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Role of regulatory T cells and Th17 cells during immune maturation for the development of atopic diseases in childhood influenced by genetic and environmental factors

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1 Introduction

1.1 Atopic diseases

Atopy - derived from the Greek word “atopia”, meaning “unusual” or “out of place” - refers to a predisposition towards an aberrant production of IgE as a response to common allergens. The prevalence of atopic diseases increased during the last half century (Devereux 2006). Clinical manifestations of atopic diseases may include atopic eczema, allergic asthma and/or allergic rhinitis (hay fever) (Biedermann *et al.* 1999).

Atopic eczema (AE) is an immune-mediated inflammatory skin disease (Biedermann *et al.* 1999) characterized by a defective skin barrier (Kliegman *et al.* 2011). Mainly younger children are affected (Leung 2003; Kliegman *et al.* 2011). While in industrialized countries 15 to 30% of children develop AE about 2 to 10% of adults suffer from the disease (Bieber 2008). AE patients present with intense pruritus and cutaneous reactivity, especially on the face, scalp and extremities. The chronic phase is characterized by lichenification or thickening of the skin (Kliegman *et al.* 2011).

Currently, 235 million people suffer from asthma worldwide. It is the most common chronic disease among children (WHO 2011) causing significant morbidity (Kliegman *et al.* 2011). Allergic asthma is a chronic inflammatory disease of the bronchial airways that results in episodic airflow obstruction. The clinical symptoms include wheeze, cough and shortness of breath. Allergic asthma commonly reveals its onset early in childhood and often persists in adulthood (Leung 2003; Kliegman *et al.* 2011). Although the cause of the disease is not clear to date, it has been associated with environmental exposures combined with inherent biologic and genetic susceptibilities (Kliegman *et al.* 2011).

Allergic rhinitis, also known as hay fever, is an allergic reaction in the nasal mucosa that causes nasal congestion, runny nose, itching and often conjunctival irritation and sneezing (Kliegman *et al.* 2011). About 10 to 20% of the global population are affected by allergic rhinitis (Brozek *et al.* 2010) which commonly begins in childhood but often develops in early adulthood (Leung 2003). The severity of

the disease is more pronounced in adults in their twenties and thirties (Biedermann *et al.* 1999). Allergic rhinitis can be elicited by either seasonal allergens including tree pollens or perennial allergens such as house dust mite. However, atopic diseases are often related and nearly 80% of infants with atopic eczema subsequently develop hay fever or asthma later in childhood (Leung 2003).

1.2 Immune regulation of atopic diseases

Atopic diseases (ADs) share similar mechanisms of allergic tissue inflammation but target different organs including the skin, lung and nasal mucosa. Several components of the immune system are involved in the development of ADs including T cells, antigen-presenting cells (APCs), IgE production, eosinophils and mast cells (Kliegman *et al.* 2011).

1.2.1 Immune regulation of atopic eczema

Atopic eczema (AE) patients may reveal two types of skin lesions due to inflammation: acute or chronic. Skin lesions observed in AE patients are a consequence of secretion of proinflammatory cytokines and chemokines by local tissue leading to extravasation of inflammatory cells. T heper (Th) cells, including Th1 and Th2 cells, play a crucial role in this inflammatory process. While cytokines released by Th2 cells are involved in both forms, Th1 cytokines are only increased in chronic skin lesions (Leung 2003; Kliegman *et al.* 2011). Inflammatory cells are attracted by chemokines to the sites of injury leading to accumulation of eosinophils, macrophages and Th2 cells in these regions (Kliegman *et al.* 2011).

Acute AE is characterized by an increased intercellular edema of the epidermis. APCs and macrophages coated with IgE are key in presenting the allergen to Th2 cells. In general, mast cells are found in normal numbers but at different stages of degranulation and an increase of perivenular T cell infiltrate is observed in the skin.

In contrast, chronic AE is characterized by a lichenified skin with hyperkeratosis, hyperplastic epidermis and minimal edema. IgE-coated Langerhans cells, dendritic APCs in the epidermis, accumulate in this region and macrophages in the dermal mononuclear cell infiltrate. Both, eosinophils and mast cells, reveal increased cell numbers. Hence, mast cells are degranulated and eosinophils release mediators causing tissue injury (Leung 2003; Kliegman *et al.* 2011). The chronic inflammatory response leads to tissue remodelling and fibrosis (Murphy *et al.* 2008; Kliegman *et al.* 2011).

1.2.2 Immune regulation of allergic asthma

In allergic asthma the presence of an allergen in the lower airways induces the activation of submucosal mast cells. Subsequently, an infiltration of exudates containing high amounts of eosinophils and other cell types including monocytes, lymphocytes, neutrophils, mast cells and basophils is induced (Kliegman *et al.* 2011). Th cells and other immune cells are activated to produce proinflammatory cytokines including IL-4, IL-5 and IL-13 and chemokines (Kliegman *et al.* 2011) which lead to the constriction of the bronchial tree and to an excessive production of mucus (Murphy *et al.* 2008). The continuous presence of proinflammatory molecules causes chronic inflammation of the lung and airway remodelling (Murphy *et al.* 2008).

1.2.3 Immune regulation of allergic rhinitis

The manifestation of allergic rhinitis is divided in two phases. In the early phase the contact of allergen with the IgE-coated mast cells leads to their degranulation (Leung 2003; Kliegman *et al.* 2011). The released mediators promote the adhesion of circulating leukocytes to the endothelial cells, whereas the respective chemoattractant cytokines promote the infiltration of inflammatory cells (lymphocytes, macrophages, eosinophils, neutrophils and basophils) through the nasal mucosa (Leung 2003; Kliegman *et al.* 2011). The late phase arises between 4 to 8 hours upon allergen exposure. While eosinophil released-mediators damage the epithelium, Th2 cells lead to an allergic response with an

increase in IgE production, mast cell recruitment and eosinophil chemoattraction (Leung 2003). It is very difficult to clinically differentiate the early- from the late-phase of allergic rhinitis (Leung 2003).

Traditionally, ADs have been described as immune-mediated diseases with a higher prevalence of Th2 cells, a subset involved in antibody switching to IgE (Biedermann *et al.* 1999). Yet, in AE both Th1 and Th2 cells seem to play a role in the immunopathogenesis (Murphy *et al.* 2008), suggesting that the immune mechanisms involved in atopy *per se* may differ depending on which type of AD is developed. Nevertheless, this pattern may be too simple and recent evidence suggests that other T cell subsets such as regulatory T cells (Treg) and Th17 cells are involved in the immunopathogenesis of ADs (Kliegman *et al.* 2011). However, the specific role of these T cell subsets in the development and persistence of these diseases is not fully understood to date. To increase the knowledge in this field, potential influences that can modulate the development of ADs need to be considered including genetic predisposition and changes in environmental factors (Kliegman *et al.* 2011).

1.2.4 The function of T cell subsets

1.2.4.1 The function of Th1 and Th2 cells

The T cell subsets - Th1 and Th2 - were first described in 1986 (Mosmann *et al.* 1986). In the early ’90s several evidences suggested the importance of Th2 cells in ADs (Romagnani 1994).

