoides pteronyssinus* 1 and Lipid A; ↑: expression upregulated; ↓: expression downregulated. P values analyzed by Kruskal-Wallis-test (overall, 3 categories) or Wilcoxon-test (recessive model, 2 categories). Genotype comparison includes the comparison of the respective genotype groups used for statistical analysis of mRNA expression; statistics performed by generalized Wilcoxon test. Significance ($p \leq 0.05$) is marked in bold (Casaca *et al.* 2012).

3.1.3 Association of Th2 *STAT6*, Th1 *TBX21* and *HLX1* polymorphisms with early disease development

When children turned 3 years, parents completed extensive questionnaires to assess allergic and respiratory diseases. Next the influence of Th1/Th2 polymorphisms in relation to clinical outcomes was assessed.

As shown in Table VIII, children that carried the *STAT6* rs324011 SNP showed a significant lower risk for doctor's diagnosis of atopic dermatitis (AD) ($p = 0.046$). AD prevalence was 20.6 % in the wildtype, 11.5 % in the heterozygous and 4.3 % in the SNP homozygous carriers group. Furthermore, wildtype children also showed the highest risk of a doctor's diagnosis of obstructive bronchitis with 19.1 % compared to 11.5 % of heterozygous and 0 % of the homozygous. When applying the recessive and dominant models ($\chi 2$ test), in relation to the clinical outcomes the significance of the results before mentioned remained (not shown). No associations were found between *STAT6* rs1059513 and respiratory and allergic diseases at age 3 years.

Table VIII. Prevalence of clinical outcomes in children in relation to *STAT6* rs324011 genotypes.

Phenotype	Genotypes % (n/N)			P value*
	WT	HT	SNP	
Atopic dermatitis	20.6 (13/63)	11.5 (10/87)	4.4 (1/23)	**0.046**
Food allergy	1.6 (1/63)	2.3 (2/87)	0.0 (0/23)	0.951
Symptoms of Food allergy	11.1 (7/63)	10.3 (9/87)	4.4 (1/23)	0.629
Obstructive bronchitis	19.1 (12/63)	11.5 (10/87)	0 (0/23)	**0.048**

Data are shown in percentages and number of cases within the respective groups. *Differences between groups were calculated with Mantel-Haenszel test. WT = wildtype, HT = heterozygous, SNP = SNP homozygous. Atopic dermatitis = Doctor's diagnosis of AD; Food allergy = Doctor's diagnosis of food allergy; Symptoms of food allergy = parental report of symptoms of food allergy; Obstructive bronchitis = Doctor's diagnosis of asthma ever or repeated obstructive bronchitis. Outcome data assessed within the group of children with available genotyping data (Casaca *et al.* 2013).

As for Th1 polymorphisms, *TBX21* rs11079788 SNP carriers showed less symptoms of atopic dermatitis compared to the heterozygous and wildtype group (19 % *vs* 23 % *vs* 36 %, p = 0.03). No other significant associations were found for the rest of the studied polymorphisms.

3.1.4 Th1 and Th2 polymorphisms and maternal atopy

It was shown that the atopic status of the mother can modulate SNP-mediated immune responses (Liu, Rädler *et al.* 2011). Consequently the potential role of maternal atopy on the Th2 and Th1 polymorphism-modulated immune responses was aimed to be investigated.

Stratification of the *STAT6* polymorphisms showed similar MAF in neonates from atopic and non-atopic mothers, thus no association of the maternal atopy was expected (Table IX). For the Th1 polymorphisms, the MAF differed slightly (Table X). Comparison of the influence of the maternal status showed no differences on the Th1 polymorphisms immune modulation. However stratification by the maternal atopic status lead to very low numbers of homozygous carriers.

Table IX. Distribution of minor allele frequencies of the *STAT6* polymorphisms in neonates from atopic and nonatopic mothers.

Polymorphism	Maternal atopy	WT/HT/SNP (n)	MAF
STAT6 rs1059513	No	100/21/1	0.09
	Yes	55/12/2	0.11
STAT6 rs324011	No	50/55/17	0.36
	Yes	24/37/8	0.38

*P value calculated with Mantel-Haenszel test. MAF = Minor Allele Frequency. (n) = number of children. WT = wildtype, HT = heterozygous, SNP = SNP homozygous (Casaca *et al.* 2013).

Table X. Distribution of minor allele frequencies of the *TBX21* and *HLX1* polymorphisms in neonates from atopic and non-atopic mothers.

Gene/rs number	Maternal atopy	WT/HT/SNP (n)	MAF
TBX21 rs17250932	No	68/46/7	0.25
	Yes	48/18/3	0.17
TBX21 rs11079788	No	53/58/10	0.32
	Yes	40/23/6	0.25
HLX1 rs2738751	No	88/32/2	0.15
	Yes	50/16/2	0.15
HLX1 rs3806325	No	84/36/2	0.16
	Yes	43/23/3	0.21
HLX1 rs12141189	No	66/47/8	0.20
	Yes	42/24/4	0.23

*P value calculated with Mantel-Haenszel test. MAF = Minor Allele Frequency. (n) = number of children. WT = wildtype, HT = heterozygous, SNP = SNP homozygous (Casaca *et al.* 2012).

3.2 Role of regulatory T cells in the context of farm exposures and allergic disease and asthma

The PASTURE (Protection against allergy: study in rural environments) study and the follow-up study EFRAIM (Mechanisms of Early Protective Exposures on Allergy Development) is a large international birth cohort study of children from Austria, Finland, France, Germany and Switzerland (von Mutius *et al.* 2006) designed to investigate the protective factors against the development of allergies in early life. The PASTURE/EFRAIM cohort comprises more than 1000 children.

Several studies have repeatedly shown that farm environments have a protective effect against the development of asthma, atopy and wheeze in childhood (von Ehrenstein *et al*. 2000; Alfvén *et al.* 2006; Douwes *et al.* 2008; Riedler *et al.* 2001; Riedler *et al.* 2000). This effect may start already *in utero* through exposure to the maternal farm lifestyle (Schaub *et al.* 2009). The mechanisms proposed to date include modulation of regulatory T cell number and function (Schaub *et al*. 2009; Douwes *et al.* 2008).

Previously it was shown that the neonates from farming mothers had increased Treg cell frequency and also higher Treg suppressive capacity (Schaub *et al.* 2009). Furthermore, it was recently shown that this pattern of higher Treg cells persists in farm children of the PASTURE/EFRAIM study until the first 4.5 years of life (Lluis, Depner *et al*, 2013).

With age, maturation of the immune system continues to develop, also the number of environmental exposures continues to accumulate and the clinical phenotypes become rather stable. However no information is known about the protective Treg-farm association after the early years. Thus, a subgroup of German children from the PASTURE/EFRAIM international birth cohort was studied at age of 6 years to investigate this matter.

Here, the frequency of regulatory T cells at the age of 6 years in the entire population is presented, then Treg cell frequency and quality is shown in relation to farm/farm exposures and in relation to clinical outcomes. Lastly the influence of farm on clinical outcomes was studied.

3.2.1 Population characteristics

A total of 143 children, with Treg cell data available were studied. Table XI displays the main population characteristics. The percentages of children exposed to specific farm elements such as contact to hay, staying in stables, drinking farm milk was higher in the farming children group (approximately 70-90% *versus* 20-25%).

Parental atopy was higher in the non-farming group and in general also the percentage of children sensitized to specific allergens (using 3 IgE concentration cut-offs: class I $\geq$ 0.35 IU/ml; class II $\geq$ 0.7 IU/mL and class III $\geq$ 3.5 IU/ml.). The asthma prevalence was slightly higher on the non-farming group.

Table XI. PASTURE/EFRAIM population characteristics at the age of 6 years.