The effector functions of Th1 cells include activation of infected macrophages, clearance of intracellular pathogens and support of B cells to produce antibody isotypes. Th2 cell functions include promoting IgE production by B cells and activation of eosinophils that serve to eradicate helminthic infections (Murphy *et al.* 2008; Korn *et al.* 2009; Abbas *et al.* 2012). IFN-γ and IL-12 are required for Th1 differentiation while IL-4 triggers the differentiation of Th2 cells. Both T cell subsets express certain transcription factors (TFs) after their activation including *T-bet* (Th1) and *GATA-3* and *STAT6* (Th2). Th1 cells secrete high amounts of IFN-γ and Th2 cells are characterized by the secretion of IL-

4, IL-5, IL-10 and IL-13 (Murphy *et al.* 2008). The differentiation of both subsets is mutually exclusive since IFN-γ and IL-4 antagonize each other on different levels (Korn *et al.* 2009).

1.2.4.2 The function of regulatory T cells

Although the existence of a suppressor T cell was already suggested in the 1970s, regulatory T (Treg) cells were only distinguished in the late '90s. Within the past decade several types of Treg cells were identified, suggesting that this T cell subpopulation is a heterogeneous group of T cells with immune regulatory properties and different developmental origins (Murphy *et al.* 2008; Sakaguchi *et al.* 2010). Natural Treg cells (nTreg) are produced in the thymus. These $CD4^+$ T cells express high amounts of surface CD25 (IL-2 receptor) and the intracellular transcription factor FOXP3. When activated, nTregs secrete IL-10 and TGF-β (Murphy *et al.* 2008). They are anergic cells with suppressive capacity (Shevach 2006).

Other T cell populations also possess regulatory activity, named adaptive or induced regulatory T cells (iTregs). These iTregs are committed to a regulatory fate in the periphery following specific antigenic stimulation (Sakaguchi *et al.* 2010). This subset of cells encompasses IL-10-secreting Tr1 cells, TGF-β-secreting Th3 cells, and certain subsets of $CD8^+$ and $CD4^-CD8^-$ T cells that have previously also been related to adaptive regulatory functions (Shevach 2006; Sakaguchi *et al.* 2010). Th3 cells are produced in the mucosa and have been shown to play a role in oral tolerance (Murphy *et al.* 2008). In contrast, Tr1 cells are antigen-specific cells controlling inflammation and collateral tissue damage (Shevach 2006). Both subsets of T cells do not express FOXP3 (Curotto de Lafaille *et al.* 2009).

$CD4^+CD25^{high}$ and $CD4^+CD25^{high}FOXP3^+$ are used as descriptive markers for nTregs. Nevertheless, some Treg cells express "intermediate" amounts of CD25 and activated effector T cells can also express high amounts of CD25 and even transiently FOXP3 without being anergic or suppressive (Shevach 2006). Other markers are used to delineate the specificity of Treg cells in addition to $CD4^+CD25^{high}$, including low expression of IL-7 receptor ($CD127^{low/-}$), expression of CTLA-4 or

GITR. As these markers are also expressed by other activated effector T cells (Shevach 2006), the suppressive capacity is the most specific factor to identify a functional nTreg cell to date.

In the last years the methylation status of CpG motifs in *FOXP3* was demonstrated as a reliable marker for Treg cells in adults and mice (Baron *et al.* 2007; Floess *et al.* 2007). An evolutionary conserved region upstream from exon 1 with transcriptional activity, named Treg-specific demethylated region (TSDR), was identified. This TSDR region was fully demethylated specifically in nTregs but not in $CD4^+CD25^-$ T cells, *in vitro* induced $FOXP3^+$ Treg cells or transiently activated $FOXP3^+$ T cells (Baron *et al.* 2007; Floess *et al.* 2007).

1.2.4.3 The function of Th17 cells

In contrast to Treg cells, Th17 cells are important for the host defense against extracellular bacteria. However, the pathogenesis of some immune-mediated diseases including ADs is also regulated by this T cell subset population (Stockinger *et al.* 2007; McGeachy *et al.* 2008; Cosmi *et al.* 2011).

The main transcription factors of human Th17 cells are RORC2 (also known as RORC isoform b) and RORα (Korn *et al.* 2009). Other transcription factors, including STAT3 and aryl hydrocarbon receptor (AHR), also play an important role in Th17 cell regulation (Dong 2011). The cytokine milieu required for Th17 cell differentiation comprises TGF-β, IL-6, IL-21, IL-1β and IL-23 (Volpe *et al.* 2008; Korn *et al.* 2009). However, the latter cytokine and its receptor, IL-23R, have been suggested to be required for stabilization and commitment to the Th17-lineage rather than for differentiation (Korn *et al.* 2009). Th17 cells themselves characteristically secrete IL-17A, IL-17F, IL-21, IL-22 and IL-26 (Korn *et al.* 2009).

Th17 cells show an apparent plasticity and the T cell subset has been related to Th1, Th2 and Treg cells. Indeed, IFN-γ^+IL-17^+ T cells have been detected in human tissues expressing both *RORC2* and *T-bet* (Annunziato *et al.* 2007); IL-17^+IL-4^+ T cells were also produced by T cell clones of human circulating T cells (Cosmi *et al.* 2010). Furthermore, *in vivo* studies demonstrated that Treg cells can

adopt a Th17-like phenotype in the periphery, expressing *IL-17*, *FOXP3* and *RORC2* with suppressive function (Voo *et al.* 2009). Although the basis for this flexibility on maintenance and reprogramming the T cell fate are not clear yet, epigenetic modifications may potentially be involved (Dong 2011).

1.2.5 Role of regulatory T cells and Th17 cells in atopic diseases

1.2.5.1 Regulatory T cells in atopic diseases

Several studies suggest that an altered number or function of Treg cells may contribute to the exacerbated immune response observed in atopic individuals. However, present data is contradictory and the underlying mechanisms remain unclear (Ryanna *et al.* 2009). A deficiency in Treg suppression activity was observed in atopic subjects compared to non-atopic controls (Ling *et al.* 2004). In this study Treg cell function was even further impaired in individuals suffering from hay fever during the pollen season. Shi *et al.* reported increased Treg cell numbers in atopic asthmatics during acute exacerbation compared to controls, yet with similar Treg suppressive function in both groups (Shi *et al.* 2004). Treg cell numbers were also increased in peripheral blood mononuclear cells (PBMCs) of AE patients compared to asthmatics (Ou *et al.* 2004) and healthy controls (Ou *et al.* 2004; Hijnen *et al.* 2009). Similar suppressive activity was observed in all groups (Ou *et al.* 2004) and Treg cells were present in atopic skin lesions (Honda *et al.* 2011). Recently, similar Treg cell numbers were observed in asthmatic adults compared to controls, but FOXP3 expression of $CD4^{+}CD25^{high}$ cells was impaired in asthmatics (Provoost *et al.* 2009).

Treg cells are relevant in the immune homeostasis. During immune maturation - a time window where the immune system is more susceptible to external influences - Treg cells may therefore contribute to the development of ADs (Lloyd *et al.* 2009). Maternal atopy is a known risk factor for the development of atopic diseases in childhood (Liu *et al.* 2003; Moore *et al.* 2004). In cord blood of atopic mothers, Treg cells were already impaired in respect to numbers and function in such an early stage of immune maturation (Schaub *et al.* 2008). While Treg cells were decreased and less

suppressive in the airways, in peripheral blood of asthmatic children compared to healthy controls no change was observed (Hartl *et al.* 2007). Interestingly, inhaled corticosteroid treatment increased Treg cell numbers and restored their function in asthmatic patients (Hartl *et al.* 2007).

Thus, Treg cells seem to be involved in ADs but results between studies have been inconsistent. This may be due to two reasons i) some of the studies defined Treg cells based on CD4 and CD25 expression but did not include FOXP3 as a marker. Therefore, the population under study may potentially have included activated T cells; and ii) the majority of the studies assessed Treg cells in peripheral blood, which may not reflect the actual active location of disease (Ryanna *et al.* 2009).

1.2.5.2 Th17 cells in atopic diseases

In addition to Tregs, Th17 cells have also been related to ADs. In fact, Th17 cell levels were increased in peripheral blood of AE patients and were associated with the severity of AE (Koga *et al.* 2008). Immunohistochemistry analyses demonstrated that IL-17$^+$ cells were mostly increased in acute but not in chronic skin lesions (Toda *et al.* 2003; Koga *et al.* 2008). IL-17 levels were increased in serum of severe birch allergy patients out of the pollen season (Ciprandi *et al.* 2008) and were highly correlated with clinical symptoms and eosinophil counts in allergic rhinitis patients during the pollen season (Ciprandi *et al.* 2009).

Elevated IL-17 protein levels were found in sputum and bronchoalveolar lavage fluids (BALF) of asthmatic patients (Molet *et al.* 2001) and in plasma of allergic asthmatics (Wong *et al.* 2001). The secretion of IL-17 was also shown to be related to bronchial hyperreactivity (Barczyk *et al.* 2003). Furthermore, a positive relationship between increased Th17 cell numbers in peripheral blood and allergic asthma severity has been observed (Zhao *et al.* 2010). Consistent with previous findings, IL-17 and IL-17F, another cytokine secreted by Th17 cells, were elevated in lung tissues of severe asthmatics (Al-Ramli *et al.* 2009). Other studies suggested that higher IL-17 in bronchial airways (Chakir *et al.* 2003) and in sputum (Bullens *et al.* 2006) is related to asthma severity and to

neutrophilic inflammation (Bullens *et al.* 2006; Alcorn *et al.* 2010). Neutrophilia correlates with asthma severity and is enhanced in non-eosinophilic non-atopic asthmatics (Alcorn *et al.* 2010). Furthermore, IL-17 can lead to elevated IL-8, a neutrophilic chemoattractant that recruits and activates neutrophils (Linden 2001; Bullens *et al.* 2006). Thus, Th17 cells are suggested to be a key player in the pathogenesis of severe neutrophilic asthma (Alcorn *et al.* 2010).

Little is known about Th17 cells during immune maturation in respect to the development of ADs. IL-17 protein was expressed in cord blood at very low levels independent of maternal atopy (Schaub *et al.* 2008). Others showed increased levels of *IL-17* and *IL-22* mRNA expression in PBMCs of rhinitis and asymptomatic atopic school-age children. In asthmatic children Farfariello *et al.* were unable to confirm the gene expression of *IL-17* (Farfariello *et al.* 2011). However, *IL-22* mRNA levels were increased and correlated directly to disease severity in both asthmatic and rhinitis patients (Farfariello *et al.* 2011).

In summary, Th17 cells are involved in the inflammatory process and/or immunopathogenesis of ADs. Differences exist depending on the disease, e.g. in AE Th17 cells are detected during acute skin lesions while in asthma they are rather involved in the more severe cases. The specific role of Th17 cells in the immune regulation of these diseases and the underlying mechanisms are still unknown and further investigations are required.

1.3 Impact of environmental factors on the development of atopic diseases

ADs are complex multifactorial diseases influenced by environmental factors (Bach 2002).

Maternal atopy is known as a risk factor for the development of ADs in childhood, whereas paternal atopy may have a rather minor influence on its manifestation (Liu *et al.* 2003; Moore *et al.* 2004). Since the genetic contribution of parental atopy is expected to be equivalent between both parents, the

date suggests that the effect of maternal atopy on the risk of developing ADs in childhood surpasses genetic predisposition. Hence, the maternal immune status during pregnancy, affecting the environment *in utero*, may subsequently have an impact on the child's immune development (Schaub *et al.* 2008).

In contrast to maternal atopy, maternal farming exposures and growing up on a farm are protective factors for the development of ADs (von Mutius *et al.* 2010; Lluis *et al.* 2012), a phenomenon known as the "farm effect". Living on a farm often involves the exposure to farm-related components that may modulate the child's immune system towards an "allergy-protective" status. Several of these farm-related exposures have been shown to influence the risk for ADs including microbes (Braun-Fahrlander *et al.* 2002; Debarry *et al.* 2010; Ege *et al.* 2011) and grass arabinogalactans (Peters *et al.* 2010), farm milk consumption (Riedler *et al.* 2001; Waser *et al.* 2007; Loss *et al.* 2011), staying in stables (Riedler *et al.* 2001) or contact to hay (Ege *et al.* 2007).

The length of exposure to these environmental factors is important for their influence on the immune system and its subsequent risk of developing ADs. *In utero* and in early life exposures - a time window when the immune system is immature and is more susceptible to external influences - are of special relevance (von Mutius *et al.* 2010; Lluis *et al.* 2012). Regarding the "farm effect", contact to stables and to farm milk consumption before the first year of life showed a stronger protective effect against development of ADs than children exposed between year 1 and 5 (Riedler *et al.* 2001). Maternal exposure to animal sheds during pregnancy was associated with lower IgE production against seasonal allergens already in cord blood (Ege *et al.* 2008) and maternal contact to cats and farm animals was also associated with lower risk of AE early in life (Roduit *et al.* 2011).

Thus, the influence on immune regulation also depends on the type of environmental exposure. Maternal atopy decreased Treg cell numbers and function in cord blood *per se* (Schaub *et al.* 2008). When maternal farm exposure was taken into account an association with higher and more efficient Treg cells (Schaub *et al.* 2009) and increased Th1-associated cytokines (Pfefferle *et al.* 2010) in cord blood was observed. Exposure to farming environment also increased expression of innate receptors in

cord blood (Roduit *et al.* 2011) and in school-age children (Lauener *et al.* 2002; Ege *et al.* 2006). Indeed, prenatal exposure to stables rather than current exposures increased *TLR2/TLR4/CD14* expression in school age children (Ege *et al.* 2006).

Hence, environmental factors are a key factor for the risk of developing ADs, with stronger influence in early stages of immune maturation.

1.4 Impact of genetic predisposition on the development of atopic diseases

In addition to environmental factors, ADs are influenced by genetic predisposition. Several studies demonstrated that specific polymorphisms within the Th1 and Th2 pathways can influence the risk to develop ADs (Cameron *et al.* 2006; Schedel *et al.* 2008; Schedel *et al.* 2009; Suttner *et al.* 2009).