Parameters		Farming		Non-Farming	
		n	%	n	%
Regular contact to hay	No	14	19.72	54	75.00
	Yes	57	80.28	18	25.00
Staying in stable	No	9	12.68	59	81.94
	Yes	62	87.32	13	18.06
Farm milk consumption	No	19	26.76	59	81.94
	Yes	52	73.24	13	18.06
Parental atopy	No	42	59.15	21	29.58
	Yes	29	40.85	50	70.42
Maternal atopy	No	47	66.20	45	62.50
	Yes	24	33.80	27	37.50
Paternal atopy	No	56	80.00	34	47.89
	Yes	14	20.00	37	52.11
Older siblings	0-1	43	60.56	62	86.11
	>=2	28	39.44	10	13.89
Gender	Male	44	61.97	37	51.39
	female	27	38.03	35	48.61
Smoking during pregnancy	No	70	98.59	60	83.33
	Yes	1	1.41	12	16.67
Asthma	No	56	78.87	53	73.61
	Yes	15	21.13	19	26.39
Sensitization					
Inhalant Class I	No	40	56.34	39	54.17
	Yes	31	43.66	33	45.83
Inhalant Class II	No	47	66.20	43	59.72
	Yes	24	33.80	29	40.28
Inhalant Class III	No	60	84.51	57	79.17
	Yes	11	15.49	15	20.83
Food Class I	No	40	56.34	45	62.50
	Yes	31	43.66	27	37.50
Food Class II	No	45	63.38	51	70.83
	Yes	26	36.62	21	29.17
Food Class III	No	65	91.55	68	94.44
	Yes	6	8.45	4	5.56
Seasonal Class I	No	31	43.66	33	45.83
	Yes	40	56.34	39	54.17
Seasonal Class II	No	36	50.70	38	52.78
	Yes	35	49.30	34	47.22
Seasonal Class III	No	60	84.51	57	79.17
	Yes	11	15.49	15	20.83

Total number of children n = 143. Class III ≥ 3.5 IU/ ml, Class II ≥ 0.7 IU/ ml, Class I ≥ 0.35 IU/ ml specific IgE against food, inhalant and seasonal allergens. Contact to hay, staying in stable and farm milk consumption percentages are related to current exposures (at age 6).

3.2.2 Regulatory T cell frequency in 6 year old children

To evaluate the Treg cell frequencies, regulatory T cells were defined by the expression of CD4, high levels of CD25 (the 20 % top cells) and the intracellular expression of FOXP3, the Treg specific transcription factor and master regulator. The number of cells was assessed by flow cytometry (Figure 5) and the percentages were calculated in relation to the lymphocyte population. The median percentages of Treg cells in the total population were: 0.12 (Q1 = 0.07; Q3 = 0.23) in media, 0.34 (Q1 = 0.19; Q3 = 0.67) in PI and 0.13 (Q1 = 0.06; Q3 = 0.21) in Lps.

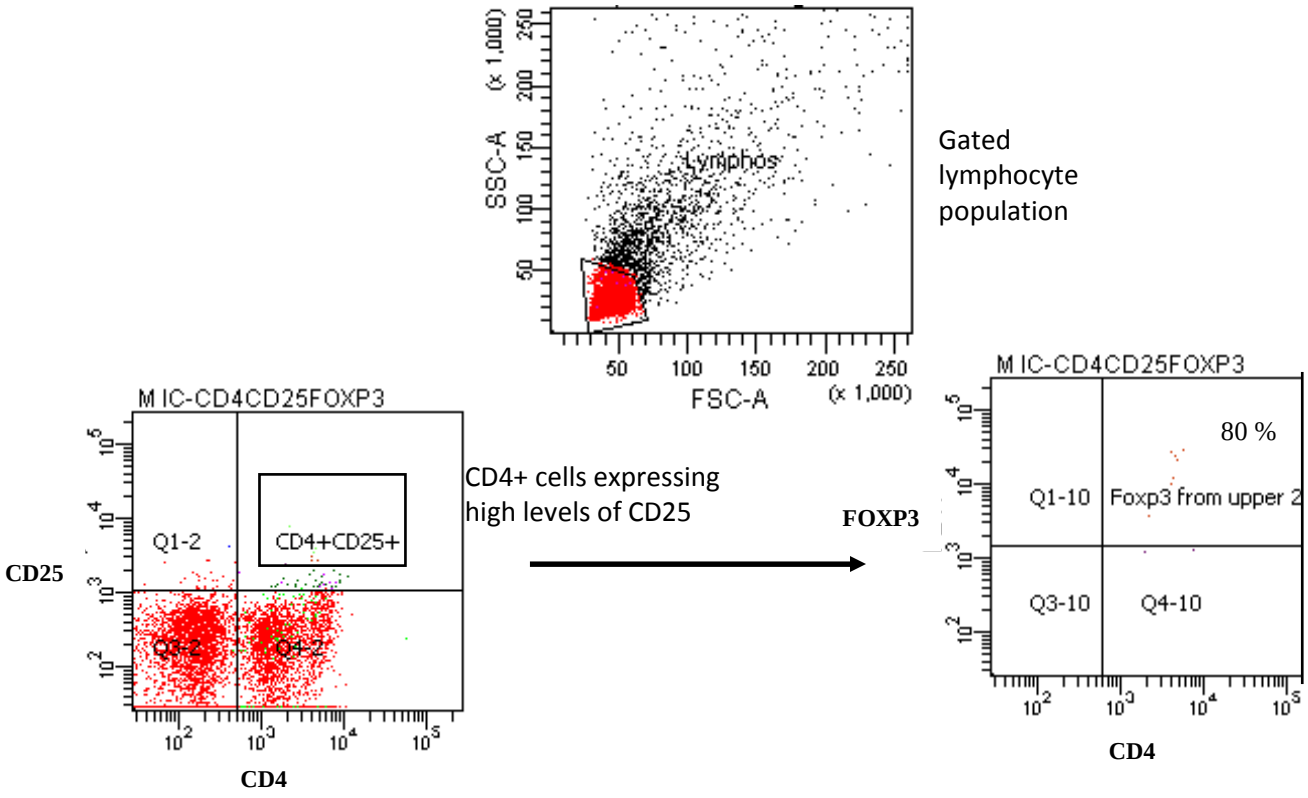


Figure 5. Representative flow cytometry plots of Treg cell makers. Treg cells were identified by CD4, high levels of CD25 (upper top 20 % cells) and FOXP3.

3.2.3 Current farm exposures and Treg cell frequency at age 6 years

In the same study population, it has been demonstrated that regulatory T cells are increased in farming children, particularly following farm milk consumption at age 4.5 years (Lluis, Depner *et al*. 2013). In this study, at age 6 years, stratification into 2 groups of farming and non-farming children showed that the farming status itself was not significantly associated with the frequency of Treg cells at the age of 6 years (in all 3 stimulation conditions) (Table XII). Next, specific farm-associated exposures such as staying in stable, regular contact to hay and farm milk consumption in the last 12 months (from the 6 year questionnaire) were examined in relation to Treg cell numbers.

Children who spent time in the stable showed a higher percentage of Treg cells at baseline, 0.16 % (p = 0.02) than children that did not have contact to the stables (0.10 %). As shown in Table XII, assessing children who had regular contact to hay and drank farm milk at the age of 6 year (consumption in the last 12 months from the 6 year questionnaire) showed a trend for higher Treg cells at baseline and lower Treg cells upon Lps-stimulation (not significant).

Table XII. Association of farm and current farm exposures with Treg cells at age of 6 years.

Parameters		Tregs M	P	Tregs PI	P	Tregs Lps	P
Farm	**No**	0.11 (0.05-0.21)	0.29	0.44 (0.20-0.70)	0.11	0.13 (0.08-0.23)	0.21
	Yes	0.15 (0.07-0.26)		0.29 (0.17-0.61)		0.13 (0.05-0.20)	
Farm milk	**No**	0.11 (0.04-0.22)	0.11	0.34 (0.19-0.67)	0.95	0.13 (0.08-0.26)	0.10
	Yes	0.15 (0.08-0.26)		0.33 (0.19-0.65)		0.12 (0.05-0.19)	
Staying in stable	**No**	0.10 (0.04-0.21)	**0.02**	0.34 (0.19-0.63)	0.41	0.12 (0.07-0.22)	0.92
	Yes	0.16 (0.08-0.27)		0.34 (0.23-0.73)		0.13 (0.06-0.20)	
Regular contact to hay	**No**	0.11 (0.04-0.21)	0.08	0.32 (0.19-0.67)	0.92	0.13 (0.08-0.26)	0.13
	Yes	0.15 (0.08-0.27)		0.36 (0.19-0.68)		0.12 (0.06-0.19)	

M = Media/baseline, PI = PMA/ionomycin, Lps = Lipopolysaccharide, 1Q and 3Q. Wilcoxon test. Significant p values are marked in bold ($p \leq 0.05$).

3.2.4 Farm milk consumption during childhood modulates regulatory T cells at age 6 years

After assessing current exposures (age 6 years) on Treg cells early exposures during childhood were assessed to study whether they influenced Treg cells at age 6 years. Shaping of the immune system during pregnancy and early in life seems to play a big role on immune modulation and disease development. Farm milk consumption has been shown to be one of the most important specific farm exposures related to disease protection (Loss *et al.* 2011; Waser *et al.* 2006). Drinking farm milk at early ages, 1 or 1.5 years was associated with higher Treg cells at baseline at age 6 years (Figure 6). On the other hand, drinking farm milk at age 4 or 5 was significantly associated with lower Treg cells at age 6 years after Lps-stimulation. The children in each age category were defined by answering “yes” or “no” drinking farm milk at that time point, thus children might oscilate overtime between groups.

Contact to hay or to stable during childhood was not significantly associated with changes in Treg cells at the age of 6 years.

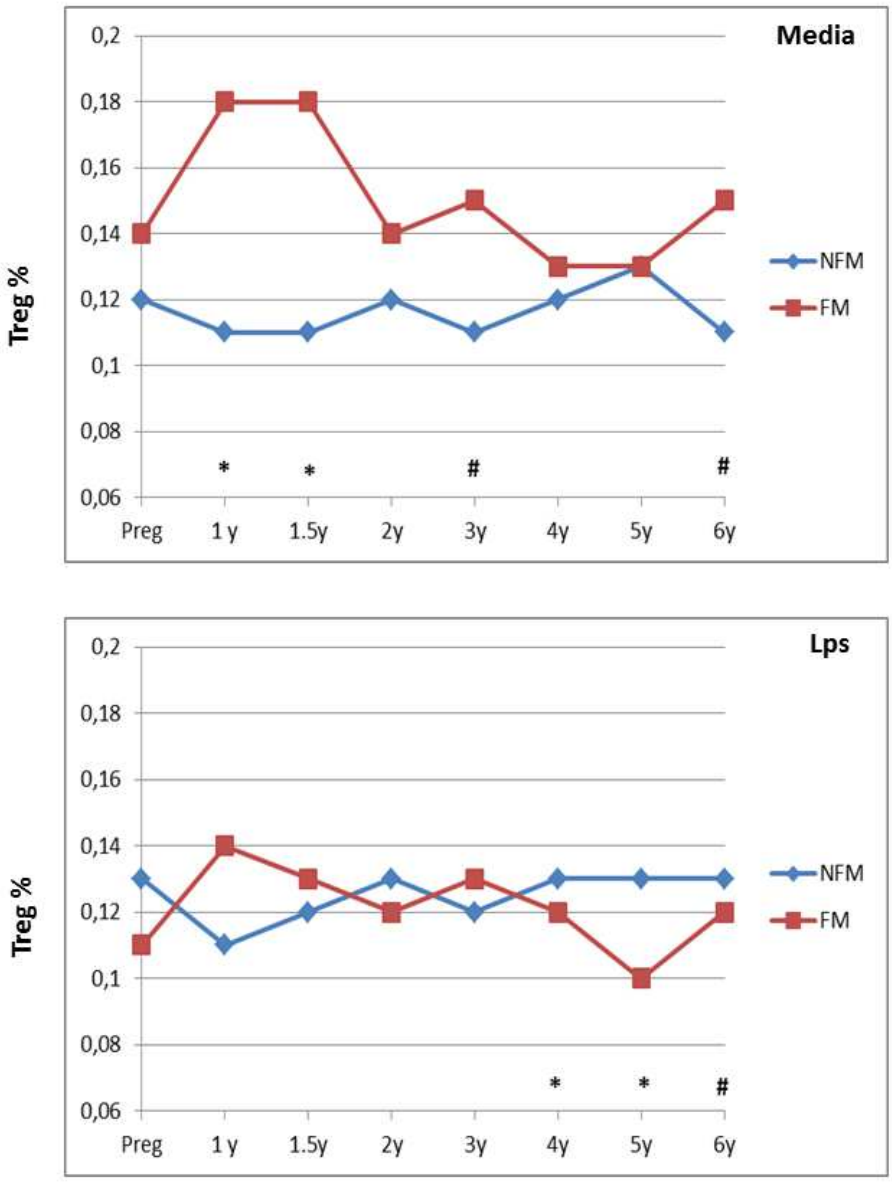


Figure 6. Association of Treg cell percentages at age 6 years with farm milk consumption from pregnancy until age of 6 years at Media (baseline) and upon Lps. Media: Number of children at the timepoints - Preg: FM=60 NFM=67. 1y:FM=45 NFM=82. 1.5y: FM=52 NFM=73. 2y: FM=60 NFM=67. 3y: FM=59 NFM=67. 4y:FM=59 NFM=68. 5y: FM= 62 NFM=63. 6y: FM=55 NFM=72. Lps: Preg: FM=64 NFM=69. 1y:FM=47 NFM=86. 1.5y: FM=53 NFM=78. 2y: FM=60 NFM=73. 3y: FM=61 NFM=71. 4y:FM=62 NFM=70. 5y: FM=67 NFM=64. 6y: FM=60 NFM=73. N at the different timepoints. * $p \leq 0.05$. # $p \leq 0.01$.

3.2.5 Regulatory T cells and suppression of effector T cell division and cytokine production in 6 year old children

In addition to the frequency of regulatory T cells we further investigated the ability of Treg cells to suppress cell division of autologous effector T cells following activation with PHA (Figure 7) in a subgroup was selected from the German PASTURE/EFRAIM population.

A subsample of the PASTURE/EFRAIM study population was selected. Antigen-presenting cells ($CD3^-$), effector T cells ($CD4^+CD25^-$) and Treg cells ($CD4^+CD25^+$) were separated and incubated at different ratios.

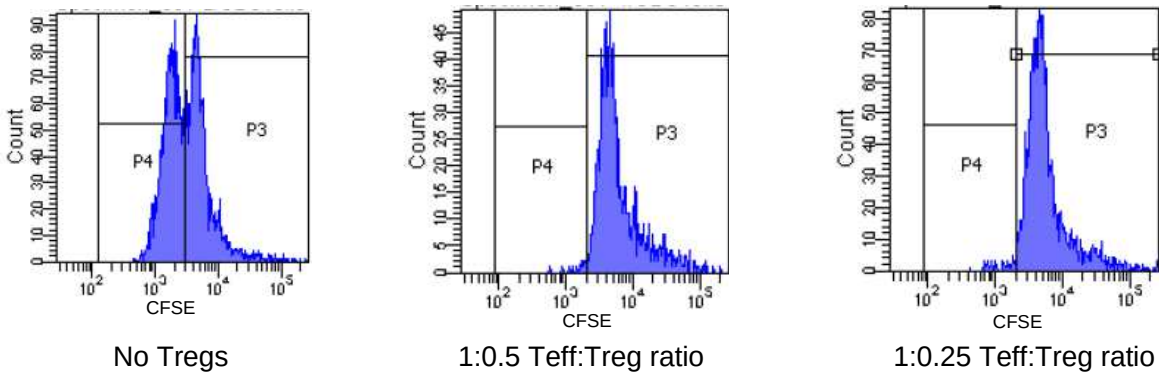


Figure 7. Representative plots. Treg suppression, measured by decrease of CFSE staining during division of effector T cells. Left plot shows division of PHA-stimulated effector T cells without addition of Treg cells. Middle and right plots show the reduction of effector T cell division (P4) with addition of Treg cells in different ratios (1:0.5 and 1:0.25, respectively).

Treg suppressive capacity was evaluated by taking the baseline of effector T cell division with PHA into account and is represented by the formula:

(Effect cell division without Tregs - Effect cell division with Tregs / Effect cell division without Tregs) x 100

In the total group if children the median of effector T cell division in response to PHA-stimulation was of 23.75 %. As shown in Table XIII, the addition of Treg cells led to an overall decrease of effector T cell division. When Treg cells were added to the culture,

effector T cells divided at a lower rate: 6.2 % (at the ratio of Effect/Treg 1:0.5) and 5.35 % (at the ratio of Effect/Treg 1:0.25).

The different ratios of Treg cells (adding $1x10^4$cells or 500 Treg cells to a total of 2000 of effector T cells) led to similar results in regards to effector T cell division.

Table XIII. Effector T cell division and Treg suppressive capacity in 6 year old EFRAIM children.

Effector T cell:Treg cell	Effector T cell division					
	n	**min**	**1Q**	**Median (%)**	**3Q**	**max**
1:0	20	6.8	12.68	23.75	35.92	50.9
1:0.5	18	1.4	5.45	6.20	9.65	26.9
1:0.25	16	1.8	4.8	5.35	8.65	38.1
	Suppressive Capacity					
1:0.5	18	10.16	51.82	63.74	78.85	94.55
1:0.25	16	0.78	41.51	64.89	77.86	93

$CD4^+CD25^-$ effector T cells ($2x10^4$ cells/well) labeled with CFSE, cultured with irradiated $CD3^-$ cells ($4x10^4$cells/well), and stimulated with PHA (0.8 μg/mL), with $CD4^+CD25^+$ Treg cells ($1x10^5$, $5x10^4$cells/well, ratio 1:0.5 and 1:0.25, respectively). After 72 hours of culture, the percentage of newly divided $CD4^+CD25^-$ effector cells was measured by flow cytometry. 1Q = 1st Quartile; 3Q = 3rd Quartile.