In the last years, Genome Wide Association Studies (GWAS) became a powerful tool to further understand the impact of specific polymorphisms on several complex diseases. For childhood asthma, two GWAS revealed a potential role for genetic variants of the 17q21 locus including *IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3*, and *GSDMA* (Moffatt *et al.* 2007; Moffatt *et al.* 2010). The association signal of 17q21 has been confirmed by many different studies (Moffatt *et al.* 2007; Bouzigon *et al.* 2008; Verlaan *et al.* 2009; Halapi *et al.* 2010; Moffatt *et al.* 2010) selectively for childhood (Moffatt *et al.* 2010). A further characterization revealed that asthma-associated polymorphisms within 17q21 influenced transcription levels of *ORMDL3* (Moffatt *et al.* 2007; Verlaan *et al.* 2009; Halapi *et al.* 2010) and *GSDMB,* (Verlaan *et al.* 2009; Halapi *et al.* 2010). However, the functional role of these genes in asthma development remains to be unravelled. The current challenge is to further understand the role of these genes, how can they affect immune regulation and subsequently influence disease development.

1.5 Aims

Much attention has been given to the Th1/Th2 unbalance in the development of atopic diseases(ADs). However, other T cell subsets including regulatory T (Treg) and Th17 cells have been shown to be involved in its pathophysiology. To date little is known about the underlying mechanisms implicated in the development and persistence of these diseases. Studying the behaviour of these T cell subsets during immune maturation, a period in which the immune system is more susceptible to external influences, is of special interest to gain knowledge on the immunopathogenesis of ADs.

The aim of the present study was to investigate the functional role of Treg and Th17 cells in relation to the development of ADs in childhood. In this context, several cross-sectional studies have demonstrated that farming lifestyle is a protective factor for the development of ADs in childhood. Maternal farming was associated with increased Treg cell numbers and higher suppressive capacity already at birth. In addition, specific maternal farm exposures were associated with *FOXP3* demethylation in the Treg-specific demethylated region (TSDR) in cord blood.

The following scientific questions are still open and were therefore addressed in this thesis:

1) To study whether *FOXP3* demethylation at the TSDR, shown as good and reliable natural Treg (nTreg) marker used in adults and mice, was an appropriate parameter to assess nTreg cell activity also in cord blood.

2) To investigate whether the differences observed in number and function of cord blood Treg cells from farming and non-farming families are persistent later in childhood and whether they may have an impact on the development of atopic diseases in a farming population at age 4.5 years.

3) To investigate whether Th17 cells, a cell population increased in atopic diseases, was influenced by maternal farm exposure, a known “allergy-protective” environment, in cord blood.

4) To study whether single nucleotide polymorphisms (SNPs) within the Th17-lineage pathway may have an influence on cord blood T cell subsets.

5) To investigate whether the previously described asthma-associated SNPs within the 17q21 region may influence T cell subsets and mRNA expression of genes located within the 17q21 region in cord blood.

2 Materials and Methods

2.1 Materials

2.1.1 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
Hydrogen chloride (HCL)	Sigma-Aldrich, Steinheim, Germany
Ionomycin	Sigma-Aldrich, Steinheim, Germany
Isopropanol 100%	Merck, Darmstadt, Germany
Lambda DNA	New England BioLabs, Ipswich, USA
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
Rotiszint® eco (LSC-Universalcocktail)	Carl Roth, Karlsruhe, Germany
RPMI 1640 + GlutaMax	Gibco, Carlsbad, USA
Sodium hydroxide (NaOH)	Sigma-Aldrich, Steinheim, Germany
Trypan Blue	Sigma-Aldrich, Steinheim, Germany
Tritium Methyl-H3-Thymidin	Amersham, Piscataway, USA
Trizma Base	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
Xylene cyanol	Merck, Darmstadt, Germany

2.1.2 Solutions and buffers

1% PFA	10g paraformaldehyde Ad 900mL ddH_2O 800µl 1N NaOH 30 min at 65°C 100mL PBS 10X until pH 7.4
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

2.1.3 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
QIAamp DNA Micro Kit	Qiagen, Hilden, Germany
QuantiTect Rev. Transcription Kit	Qiagen, Hilden, Germany
Roche LightCycler® 480 Probes Master	Roche Diagnostics, Mannheim, Germany
SYBR Green PCR Master Mix	Applied Biosystems, Foster City, USA

2.1.4 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
BZO Adhesive Optical Seal Film	Biozym Scientific, Hessisch Oldendorf, 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
S-Monovette	Sarstedt Nümbrecht, Germany
Zinsser polyvials (liquid scintillation)	Zinsser Analytic, Frankfurt am Main, Germany

2.1.5 Laboratory equipment

Additionally to the standard laboratory equipment the following devices were used:

Beta Counter LS 6000IC	Beckman Coulter, Fullerton, USA
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
FACScan	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
MoFlo XDP	Beckmann Coulter, Fullerton, USA

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

2.1.6 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
CHIP Bioinformatics Tools	http://snpper.chip.org/bio/
EndNote X3	ISI ResearchSoft, Berkeley, USA
Ensembl Genome Browser	http://www.ensembl.org/
FCS express v3	De Novo Software, Los Angeles, USA
Haploview	http://www.broad.mit.edu/mpg/haploview/
iCycler iQ Optical System Software v3.1	Biorad, Hercules, USA
International Hapmap Project	http://hapmap.ncbi.nlm.nih.gov
Microsoft Office	Microsoft, Redmont, USA
National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
SAS software package version 9.2	SAS Institute, Cary, USA
Sigmastat version 1.0	Systat Software Inc., Chicago, USA
Vector NTI 10	Invitrogen, Carlsbad, USA
Vertebrate Genome Annotation (VEGA) database	http://vega.sanger.ac.uk/
WinMDI 2.8	The Scripps Research Institute, La Jolla, USA

2.2 Methods

2.2.1 Subject recruitment

2.2.1.1 Characteristics of birth cohort studies

Two birth cohorts were used for the present study: the PAULINA (Schaub *et al.* 2008) and the PAULCHEN (Schaub *et al.* 2009) Study. Analyses of cord blood Th17 cells in a farming population and genetic polymorphisms within the Th17-lineage genes were assessed in the PAULCHEN cohort. The rest of the investigations, including regulatory T cell (Treg) demethylation and 17q21 genetic polymorphism analyses, were based on a combined dataset of both study populations.

2.2.1.1.1 The PAULINA Study (Paediatric Alliance for Unselected Longitudinal Investigation of Neonates for Allergies)

Cord blood of 161 children was collected in Munich, Germany (Schaub *et al.* 2008). Enrolment occurred from January 2005 until February 2007. Pregnant mothers were approached before delivery for consent and completed a detailed questionnaire assessing maternal and infant data. Inclusion criteria comprised healthy neonates and mothers with uncomplicated pregnancies. Exclusion criteria included preterm deliveries, multiple gestations, perinatal infections, fever around birth, maternal intake of medication and maternal chronic diseases. Approval was obtained from the local review board (Bavarian Ethical Board), Germany.

2.2.1.1.1.1 Questionnaire

Through the questionnaire potential covariates including sex, birth characteristics, race/ethnicity, siblings, maternal education, maternal smoking, parental atopic status and medication used by the parents were assessed. Maternal and paternal atopic status was determined as the presence or absence of an allergic illness through a doctor's diagnosis of asthma and/or eczema and/or hay fever (Schaub *et al.* 2008).

2.2.1.1.2 The PAULCHEN Study (Prospective Cord Blood Study in Rural Southern Germany)

84 pregnant mothers from rural southern Germany were enrolled in the study from July 2005 until September 2007 before birth (Schaub *et al.* 2009). Inclusion and exclusion criteria as well as potential covariates were equivalent to the PAULINA Study (See point 2.2.1.1.1). 82 cord blood samples were included in the study as they fulfilled all quality criteria. Informed consent was obtained from mothers for participation in the study, including cord blood collection. Approval was obtained from the local review board (Bavarian Ethical Board), Germany.