To further understand the underlying mechanisms of suppression the culture supernatants were collected and several cytokines were measured including IL-1β, IL-2, IL-5, IL-9, IL-10, IL-13 and IFN-γ (Table XIV) in cultures without Treg cells or with (at 1:0.5 ratio Effect/Treg cells). The Th2-associated cytokines IL-5 and IL-13 were strongly decreased when Treg cells were in culture. Changes in IL-10 secretion were rather mild, from 2.93 to 2.32 pg/ml. The Th1-associated cytokines IL-2 and IFN-γ were also strongly suppressed with Tregs. Furthermore, IL-9 was also limited by the Treg cells. No clear downregulation of the pro-inflammatory IL-1β was observed.

Table XIV. Cytokine concentrations with and without Treg cells.

Cytokine	Median (1Q-3Q) pg/ml	
	No Tregs	**With Treg (1:0.5)**
IL-1β	23.7 (16.02 - 29.19)	21.36 (13.32 - 44.34)
IL-2	44.68 (16.66 – 73.31)	26.3 (7.88 - 65.29)
IL-5	4.92 (1.13 - 12.15)	1.42 (0.4 - 6.49)
IL-9	2.36 (1.32 - 4.85)	1.5 (0.02 - 2.7)
IL-10	2.93 (1.09 - 3.57)	2.32 (0.39 - 3.27)
IL-13	68.74 (37.44 - 148.5)	6.15 (0.74 - 28.39)
IFN-γ	124.4 (66.87 - 280.8)	40.66 (0.02 - 58.5)

Supernatants were collected after 72 hours of culture. Cytokines were measured by Luminex Technology. Results are expressed in medians (pg/ml) and 1Q = 1st Quartile and 3Q = 3rd Quartile.

3.2.6 IL-2 is significantly modulated in non-farming children

Effector T cell division and cytokine secretion can be inhibited by Treg cells by different suppressive mechanisms. To understand whether there were qualitative differences regarding suppressive capacity, Treg suppression assays were conducted. Farming and non-farming children did not show significantly different suppressive capacity *in vitro*. Also, consumption of farm milk did not influence effector T cell suppression at age 6 years. Besides, Treg cell suppressive capacity, cytokines from the cell cultures were also assessed. Cytokines were measured with and without Treg cells in the culture.

Non-farming children showed a significant decrease in IL-2 concentration in co-culture with Treg cells; this was not observed in farming children (Figure 8).

No other changes regarding IL-1β, IL-5, IL-13, IL-10, IL-9 or IFN-γ were observed depending on the farm status of the child.

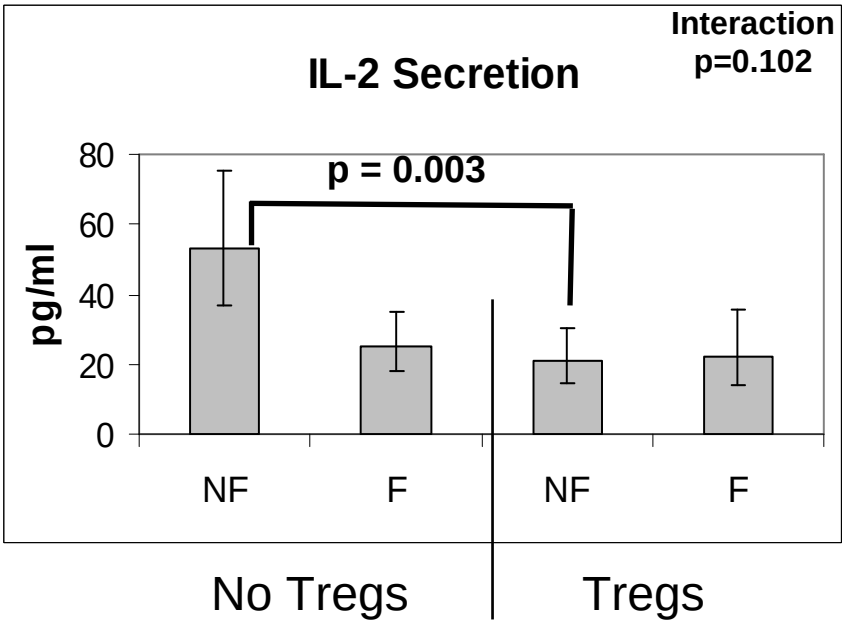


Figure 8. Concentration of IL-2 (pg/ml). NF = Non farming children; F = Farming children. NF = 10; F = 7.

3.2.7 Association of regulatory T cell frequency at age 6 years with sensitization and the asthmatic status

Impairment of Treg cells has been shown repeatedly to be associated with allergic diseases (Smith *et al.* 2008; Hinz *et al.* 2012), thus the association of Treg cells at the age of 6 and sensitization to inhalant and seasonal allergens was investigated. No associations between Treg cell numbers, in none of the stimulation conditions, and sensitization in 6 year old children were found (Table XV).

Table XV. Treg cells in sensitized and non-sensitized 6 year old children.

Outcomes		Treg M	P	Treg PI	P	Treg Lps	P
Inhalant IgE Class I	**No**	0.12 (0.07-0.21)	0.99	0.35 (0.19-0.67)	0.83	0.13 (0.06-0.21)	0.94
	Yes	0.13 (0.05-0.24)		0.32 (0.19-0.67)		0.13 (0.06-0.22)	
Inhalant IgE Class II	**No**	0.12 (0.07-0.20)	0.67	0.33 (0.19-0.65)	0.73	0.12 (0.06-0.21)	0.73
	Yes	0.16 (0.05-0.26)		0.41 (0.19-0.74)		0.13 (0.07-0.24)	
Inhalant IgE Class III	**No**	0.12 (0.07-0.21)	0.65	0.33 (0.19-0.66)	0.54	0.12 (0.07-0.20)	0.41
	Yes	0.18 (0.05-0.25)		0.45 (0.23-0.74)		0.14 (0.06-0.30)	
Seasonal IgE Class I	**No**	0.12 (0.07-0.21)	0.55	0.34 (0.19-0.67)	0.90	0.12 (0.06-0.20)	0.46
	Yes	0.16 (0.06-0.25)		0.34 (0.19-0.70)		0.13 (0.07-0.26)	
Seasonal IgE Class II	**No**	0.12 (0.07-0.20)	0.53	0.34 (0.19-0.66)	0.94	0.12 (0.06-0.20)	0.45
	Yes	0.18 (0.05-0.25)		0.37 (0.19-0.70)		0.13 (0.06-0.27)	
Seasonal IgE Class III	**No**	0.12 (0.07-0.22)	0.76	0.34 (0.19-0.67)	0.75	0.13 (0.07-0.21)	0.48
	Yes	0.21 (0.04-0.25)		0.37 (0.19-0.76)		0.15 (0.06-0.32)	

Values are shown as medians (percentages of Treg cells in relation to the lymphocyte gate) and 1st and 3rd quartiles. Class I ≥ 0.35 IU/ml; Class II ≥ 0.7 IU/ml; Class III ≥ 3.5IU/ml.

When children with current asthma were investigated, these had significantly higher Treg cells upon Lps-stimulation (0.26 *vs* 0.12 %, p = 0.02) in comparison to children without current asthma. This same pattern of higher Treg cells in children with current asthma was observed at baseline and after PI-activation, although not statistically significant (M, 0.12 *vs* 0.24 %, p = 0.27 and PI, 0.34 *vs* 0.50 %, p = 0.84).

3.2.8 *In vitro* modulation of IL-2 and IFN-γ depending on the asthmatic status

The suppressive capacity of Treg cells on effector T cells from asthmatic and non-asthmatic children did not show statistical differences. However there was an IL-2 and IFN-γ

modulation in response to PHA and Treg co-culture, depending on the asthmatic status (Figure 9).

Non-asthmatics showed a significant consumption of IL-2 in the presence of Treg cells in parallel to decreased IFN-γ ($p = 0.002$ and $p \leq 0.000$, respectively). This was not observed in the asthmatic group.

Also, asthmatic children produced significantly more IFN-γ when autologous Treg cells where added into the culture compared to non-asthmatics (in response to PHA, $p = 0.018$). APCs and effector T cells in culture (without Tregs) produced higher IFN-γ in the asthmatic group (Figure 9).