2.2.1.1.2.1 Questionnaire

Potential covariates were assessed by questionnaire including sex, birth characteristics, race/ethnicity, marital status of the mother, maternal education, maternal smoking, parental atopic status and medication used by the parents, siblings and atopic diseases of the siblings. Atopic status was determined as the presence or absence of an allergic illness through a doctor's diagnosis of asthma and/or eczema and/or hay fever (Schaub *et al.* 2009).

Furthermore, mothers completed a detailed questionnaire regarding rural lifestyle and farm-related exposures. Questions comprised whether mothers lived on a farm, if they ran and actively worked on the farm, which kind and numbers of farm animals lived on the farm, kind of fodder, specific farm-related jobs performed, time spent in the stables and/or barns during pregnancy, contact to farm animals during pregnancy, farm milk consumption during pregnancy (boiled/unboiled), children and pets living in the house and whether the mothers lived on a farm during their childhood. Maternal farm exposure was defined as the mother living and regularly working on a farm in the last 1 to 5 years and during pregnancy. Non-farming mothers lived in the same rural area but not on a farm.

2.2.1.1.3 The PASTURE/EFRAIM Study (Protection against allergy: Study in Rural Environments)

This prospective birth cohort study included children from rural areas in 5 European countries (Austria, Finland, France, Germany and Switzerland) (von Mutius *et al.* 2006). In brief, women living in rural areas were contacted during pregnancy. Women who lived on family-run livestock farms were

assigned to the farm study group, while women from the same rural areas, but not living on a farm, were recruited as the reference group.

The complete PASTURE/EFRAIM population consists of 1,133 children. For a subgroup of 325 German or French children follow-up at the age of 4.5 years was selected for measurements of immunological markers at this age. Data on Treg cells was available from 298 children including 157 German and 141 French children (91.7%). 27 children were excluded as not enough blood was available for the experiment or due to technical problems (Figure 1). According to the study design 50% of the subjects were farm children. Table I shows prevalence of typical farm exposures, potential confounders and atopic sensitization at age 4. The study was approved by the local research ethics committees in each country, and written informed consent was obtained from all parents (Lluis, Depner *et al.* Manuscript submitted).

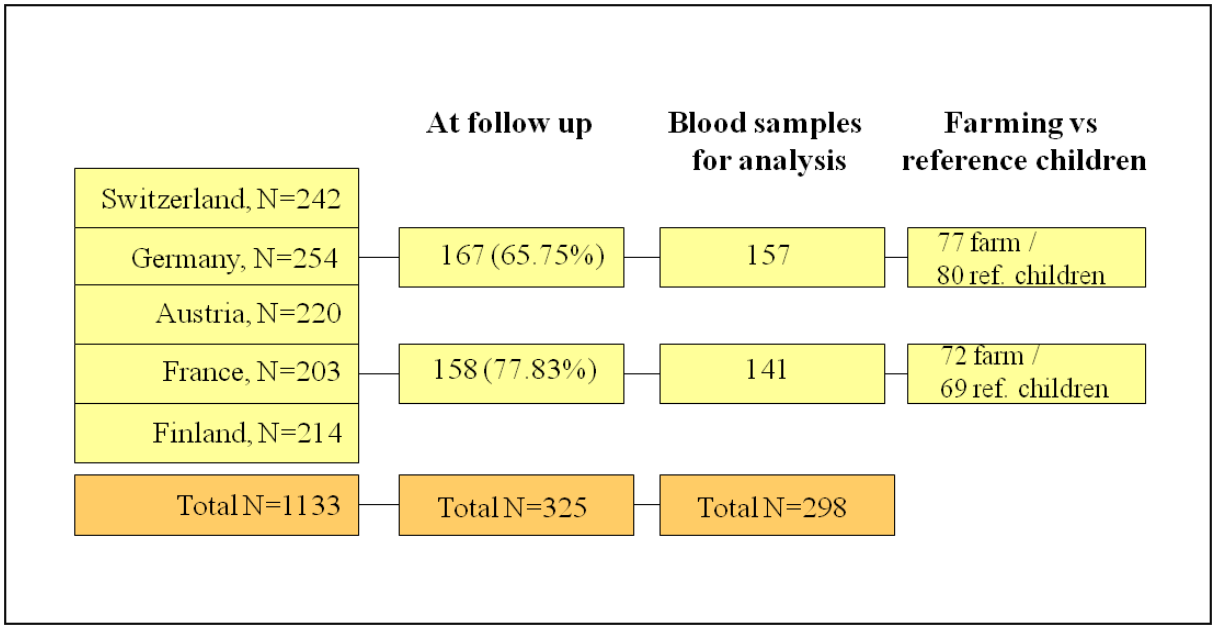


Figure 1. Study design for assessement of Treg cells at age 4.5 years.

Table I. Prevalence of exposures, potential confounders and atopic sensitization at 4.5 years.

A.

Exposures	No (N)	Yes (N)	Yes (%)
Farming	149	149	50.00
Stay in stables	127	171	57.38
Regular contact to hay	167	131	43.96
Farm milk consumption	150	148	49.66
- Only shop milk		135	47.70
- Shop or farm milk		48	16.96
- Exclusive farm milk		100	35.34
- Only shop milk		135	50.37
- Farm milk always boiled		51	19.03
- Farm milk unboiled		82	30.60

B.

Potential confounders	No (N)	Yes (N)	Yes (%)
≥ 2 older siblings	155	142	47.81
A female	202	96	32.21
Parental history of atopy	140	156	52.70
Smoking in pregnancy	254	39	13.31

C.

Atopic sensitization IgE≥3.5 IU/ml	No (N)	Yes (N)	Yes (%)
Inhalant IgE [#]	258	39	13.13
Food IgE[#]	284	13	4.38

[#] RAST class 3 ≥3.5IU/mL

2.2.1.1.3.1 Questionnaire

Parents completed questionnaires during pregnancy and at age 2, 12, 18, 24, 36, 48 months. The questions referred to the general health of the child and family, with focus on respiratory and ADs and different farm exposures. Questionnaires were based on items from previous studies including 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 study was approved by the local research ethics committees in each country, and written informed consent was obtained from all parents (von Mutius *et al.* 2006) (Lluis, Depner *et al.* Manuscript submitted).

Farm children were defined by the study design as children whose parents answered positively to the question 'does your child live on a farm?' and whose family ran the farm.

The analyses of farm-related exposures was primarily based on the questionnaire at age 4 years since Treg cell numbers were assessed at 4.5 years of age.

Analyzed farm exposures included:

a) Farm milk consumption. Children drinking at least once farm milk during the last twelve months were considered farm milk drinkers. Specific questions regarding farm/shop milk consumption and boiling status of consumed farm milk were also derived. Based on the questionnaire children were grouped into the following categories i) exclusive shop milk exposure, ii) mixed milk exposure (exposure to both shop and farm milk), and iii) exclusive farm milk exposure. The information on the boiling status of the milk drunken was used to subdivide the farm milk exposure into 'only boiled farm milk drinkers and 'any unboiled farm milk drinkers'. The latter included children consuming exclusively unboiled farm milk as well as those consuming both unboiled and boiled farm milk.