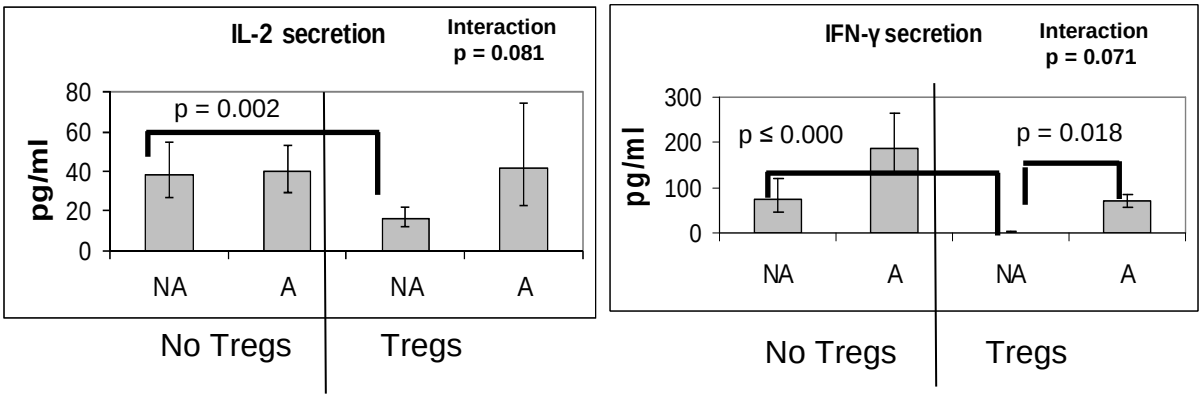


Figure 9. IL-2 and IFN-γ concentrations (pg/ml) in asthmatics and non-asthmatics at the age of 6 years. A) IL-2 secretion in response to PHA-stimulation, Antigen presenting cells, $CD3^+$ and effector T cells, $CD4^+CD25^-$ in culture. B) IFN-γ secretion in response to PHA-stimulation, Antigen presenting cells, $CD3^+$, effector T cells, $CD4^+CD25^-$ and Treg cells $CD4^+CD25^+$ in culture.

3.2.9 Farm exposures and clinical outcomes

Next, the influence of current exposures on the asthmatic current status (doctor diagnosis of asthma ever and any wheeze episodes in the last year) at the age 6 years was investigated. Contact to stable and drinking farm milk at age 6 was associated with lower current asthma prevalence (10.84 % *vs* 2.22 %, $p = 0.02$; 9.68 % *vs* 2.5 %, $p = 0.054$, respectively). Investigating how exposures affect the prevalence of asthma (a life time prevalence of a doctor's diagnosis of asthma ever and/or a repeated diagnosis of obstructive bronchitis and/or

children which are intermediate, late onset and persistent wheezers after the first 6 years and/or children with wheeze without a cold and symptoms between wheeze from age 18 months to age 6 years) during childhood overall showed that contact to stable, to hay and drinking farm milk, generally at ages of 4, 5 and 6 years, was associated with a lower prevalence of asthma (Table XVI). No association of the farm status itself with asthma prevalence was found.

Table XVI. Farm exposures during and association with the prevalence of asthma.

	Asthma prevalence % (n/N)					
	Farm milk		**Staying in stable**		**Contact to hay**	
	No	Yes	No	Yes	No	Yes
pg	29.03 (27/93)	24.42 (21/86)	31.51 (23/73)	19.59 (19/97)	28.28 (28/99)	18.57 (13/70)
p	0.48		0.075		0.147	
1y	29.91 (35/117)	20.97 (13/62)	28.72 (27/94)	22.89 (19/83)	27.56 (43/156)	21.74 (5/23)
p	0.199		0.377		0.556	
1.5y	29.25 (31/106)	23.94 (17/71)	32.91 (26/79)	21.88 (21/96)	29.13 (37/127)	22.00 (11/50)
p	0.437		0.101		0.337	
2y	29.90 (29/97)	23.17 (19/82)	34.94 (29/83)	20.00 (19/95)	29.82 (34/114)	21.54 (14/65)
p	0.312		**0.025**		0.229	
3y	35.05 (34/97)	17.28 (14/81)	34.21 (26/76)	21.57 (22/102)	31.78 (34/107)	19.72 (14/71)
p	**0.008**		0.060		0.076	
4y	34.41 (32/93)	17.65 (15/85)	35.06 (27/77)	19.80 (20/101)	32.97 (30/91)	19.54 (17/87)
p	**0.011**		**0.022**		**0.042**	
5y	37.21 (32/86)	16.67 (15/90)	37.65 (31/83)	17.20 (16/93)	31.91 (30/94)	20.73 (17/82)
p	**0.002**		**0.003**		0.094	
6y	32.26 (30/93)	18.75 (15/80)	36.14 (30/83)	16.67 (15/90)	32.95 (29/88)	18.82 (16/85)
p	**0.044**		**0.004**		**0.034**	

Results are shown in median percentages and total numbers. Pg = pregnancy, y = year(s). Significant results are marked in bold.

4. Discussion

Allergic diseases have reached epidemic proportions in the last years. There is the crucial necessity to understand the underlying mechanisms to, in the long run, develop prevention strategies and effective therapies. The complexity of these diseases relies on an intricate interplay between genetics and environmental exposures, which seems to shape the immune system at a very young age, a critical time window for the development of allergic diseases.

In this study both genetic and environmental exposures associated with the development of allergic diseases were investigated. First, the impact of genetic variants within two crucial cell players, Th1 and Th2 cells, on immune modulation early in life (at birth) and their influence on the development of allergic diseases during the first 3 years of life was investigated. Second, the role of regulatory T cells within the "allergy-protective" farm environment and their association with allergic diseases including asthma was examined in older children (6 years old).

Several studies have shown that polymorphisms in the *STAT6* gene, a Th2 transcription factor, were associated with the IgE levels in adults and children (Schedel *et al.* 2004; Duetsch *et al.* 2002; Weidinger *et al*, 2004; Weidinger *et al.* 2008). Furthermore *STAT6* represents one of the candidate genes associated with asthma development (Duetsch *et al.* 2002). Up to date, no studies have been conducted to investigate whether genetic changes in *STAT6* can shape the immune system at birth and associated with disease development within the first 3 years of life.

In this study the influence of *STAT6* polymorphisms on the modulation of immune responses at birth was assessed.

The *STAT6* rs324011 polymorphism was associated with lower gene expression of Treg-related markers, *FOXP3*, *LAG3* and *GITR* in cord blood. Both *STAT6* polymorphisms,

rs324011 and rs1059513 were associated with changes in cytokine patterns, however not with mRNA expression of *Th1*, *Th2*, *Th9*, *Th17* or *Th22*-related genes (Casaca *et al.* 2013).

Carriers of *STAT6* rs324011 produced higher amounts of IFN-γ and lower TNF-α. These carriers had lower probability to develop atopic dermatitis and obstructive bronchitis during the first 3 years of life. On the other hand, neonates carrying the *STAT6* rs1059513 SNP presented increased TNF-α and GM-CSF but no associations were observed with disease development at the age of 3 years (Casaca *et al.* 2013).

In this study decreased of *LAG3* upon Derp1 and *FOXP3*, *GITR* and *LAG3* in different conditions using the recessive model were found in *STAT6* rs324011 neonate carriers. The consistent decrease of Treg-related genes is in accordance with studies that have shown that *STAT6* plays a role in the modulation of Treg cells (Milkova *et al.* 2009). Nonetheless, the exact mechanism is unknown and further studies are required. It could however be speculated that this finding might be related to changes in the NF-κB pathway. The *STAT6* rs324011 polymorphism creates a binding site for NF-κB T-allele specific in the *STAT6* gene which is not present in the wildtype (Schedel *et al.* 2009). Studies have shown that blocking NF-κB pathway inhibited upregulation of *FOXP3* expression (Milkova *et al.* 2009). Thus, it is possible that in the polymorphic allele carriers more NF-κB molecules will be bound to the specific binding site created by the rs324011, diminishing the availability of NF-κB which is required in the Treg-pathways. Another possible mechanism to explain the downregulation of Treg-related genes could involve direct binding of *STAT6* to the *FOXP3* gene and subsequent lower *FOXP3* promoter-activation (Takaki *et al.* 2008). This is plausible as *STAT6* rs324011 was shown to increase *STAT6* expression (Schedel *et al.* 2009). Of note, using the recessive model not only *LAG3* was downregulated but also *FOXP3* (Ppg/Derp1) and *GITR* (Ppg).