For farm milk consumption an additional variable "farm milk in childhood" was defined in which children with farm milk consumption in the 2^{nd}, 3^{rd} or 4^{th} year of life were stated as farm milk consumers during childhood while children who did not drink farm milk at that age were defined as the control group. A four-categorical variable showing the pattern of farm milk consumption in childhood and/or pregnancy was also created.

b) Being in stables. Children with a single contact to the stable were classified as children with stable contact.

c) Regular contact to hay. The group included children that were at least once a week in contact with hay.

Other relevant exposures were also assessed including type of farm (milkers, pigs, poultry or other animals), number of animal species, number of older siblings, maternal smoking during pregnancy, duration of breastfeeding and parental allergic status. Parental allergic status was defined as at least one of the parents suffering from asthma, allergic rhinitis and/or atopic eczema (Lluis, Depner *et al.* Manuscript submitted).

2.2.1.2 Cord blood samples for methylation study of regulatory T cells and for stimulation study with retinoic acid

Additional cord blood donors (N=25) were recruited randomly during January 2009 until January 2011 at the Munich University Maternity Hospital, Munich, Germany. Samples were used for cell isolation and regulatory T cell (Treg) methylation analyses (Liu *et al.* 2010) and for studying cord blood T cell subsets upon retinoic acid (RA) stimulation (Lluis *et al.* Manuscript submitted).

2.2.2 Blood collection and DNA extraction

2.2.2.1 Cord blood samples

Blood was collected in sodium heparin or EDTA containing tubes. Sodium heparin tubes were processed within the first 24h after blood collection with Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, USA) and cord blood mononuclear cells (CBMCs) isolated for further experiments. EDTA tubes were frozen at -80°C within the first 24h upon blood withdrawal. Tubes were subsequently thawed and DNA was extracted with the FlexiGene DNA Kit following manufacturer's instruction (Qiagen, Hilden, Germany).

2.2.2.2 The PASTURE/EFRAIM Study

Processing of blood samples occurred within the first 24h upon withdrawal. Blood was kept in a serum-gel tube and sodium heparin tubes. The serum-gel tube was centrifuged, serum aliquoted and kept at -80°C for IgE measurements. Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, USA) (Lluis, Depner *et al.* Manuscript submitted).

2.2.3 IgE measurements

For the PASTURE/EFRAIM study, specific immunoglobulin (Ig)E for six food and 13 common inhalant allergens was assessed at the age of 4.5 years in serum by the Allergy Screen test panel for atopy (Mediwiss Analytic, Moers, Germany) in a central laboratory. Food allergens included hen's egg, cow's milk, peanut, hazelnut, carrot, and wheat flour; inhalant allergens comprised *D. pteronyssius*, *D. farinae*, cat, horse, dog, Alternaria, mugwort, plantain, alder, birch pollen, hazel pollen, rye pollen, and a grass pollen mix. Sensitization against inhalant or food IgE was defined using RAST class 3 (≥3.5IU/mL) (Lluis, Depner *et al.* Manuscript submitted).

2.2.4 Isolation and culture of mononuclear cells

2.2.4.1 Birth cohort samples

CBMCs from the PAULINA and the PAULCHEN study population were isolated within 24h after blood collection by density gradient centrifugation with Ficoll-Paque™ PLUS following manufacturer's instructions (GE Healthcare, Piscataway, USA). Cells were washed with RPMI 1640 + GlutaMax (Gibco, Carlsbad, USA) and resuspended in medium 10% human serum (Sigma-Aldrich, Steinheim, Germany) at a final concentration of $5x10^6$cells/mL. Cells were stimulated with phytohemagglutinin (PHA, 5µg/mL), lipid A (LpA, 0.1µg/mL), peptidoglycan (Ppg, 10µg/mL) (all Sigma-Aldrich, Steinheim, Germany) and *Dermatophagoides pteronyssinus 1* (Derp1, 30µg/mL)

(Indoor Biotechnologies, Charlottesville, USA) for 3 days and compared to unstimulated cells, which were processed immediately. Endotoxin concentrations in Ppg, Derp1 and PHA were measured by Limulus assay. Analyses indicated low levels of endotoxin (<0.01EU/m=0.002ng/mL) which did not significantly change cytokine secretion. After incubation at 37°C in a humidified 5% CO_2 incubator cells were collected for flow cytometry analyses and/or mRNA extraction; and supernatants of CBMCs were frozen for cytokine measurements (Schaub *et al.* 2008; Schaub *et al.* 2009).

2.2.4.2 The PASTURE/EFRAIM Study

PBMCs from the PASTURE/EFRAIM study were isolated within 24h after blood collection by density gradient centrifugation with Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, USA). Cells were washed with RPMI 1640 + GlutaMax (Gibco, Carlsbad, USA) and resuspended in medium 10% human serum (Sigma-Aldrich, Steinheim, Germany) at a final concentration of $5x10^6$cells/mL. Cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24h unstimulated or stimulated with PMA (5ng/mL) and Ionomycin (1µg/mL) (PI) or lipopolysaccharide (LPS, 0.1µg/mL) (Sigma-Aldrich, Steinheim, Germany). After incubation cells were collected for flow cytometry analyses.

2.2.5 Cytokine expression

The concentration of cytokines in supernatants of CBMCs (PAULINA and PAULCHEN study) were measured with a Human Cytokine Multiplex Assay Kit, polystyrene bead-based, according to the manufacturer's instructions using LUMINEX technology (Biorad, Hercules, USA).

The lower limit of detection of the assay (pg/mL) was 1.1 (IL-2), 1.8 (IL-5), 0.5 (IL-6), 3.0 (TNF-α), 0.9 (IL-10), 2.1 (IL-13), 4.2 (IL-15), 0.2 (IL-17), 1.3 (IFN-γ), and 1.0 (GM-CSF) (Lluis *et al.* 2011) (Lluis *et al.* Manuscript submitted).

2.2.6 Flow cytometry analyses

2.2.6.1 Birth cohort studies

CBMCs from the PAULINA and the PAULCHEN study population were stained for surface markers of Treg cells (CD4, CD25) under stimulated (PHA, LpA, Ppg; incubated for 72h) (Sigma-Aldrich, Steinheim, Germany) and unstimulated (processed immediately) conditions.

Cells were resuspended in RPMI 1640 + GlutaMax (Gibco, Carlsbad, USA) at $1x10^7$cells/mL. 25µL were incubated with 22µL FACS buffer, 2µL CD4-FITC and 1µL CD25-PC5 (Beckman Coulter, Fullerton, USA) antibodies for 30min at 4ºC. The corresponding isotype controls IgG1-FITC (Dako Cytomation, Glostrup, Denmark) and IgG2a-PC5 (Beckman Coulter, Fullerton, USA) were used for single staining. The samples were washed twice with 1mL FACS buffer and resuspended in 200µL FACS buffer + 200µL 1% PFA (Sigma-Aldrich, Steinheim, Germany) for flow cytometry acquisition. Data were acquired by FACScan with CellQuest Software (Beckton-Dickinson, Heidelberg, Germany) and post-acquisition analyses were performed with the WinMDI 2.8 software (The Scripps Research Institute, La Jolla, USA).

2.2.6.2 The PASTURE/EFRAIM Study

PBMCs from the PASTURE/EFRAIM study population (from samples recruited in Germany and France) were incubated for 24h unstimulated or PI- and LPS-stimulated (Sigma-Aldrich, Steinheim, Germany).