At protein level, Th1, Th2 and pro-inflammatory cytokine secretion was altered depending on the *STAT6* genotypes. This is in accordance with our previous reports showing cytokine

modulation already at birth depending on genetic variants located in allergy-associated genes (Casaca *et al.* 2012; Liu, Raedler *et al.* 2011; Lluis *et al.* 2011).

In this study the carriers of *STAT6* rs324011 not only had lower Treg-associated gene expression but also lower levels of TNF-α and skewed IFN-γ increased response. Taken altogether, these patterns of neonatal immune responses may be suggestive for an atopy protective phenotype.

Analysis of the relation between Treg modulation and cytokine secretion showed that homozygous carriers of the *STAT6* rs324011 had a characteristic pattern of correlation not observed in the heterozygous and wildtype neonates: they presented a high negative correlation of Treg markers with IFN-γ (statistical trend). In regards to the relation of Treg with Th2 and pro-inflammatory cytokines, the homozygous carriers of *STAT6* rs324011 showed a positive correlation of *FOXP3* expression with IL-5 and TNF-α. On the other hand, heterozygous and wildtype showed a negative correlation. These findings may reflect a potential combined regulation of Treg cells and the cytokine profile in cord blood, associated with the presence of both polymorphic alleles (rs324011) in the *STAT6* gene (Casaca *et al.* 2013). These patterns of correlation of the *FOXP3* findings were not observed in relation to the second *STAT6* SNP, rs1059513, indicating potential different modulation mechanisms than for *STAT6* rs324011.

In regards to the development of atopic diseases in relation to genetic changes in Th2 transcription factor, it was shown that children carrying *STAT6* rs324011 had a lower propensity to develop atopic dermatitis and obstructive bronchitis.

STAT6 polymorphisms have been associated with food allergy (and with food sensitization (Amoli *et al.* 2002; Hancock *et al.* 2012) however there was no association with diagnosis of food allergy in the present study. The missing effects may potentially be related to lack of power as only 3 children had a doctor diagnosis of food allergy, for this reason, it would be of great value to investigate the role of *STAT6* polymorphisms in a high-risk cohort.

Previous studies have shown that decreased Treg cell numbers at birth were associated with increased risk of developing egg allergy in early childhood (Smith *et al.* 2008; Hinz *et al.* 2012), and that *STAT6* rs324011 polymorphism was associated with increased IgE both in children and adults (Schedel *et al.* 2004, Duetsch *et al.* 2002; Weidinger *et al*, 2004; Smith *et al.* 2008). However, in the present study children with this polymorphism (also with decreased gene expression of Treg-genes at birth) had a lower association with atopic dermatitis and obstructive bronchitis.

The apparent discrepancy between our results and previous findings (Schedel *et al.* 2004, Duetsch *et al.* 2002; Weidinger *et al*, 2004; Smith *et al.* 2008) may be related to the fact that 1) disease development was assessed in the present study while the other studies investigated IgE levels (Schedel *et al.* 2004, Duetsch *et al.* 2002); 2) different stages of immune maturation, during the first 3 years of life (our study) *versus* adult (Duetsch *et al.* 2002) and 9-11 year old children (Schedel *et al.* 2004) and 3) differences in Treg assessment (Smith *et al.* 2008). In our study, despite having lower Treg-related gene expression these neonates had a robust Th1 response with high secretion of IFN-γ which might be associated with the observed lower risk of developing atopic dermatitis and obstructive bronchitis. In agreement with these findings, other studies have shown that decreased IFN-γ levels at birth were associated with increased risk for atopic diseases and wheeze (Herberth *et al.* 2010; Guerra *et al.* 2004). In the present study no associations were found for the other *STAT6* polymorphism rs1059513 and the development of atopic dermatitis, food allergy and obstructive bronchitis (Casaca *et al.* 2013).

In addition to study the role of Th2-related polymorphisms early in life, important Th1 SNPs were also examined. Polymorphisms in the crucial Th1 transcription factors *TBX21* and *HLX1*, showed altered cord blood cytokine patterns depending of the genotypes. IL-5 and IL-13, both Th2 cytokines, were up- or downregulated depending on the *HLX1* and *TBX21*

polymorphims after innate stimulation. Furthermore, gene expression of Th1 and Th2-related genes partially correlated with the cytokine patterns (Casaca *et al*. 2012).

Genetic variants in the *TBX21* gene were previously shown to be associated with asthma (Chung *et al.* 2003; Munthe-Kaas *et al.* 2008; Akahoshi *et al.* 2005), confirming the relevance of the Th1/Th2 balance on Th2-related allergic diseases.

Previous experiments have shown that *TBX21* rs17250932 leads to increased promoter activity of the *TBX21* gene (Suttner *et al.* 2009). This may potentially explain why the carriers of *TBX21* rs17250932 showed lower IL-5 and a trend for lower IL-13, as *TBX21* suppresses Th2 responses. Using retroviral gene transduction, Szabo and colleagues showed that overexpression of *TBX21* in Th2 cells decreased IL-4 and IL-5 production (Szabo *et al.* 2000). Another study showed that TBX21 could also suppress the expression of IL-13 and its promoter activity (Suzuki *et al.* 2008). Consistent with those studies, *TBX21* knockout mice had high production of Th2 cytokines (IL-4, IL-5, IL-13) (Lakos *et al.* 2006). Furthermore, these carriers of *TBX21* rs17250932 presented lower expression of *STAT6e* (Th2), an isoform which is a splicing variant that includes intron 17 and intron 18 (Schedel *et al.* 2009).

In the present study no changes in the cytokine responses were observed depending on *TBX21* rs11079788, however significant increased frequency of activated T cells and accordingly higher expression of Th1 and Th2 genes (*GATA3*, *HLX1*, *IRF1*) were found.

Similarly to *TBX21* polymorphism rs17250932, carriers of *HLX1* rs2738751 showed decreased IL-5, IL-13 and TNF-α representative of Th2 and pro-inflammatory responses.

An impaired Th1 response, characterized by lower IFN-γ secretion, was associated with the presence of *HLX1* rs12141189 SNP. Also at mRNA level, the homozygous neonate carriers had decreased *TBX21* expression. Since *HLX1* can be induced by *TBX21* (Mullen *et al.* 2002), the lower expression of *TBX21* observed in our study could potentially lead to a downregulation of *HLX1*. This polymorphism was also associated with a skewed Th2 response (higher IL-5, IL-13). Previously *HLX1* had been associated with an asthma-

protective effect observed in school-age children (Suttner *et al.* 2009). At a first glance these observations in the immune responses in neonates seem to be in contrast with an "asthma protective phenotype" as a diminished Th1 and exacerbated Th2 responses were observed. However direct comparisons cannot be done as immune maturation and environmental exposures during childhood need to be taken into account. Also, a possible feedback mechanism of subsequent reduction of Th2 is possible.

Similarly, *HLX1* rs3806325 was associated with increased *GATA3* and *STAT6* gene expression upon innate stimuli, which may indicate a Th2-biased response. In parallel, neonates carrying this SNP had increased levels of IL-3 and IL-6. This SNP was previously associated with a higher risk of asthma development in school-age children (Suttner *et al.* 2009). Of note, it has been demonstrated that this genetic variant has functional relevance and that it leads to higher expression of the *HLX1* gene by altering transcription factor binding of the *HLX1* promoter (Suttner *et al.* 2009).

The application of the recessive model, which compares homozygous SNP carriers *versus* heterozygous and wildtype, showed more significant differences, suggesting that the effect is stronger when carrying both polymorphic alleles. Taken together, it is important to note that different factors account for the development of allergic diseases and changes in the immune system due to maturation and specific exposures must be considered (Casaca *et al.* 2012).

The majority of the changes depending on *TBX21* and *HLX1* were upon innate stimulation (mainly LpA), thus the effects may be related to TLR4 pathway stimulation. Functional studies are necessary to better understand the mechanisms involved.

Regarding the polymorphisms within the Th1 pathway only one *TBX21* SNP rs11079788 was associated with less symptoms of atopic dermatitis.

Thus, it is necessary to further investigate these findings in larger cohorts in order to better understand the impact of these genetic variations in the development of allergic diseases in childhood. Nonetheless, more information will be obtained from our cohort during the follow-

up until the age of 6 years. This information will be critical since the allergic phenotypes are still evolving during the first years of life. Furthermore, lung function will be available to objectively assess asthma including airway obstruction at the age of 6 years.

It was shown in previous studies that the maternal atopic status can affect the impact of the polymorphisms on immune regulation (Liu, Raedler *et al.* 2011). In the present study, additional modulation by the maternal atopic status was not found in relation to the Th1 polymorphisms. However stratification of the data led to very small groups for some of the polymorphisms, which might constitute a limitation. In relation to Th2 polymorphisms, the small number of *STAT6* rs1059513 carriers did not allow stratification by the maternal status. Nevertheless the MAF was similar (for both for *STAT6* rs1059513 and rs324011) in the neonates from atopic and non atopic mothers, thus no differences were expected.

Overall, a few limitations of this study need to be considered. The cytokine secretion was measured in the supernatant of PBMCs bulk culture not being possible to identify the specific cell origin. Due to low cell availability, Treg cell numbers were only measured in little number of children, thus no being feasible to stratify by genotypes and properly analyse it. However in this study, this would have been valuable together with Treg-related gene expression in relation to the genotypes. In some analyses the statistical power might have been limited to the low frequency of polymorphisms. This could partly explain missing effects and stresses the need to replicate the study in larger high-risk cohorts as previously mentioned.

Multiple testing is also an important issue, particularly regarding the polymorphisms and birth immune outcomes. However adjustment for multiple testing would have not been valuable in the present analysis as the immunological parameters were highly correlated, and expression of Treg gene markers (*FOXP3*, *GITR* and *LAG3* with each other) and pro-inflammatory and Th2 cytokines were correlated.

The strength of this work is a comprehensive study of how the Th1/Th2 polymorphisms are associated with neonatal immune responses, assessed at protein and mRNA levels under several stimulation conditions, and further influence the development of allergic diseases at a very young age. Furthermore, continuation of the follow-up of this 200 children will provide further information on whether the polymorphisms can further shape disease development in childhood.

While in the first part of the thesis the role of genetic variants in immune modulation and allergy development early in life was presented, in the second part the relevance of environmental exposures such as the allergy-protective farming environment was investigated. The aim was to investigate whether farm itself and specific farm exposures modulate regulatory T cells at age 6 years and its relation to development of allergic diseases and asthma.

The German children assessed in this work belonged to the large international birth cohort PASTURE/EFRAIM study, which includes over a total of 1000 children across Austria, Finland, France, Germany and Switzerland.

An extensive amount of studies in different countries has consistently shown that children growing up on a farm or exposed to farm environment develop less atopy and asthma (Braun-Fahrländer *et al*. 1999; von Ehrenstein *et al*. 2000; Riedler *et al*. 2000; Klintberg *et al.* 2001).

Several pathways and cell sub-types are thought to participate in this phenomenon. It was shown that blood cells from farming children express significantly increased amounts of CD14 and Toll-like receptor 2 in comparison to non-farming children (Lauener *et al.* 2002), suggesting an important role for the innate immune system.

Furthermore, Treg cells, crucial for the maintenance of immune homeostasis, have also been implicated in the “allergy-protective farm effect”. Previously it was demonstrated that newborns from farming mothers have a higher number of Treg cells and also that Treg cells have an increased suppressive capacity in controlling the responses of effector T cells (Schaub

et al. 2009). A different study conducted in Australia with newborns from Papua New Guinean mothers, representing a traditional lifestyle with high microbial burden in comparison to neonates from Australian mothers, representing a western lifestyle showed no differences between the Treg suppressive capacity within both groups, and increased Treg frequency in the Australian neonates (Lisciandro *et al.* 2012). The differences between the studies could be due to different genetic background, different environmental exposures or technical differences in cell assessment.

Farms are known to be microbial heavy-loaded environments. In the GABRIELA study, bacterial and fungal taxa cultured in the settled mattress dust were more prevalent among children living on farms than children in the reference group. In the PARSIFAL study, the percentage of samples of mattress dust that were positive for bacteria was higher on mattresses from farm children (Ege *at al.* 2011).

A recent study in the same cohort has demonstrated that levels of Treg cells are still increased during early childhood years (4.5 years) (Lluis, Depner *et al*. 2013). In the present study Treg cell frequency was not associated with the farm status of the child at the age 6 years. However despite no observed differences between farming and non-farming children regarding Treg cell frequency, the investigation of current farm exposures at the age of 6 years, in particular spending time in the stable, was significantly associated with increased levels of Treg cells at baseline. Drinking farm milk and contact to hay showed the same direction of increased Treg cells at baseline, although effects were not significant.

In this context it is possible that higher Treg cell numbers, also shown in the aforementioned studies with younger children and newborns, might be indicative of an immune regulatory mechanism in order to suppress potential high T cell responses to the extremely rich microbial burden present in the farm environment.

In this study, investigation of the suppressive capacity of Treg cells also revealed no significant differences depending on the farming status. Assessing cytokines produced in

culture, non-farming children showed a higher decrease in IL-2 when autologous Treg cells were in culture in comparison to the farm children. Cytokine competition between different cell populations is known to be an important factor determining whether the immune system responds or tolerates an antigen (Höfer *et al*. 2012). IL-2 secretion and uptake is one of the best models of cytokine consumption as a balance mechanism (Höfer *et al*. 2012). Pandiyan and colleagues have used *in vitro* and *in vivo* (mouse) models to investigate the apoptosis of effector T cells as a consequence to the lack of cytokines resulting from consumption by Treg cells. It was shown that despite the fact that Treg cells did not affect the early activation or proliferation of effector T $CD4^+$ T cells, they induced effector T cell inhibition by cytokine deprivation. IL-2 concentration in culture supernatants with both effector and Treg cells was reduced in comparison to single effector T cell culture (Pandiyan *et al.* 2007).

In the present study while non-farming children showed a significant decrease of IL-2 in culture with Treg cells, farming children showed similar concentrations before and after addition of Treg cells. One potential explanation is that non-farming children might tend to respond strongly when cells are stimulated. Although not significantly different from the farm group, non-farm children had a stronger IL-2 secretion in response to PHA. Thus, Treg cells might activate the mechanism of IL-2 consumption in order to stop this exacerbated effector T cell response while the response and suppression is not so strong in the farm children group.

Several components have been suggested to explain the protective farm effect, including the microbial diversity (Ege *et al*. 2011), contact to animals (Remes *et al*. 2003), endotoxin levels (Braun-Farländer *et al*. 2002) and farm milk consumption (Riedler *et al*, 2001; Waser *et al.* 2007). Raw farm milk consumption was shown to be associated with a decreased risk for asthma and atopy (von Mutius, 2012) and early farm milk drinking (during the first year of life) showed an association with lower risk of asthma, atopic sensitization and hay fever (Riedler *et al.* 2001). A study that aimed to determine the key farm milk components that are associated with the protective effect has shown that total viable bacterial counts and total fat

content of the milk was not associated with asthma or atopy, however the increased levels of whey proteins seemed to be the most relevant components (Loss *et al.* 2011). In this study it was also shown that raw but not boiled milk was associated with asthma, atopy and hay fever.

In the present study, looking in more detail into specific farm exposures during childhood, drinking farm milk during the early years (1 and 1.5 years) showed a significant association with higher Treg cell frequency at baseline at the age of 6 years. Relation between drinking farm milk at such young ages might be reflected at 6 years. However careful interpretation is needed as drinking farm milk at that specific age does not exclude farm milk consumption before and after that timepoint. On the other hand, children who were drinking farm milk at the ages 4 and 5 years showed mildly lower levels of Treg cells when exposed to Lps in culture. Lps is ubiquitous in farm environments. This component is part of the outer wall of gram-negative bacteria, thus being a constant exposure to farm children for instance through animal contact (Braun-Faerländer *et al.* 2002).

A study by Tulic *et al.* has shown that the timing of the Lps contact (exposure) leads to different mechanisms. Exposing sensitized mice with Lps in the first days of sensitization abolished the hyperresponsiveness while exposure on later time points exacerbated the allergic responses (Tulic *et al.* 2000). On this note, children who drink farm milk, the majority farming children, might be exposed to higher levels of Lps since an early age through microbial contact. With continuous immune maturation they might potentially become less sensible to Lps exposure thus showing lower levels of Treg cells *in vitro*.

Moreover, this modulation through farm milk consumption is in line with the concept that farm milk might be one of the most important specific farm exposures for shaping the immune system.

In the present study no associations were found between Treg cell frequency and atopic sensitization measured by specific IgE against common allergens. Even applying a less strict cut-off such as Class I $\geq$ 0.35 IU/ml, no significant results were found. Studies from our lab

have shown that neonates from atopic mothers (risk factor) have decreased Treg cell numbers, Treg-marker expression and function (Schaub *et al.* 2008). At age 4.5 years Treg cell frequency was inversely associated with perennial IgE levels (Lluis, Depner *at al.* 2013). Tulic and colleagues have investigated the maturation of thymus-derived Treg cells from birth until 14 years and found that overall these cells could suppress proliferative responses of effector T cells. However, in nonatopic children Treg cell turnover and suppressive function increased with age and paralleled the increase in global thymic *FOXP3* mRNA expression. This development was delayed in atopic children and these presented lower levels of Treg cells (Tulic *et al.* 2012). However this was investigated within thymocytes which might not directly reflect the levels of Treg cells in peripheral blood and different geographical populations (Australian children).