Treg cells were stained using the CD4, CD25 and CD127 markers for a surface staining or by intracellular staining with the markers CD4, CD25 and FOXP3. Surface staining was assessed only for German samples and intracellular staining was performed for samples collected in Germany and France.

Cells were resuspended in RPMI 1640 + GlutaMax (Gibco, Carlsbad, USA) at $1x10^7$cells/mL and different cell subsets were analyzed including activated T cells ($CD4^+CD25^+$) and Treg cells (defined

as $CD4^+CD25^{high}CD127^{low/-}$ and $CD4^+CD25^{high}FOXP3^+$ in addition to $CD4^+CD25^+CD127^{low/-}$ and $CD4^+CD25^+FOXP3^+$).

For surface staining, $2.5x10^5$ cells were stained with 2µL CD4-FITC, 1µL CD25-PC5 (Beckman Coulter, Fullerton, USA) and 2µL CD127-PE (eBioscience, San Diego, USA) antibodies. The corresponding isotype controls IgG1-FITC, IgG1-PE (Dako Cytomation, Glostrup, Denmark)) and IgG2a-PC5 (Beckman Coulter, Fullerton, USA) were used.

For intracellular FOXP3 staining the Human Regulatory T Cell Staining Kit (eBioscience, San Diego, USA) or Human FoxP3 Buffer Set (BD Pharmingen, Franklin Lakes, USA) was used. 10^6 cells were stained with 8µL CD4-FITC, 4µL CD25-PC5 antibodies (Beckman Coulter, Fullerton, USA) and 10µL FOXP3-PE (eBioscience, San Diego, USA) in Germany or 3µL CD4-FITC, 3µL CD25-APC and 3µL FOXP3-PE (BD Pharmingen, Franklin Lakes, USA) in France. Corresponding FOXP3 isotype antibodies IgG2a-PE (eBioscience, San Diego, USA) or IgG1-PE (BD Pharmingen, Franklin Lakes, USA) were used as controls, respectively. Acquisition was performed with CellQuest or CellQuest Pro Software for FACScan and FACSCalibur (Becton-Dickinson, Heidelberg, Germany) and with FACS Diva software for FACS Canto II (Becton-Dickinson, Heidelberg, Germany). Analyses were performed with FCS express v3 (De Novo Software, Los Angeles, USA) and FACS Diva software (Becton-Dickinson, Heidelberg, Germany).

To objectively quantify Treg cells, $CD4^+CD25^{high}$ cells were defined as the upper 20% of $CD4^+CD25^+$ ($Upper20\%CD4^+CD25^+$). In Germany, a subjective gate defined by the investigator, named $CD4^+CD25^{high}$, was used for surface staining analyses. $CD127^{low/-}$ included the population where CD127 was lowly expressed and the negative CD127 population.

2.2.7 Isolation and suppressive function of regulatory T cells

To assess the suppressive function of regulatory T (Treg) cells, the division and the proliferation of effector T cells (Teff) were analyzed (Liu *et al.* 2010).

Antigen presenting cells (APCs) were isolated after positive depletion of $CD3^+$ cells with CD3 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and irradiated (30 Gy, 3000 rad, 10min). $CD4^+CD25^-$ effector T cells and $CD4^+CD25^+$ T cells were isolated (in a two-step-procedure) using depletion of non-$CD4^+$cells followed by positive selection (run twice) of $CD4^+CD25^+$ T cells with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). For the analyses of cell division, $CD4^+CD25^-$ T cells (2×10^4/well) labelled with 5µM CFSE (carboxyfluorescein diacetate succinimidyl ester) (Invitrogen, Carlsbad, USA) were incubated for 3 days with irradiated $CD3^-$ cells (4×10^4/well) in co-culture with/without $CD4^+CD25^+$ T cells (2×10^4/well) with 0.8µg/mL PHA stimulation (Sigma-Aldrich, Steinheim, Germany). Division was assessed by flow cytometry.

For proliferation analyses of effector cells $CD4^+CD25^-$ T cells (2×10^4/well) were incubated for 3 days with irradiated $CD3^-$ cells (4×10^4/well) in co-culture with/without $CD4^+CD25^+$ T cells (2×10^4/well) with 0.8µg/mL PHA stimulation (Sigma-Aldrich, Steinheim, Germany). Upon 72h, 25µL tritium methyl-H3-thymidin (1uCi/well) (Amersham, Piscataway, USA) was added. After additional 8h incubated 3-[H]-thymidin incorporation was assessed. The suppressive capacity of Treg cells was defined as the following:

$$\frac{(\textit{division or prolif. of Teff cells without Tregs}) - (\textit{division or prolif. of Teff cells with Tregs})}{\textit{division or prolif. of Teff cells without Tregs}}$$

2.2.8 FACS sorting of regulatory T cells and T effector cells

FACS sorting was performed to determine *FOXP3* demethylation at the Treg-specific demethylated region (TSDR), a marker for Treg cells, in isolated $CD4^+CD25^{high}$ cells, namely Treg cells; and in $CD4^+CD25^-$ cells of CBMCs (Liu *et al.* 2010).

Upon isolation of CBMCs using Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, USA) within the first 24h after blood collection, cells were stained with the surface markers anti-human CD4-FITC (40µL) and CD25-PC5 (20µL) (Beckman Coulter, Fullerton, USA) per 1×10^7 cells and sorted by

FACS MoFlo XDP (Beckman Coulter, Fullerton, USA) in two subpopulations: $CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{high}$ cells. DNA extraction was performed with QIAamp DNA Micro-Kit following manufacturer's instructions (Qiagen, Hilden, Germany) and *FOXP3*-methylation specific real-time PCR as described in section 2.2.9.

2.2.9 *FOXP3* methylation analyses

FOXP3 methylation analysis was performed in the Treg-specific demethylated region (TSDR) of *FOXP3* locus in cord blood EDTA and in isolated T cell subsets (Liu *et al.* 2010).

Genomic DNA was isolated from EDTA-handled cord blood with FlexiGene DNA Kit following manufacturer's instructions (Qiagen, Hilden, USA). From 2mL EDTA-blood, a recovery of 300–900µg/µL DNA was sufficient for the 20ng of DNA required for methylation analyses. In case of FACS sorted samples, DNA was isolated with QIAamp DNA Micro-Kit (Qiagen, Hilden, USA) (see section 2.2.8). For measurement of the *FOXP3* TSDR real-time-PCR was performed by a collaborator (Epiontis GmbH, Berlin, Germany) in 20µL using Roche LightCycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany) containing 15pmol of methylation or non-methylation-specific forward and reverse primers for TSDR, 5pmol hydrolysis probe, 200ng lambda-DNA and 30ng bisulfite-treated genomic DNA template or respective amount of plasmid standard (Baron *et al.* 2007). Each sample was analyzed in triplicate using a Light-Cycler 480 system (Roche Diagnostics, Mannheim, Germany). Of note, *FOXP3* is a X-linked gene. In females, having two X-chromosomes, Treg cell X-inactivation leads to complete inactivation and methylation of the bar body and one out of the two X-chromosomes in Treg cells of female subjects remains fully methylated. The other "open" X-chromosome is demethylated in female Treg cells. This does not apply to male subjects as they carry only one X chromosome which is therefore demethylated. For calculation of Treg ratios in a given cell sample, it is necessary to multiply with a factor of 2 in all samples of female subjects in order to determine the Treg number (Liu *et al.* 2010).