In our study, investigation of children with current asthma revealed an association with increased Treg cells (the lifetime prevalence of asthma showed the same pattern of Treg association, yet this was not statistically significant). Qualitatively, the suppressive capacity to abolish $CD4^{+}CD25^{-}$ effector T cell division *in vitro* showed no significant differences between non-asthmatics and asthmatics, however the asthmatic group produced higher levels of IFN-γ at age 6 years. Previously it was shown that low IFN-γ secretion at birth was associated with allergen-specific IgE antibodies (Kondo *et al.* 1998; Pfefferle *et al.* 2008) and thus linked to sensitization. Over all, this emphasizes the importance of the timing, as children grow up and the phenotypes become established it is possible that the immune system tries to counteract the biased allergic responses. At age 6 years it is possible that Th1 cells from the asthmatic children counter-regulate *in vivo* in order to control the Th2 exacerbation immune response typical in asthmatics, thus adopting an increased IFN-γ production *in vitro*. While a decrease of IFN-γ at birth is associated with increase sensitization (Pfefferle *et al.* 2008).

In this study besides IFN-γ modulation in asthmatic children, IL-2 concentrations were also affected. As mentioned before, IL-2 consumption is a mechanism used by Treg cells to

suppress effector T cell responses (Höfer *et al.* 2012). The non-asthmatic children showed a significant decrease of IL-2 with Treg cells in co-culture; this was not observed in the asthmatic group. Consequently, this regulatory mechanism could be potentially impaired on the asthmatic group.

The inverse relation between growing up on farms and asthma development has been shown in several epidemiological studies (reviewed in von Mutius, Vercelli, 2010). Additionally, several studies have shown the importance of different farm exposures, such as farm milk consumption during pregnancy and early years, the number of animal species, contact to hay and spending time in stables, on asthma development (reviewed in Mutius, Vercelli, 2010). In the PARSIFAL study, Ege and colleagues showed that the asthma-protective effect of being raised on a farm could be attributed to pig farming, feeding silage, child's involvement in haying, farm milk and regular stay in animal sheds and barns (Ege *et al.* 2007).

The children who were in contact to hay, spending time inside the stables and also drinking farm milk, showed only half of the asthma prevalence rate of non-exposed children. Thus the present investigation reinforces the concept of farm exposures and the inverse relation to asthma development.

A few potential limiting aspects of the study need to be considered. For the Treg cell functional assays the number a few number of children were included due to cell availability, resulting in limited number of children. This did not allow further stratification into subgroups (e.g. asthmatic farmer *vs* asthmatic non-farmer). Confirmation is needed using a larger number of subjects. The cytokine concentrations give an overall picture, however it does not specify the origin cell, for instance, IL-10 can be both secreted by Th2 and Treg cells.

Further specific questions have risen and are planned for further analysis such as further stratifications in regards to the Treg cell analysis, the influence of boiling the milk, etc. A follow-up of this cohort at the age 10.5 years is currently ongoing.

The strengths of this study rely on the combination of a comprehensive investigation of 143 German children from a large international cohort at age 6 years with the assessment of regulatory T cells, both quantitatively and qualitatively (frequency and suppressive capacity), the investigation of sensitization and asthma including lung function test and an in-depth information obtained from detailed questionnaires assessing a wide range of farm exposure related questions, disease and clinical outcomes.

In conclusion, farm exposures in particular farm milk drinking and contact to hay were associated with the frequency of regulatory T cells. The specific farm exposures assessed, contact to hay, staying in stables and farm milk drinking, were significantly associated with lower asthma prevalence. Children with current asthma showed increased Treg cells but no significant association with sensitization. The farm or asthma status was not associated with Treg suppressive capacity. A modulation of IL-2 and IFN-γ may however play a major role in regulatory suppression of effector responses and requires further functional studies. Our results reinforce the concept of farm exposures in the protection of asthma.

This work has shown for the first time the importance of *STAT6*, *TBX21* and *HLX1* on shaping immune responses, at cytokine and gene expression levels, at birth and disease development during early years, in particular with atopic dermatitis and obstructive bronchitis. In addition, the present data has shown that farm exposures are inversely associated with asthma prevalence and Treg cells are increased in children with current asthma. Also, consumption of farm milk during particular childhood time point might be reflected on Treg cell levels at age 6 years. Furthermore, IL-2 consumption might be regulatory mechanism of suppression differently regulated in non-farming and non-asthmatic children.

Additional investigations in larger cohorts and functional studies are required to support the present report and further understand these and other immune mechanism of allergic diseases in children.

5. SUMMARY

Allergic diseases have exponentially increased during the last decades. The complexity of its aetiology is due to multifaceted interactions between genetic and environmental factors on the development of the immune system. While advances of technology have identified allergy susceptibility genes, functional assays are needed to better understand the underlying mechanisms. Epidemiological studies have consistently shown that rural/farm environments are protective for the development of allergic diseases, including asthma and atopic sensitization. Importantly, prenatal and early life exposures have been shown to confer the strongest protection effects. The mechanisms of how farming modulates the immune system are still not disentangled in detail but include regulation of innate receptors and Regulatory T cells.

In the herewith presented thesis, the following main findings were achieved in the context of genetic and immunological influences on development of allergic disease in two different birth cohort studies:

First, 200 neonates were assessed for genetic influence of polymorphisms on neonatal immune responses and development of allergic diseases in childhood. The present study suggested a role for polymorphisms in the Th2-pathway, particularly for *STAT6* rs324011, on immune regulation at an early stage of immune maturation, namely significantly lower Treg-associated gene expression and Th1-polarization. Polymorphisms in the Th1-pathway, namely the transcription factors *TBX21* and *HLX1*, were shown to be relevant in shaping early immune responses and mainly Th2 cytokines at birth. Th1 and Th2 genotype-related immune responses at birth were partially associated with development of allergic diseases and/or protection during early life. These children are currently followed until the age of 6 years to further investigate allergic and respiratory disease during age-related immune maturation.

Secondly, almost 150 children were investigated at the age of 6 years to assess the role of regulatory T cells in relation to farm exposures and clinical outcomes of allergic diseases. Our data indicated an inverse association of farm exposures and the prevalence of asthma during childhood. Children exposed to hay, stable and farm milk had a lower prevalence of asthma. Regarding underlying immune mechanisms, we have detected that children with contact to hay have increased levels of Treg cells and that farm milk intake earlier during childhood can still be partially reflected on Treg cells levels at age 6 years. Assessing Treg functional mechanisms, changes in cytokine secretion were observed depending on the farming and asthmatic status of the children, however confirmation in a larger number of children is required

In summary the present work indicated that Th1 and Th2 polymorphisms were associated with modulated immune responses already at birth and influenced allergic disease development during the first three years of life. Furthermore, farm exposures were associated with a lower prevalence of asthma and associated with modulation of regulatory T cell frequency in German children at age 6 years.

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7. TABLE INDEX

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8. FIGURE INDEX

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9. ABBREVIATIONS

Abs = Antibodies
AD = Atopic dermatitis
CBMCs = Cord blood mononuclear cells
CI = Confidence interval
CT = Cycle threshold
Derp1 = *Dermatophagoides pteronyssinus*
FOXP3 = Forkhead-Box-Protein P3
GATA3 = GATA-binding protein 3
GITR = Glucocorticoid-induced tumor necrosis factor receptor
GM-CSF = Granulocyte macrophage colony-stimulating factor
GMR = Geometric mean ratio
HT = Heterozygotes
HWE = Hardy-Weinberg equilibrium
IFN-γ = Interferon gamma
LAG3 = Lymphocyte-activation-gene 3
LpA = Lipid A
LPS = Lipopolysaccharide
MAF = Minor allele frequency
NF-κB= Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
OR = Odds Ratio
PBMC = Peripheral blood mononuclear cells
PI = PMA/Ionomycin;
PMA = Phorbol 12-myristate 13-acetate
Ppg = Peptidoglycan
RAST = Radioallergosorbent test
SD = Standard deviation;
SNP = Single nucleotide polymorphism
STAT6 = Signal transducer and activator of transcription 6
TCR = T-cell receptor
TGF-β1 = Transforming growth factor beta1
Treg cells = Regulatory T cells
U = Unstimulated
WT = Major allele homozygotes

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