2.2.10 Real-time RT-PCR

Total RNA, isolated with TRIzol Reagent (Invitrogen, Carlsbad, USA) and reverse transcription of 1µg RNA with QuantiTect Rev. Transcription Kit were performed according to the manufacturers´ instructions (Qiagen, Hilden, Germany). mRNA-specific oligonucleotide primers (Forward/Reverse) of housekeeping gene *18S rRNA*, the Treg specific transcription factor *FOXP3*, and Th17-lineage related markers *RORC* (isoform b, primers "RORC3" and isoforms a+b, primers "RORC4"), *RORA, IL-17, IL-17F, IL-22* and *IL-23R* and the genes located within the asthma-associated 17q21 region *ORMDL3, GSDMA, GSDMB, ZNFN1A3, ZPBP2* and *PSMD3* were designed with Vector NTI Advance10 (Invitrogen) (Table I). Direct detection of the PCR product with iCycler iQ™ Real Time PCR (Biorad, Hercules, USA) was monitored by measuring the increase in fluorescence caused by binding of SYBR Green (Applied Biosystems, Foster City, USA) to double-stranded DNA. The determined cycle threshold (CT) was set in relation to the amplification plot of *18S rRNA*. The CT is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value, which was set to the log-linear range of the amplification curve. For the calculation of delta CT (Δct), the difference in CT values of two genes was used. A higher Δct resembles lower mRNA expression (Gibson *et al.* 1996; Liu *et al.* 2010; Lluis *et al.* 2011) (Lluis *et al.* Manuscript submitted).

Table II. Primer sequences (Forward and Reverse).

Gene	Primers
18SrRNA	5'-AGTCCCTGCCCTTTGTACACA-3'
	5'-GATCCGAGGGCCTCACTAAAC-3'
FOXP3	5'-ACCTTCCCAAATCCCAGTGC-3'
	5'-GAAGATGGTCCGCCTGGC-3'
GITR	5'-GCACCACCCTTGTCCCCC-3'
	5'-CAGCGTTGTGGGTCTTGTTCC-3'
LAG-3	5'-CAATGGCGACTTTACCCTTC-3'
	5'-CCTCTGGGATGGGGTGTC-3'
RORC3 (Isoform b)	5'-GGCTCAGTCATGAGAACACAAATTGAAG-3'
	5'-ATCGGTTTCGGCTGGTGCG-3'
RORC4 (Isoform a+b)	5'-CATCTTTGACTTCTCCCACTCCC-3'
	5'-TGGCTACACAGGCTCCGAAG-3'
RORA	5'-GCTTCTTTCCCTACTGTTCGTTCAC-3'
	5'-GCAGGTTTCCAGATGCGATTTAG-3'
IL-23R	5'-TGATCGTCTTTGCTGTTATGTTGTC-3'
	5'-TGTAGGCTTGTGTTCTGGGATG-3'
IL-17	5'-CACAATCCCACGAAATCCAGG-3'
	5'-CACTTTGCCTCCCAGATCACAG-3'
IL-17F	5'-TGTGCCAGGAGGTAGTATGAAGC-3'
	5'-GGTCTCTTGCTGGATGGGAAC-3'
IL-22	5'-CTCAGCAACAGGCTAAGCACAT-3'
	5'-GGAGTTTGGCTTCCCATCTTC-3'
ORMDL3	5'-GGACCAGGGCAAGGCGAG-3'
	5'-CACGCTCATCAGGGACACGG-3'
GSDMA	5'-GAAGGTGAAGGGAACGGCAG-3'
	5'-CGGCTCGCTCCAGTGTGAC-3'
GSDMB	5'-TTTCAGGCAGTTTCCAGGGC-3'
	5'-CGGTCGCTTTTCAGGGTTTC-3'
ZNFN1A3	5'-CCCAGCCAATGAAGATGAAGAC-3'
	5'-GTTCATCTTTCCACTGGTTGGC-3'
ZPBP2	5'-GATTTTTTGGAGCCTTTGTCTGG-3'
	5'-GTATCTTCACAGTCAACAGATCCATTG-3'
PSMD3	5'-GCCAGCATCAACCACGAGAAG-3'
	5'-TGCTGTTCTCGCTCACGCC-3'

2.2.11 Retinoic acid stimulation in cord blood mononuclear cells

Th17 and Treg cells were analyzed in CBMCs upon stimulation with retinoic acid (RA), a known modulator of Treg/Th17 regulation in mice and adults (Mucida *et al.* 2007; Bai *et al.* 2009).

Both unstimulated or PHA stimulated (5ug/mL) CBMCs were incubated with or without RA. Three different doses of retinoic acid were used: 2.5nM, 50nM, 100nM. Cells were harvested after 48h for gene expression analyses of *FOXP3*, *RORC* and *IL-23R*. (Lluis *et al.* Manuscript submitted).

2.2.12 Single nucleotide polymorphisms selection and genotyping

Genotype data for seven single nucleotide polymorphisms (SNPs) within the Th17-lineage genes *RORC*, *IL-23R* and *IL-17* including approximately 5kb boarders up- and downstream of each genetic region was extracted from the MAGICS/ISAAC discovery dataset (Moffatt *et al.* 2007) using the PLINK software package version 1.07 (Purcell *et al.* 2007). SNPs associated with asthma in the MAGICS/ISAAC discovery dataset were identified (Moffatt *et al.* 2007) and genotyped in the PAULCHEN study population for functional assessment. Additionally, rs2275913 was included in this study due to its potential functional relevance in IL-17 regulation and asthma (Chen *et al.* 2010; Espinoza *et al.* 2011) (Lluis *et al.* Manuscript submitted). Furthermore, 10 SNPs covering the 17q21 locus were selected based on the previous GWAS where these 17q21 polymorphisms were associated with the development of asthma in 2 independent study population of children (Moffatt *et al.* 2007) and genotyped in the PAULCHEN and PAULINA study. Genotyping was performed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Ding *et al.* 2003; Schedel *et al.* 2004) at the Institute of Epidemiology (Helmholtz Centre Munich, Neuherberg, Germany). The genotyping success-rate was at least 94%, whereas rs2290400 in 17q21 region was excluded from all analyses because genotyping was not successful. None of the polymorphisms significantly deviated from Hardy-Weinberg equilibrium (HWE) with the level of significance set to $p \leq 0.05$ (Lluis *et al.* 2011).

2.2.13 Statistical analyses

Data analyses were performed with SigmaStat 1.0 and SAS 9.2 software as depicted below in detail.

2.2.13.1 **FOXP3** *methylation analyses*

Correlations of *FOXP3* demethylation with other Treg markers including *FOXP3* gene expression and $CD4^{+}CD25^{high}$ T cells were analyzed using Pearson Product Moment Correlation or Spearman Rank Order Correlation based on distribution of the data. The comparison of the suppressive capacity of Treg cells (%) in the samples below and above the median of *FOXP3* demethylation (%) was analyzed with T-test. Statistical significance was defined by $p \leq 0.05$. Analyses were performed with Sigmastat version 1.0.

2.2.13.2 Regulatory T cells in 4.5 year-old farming children (the PASTURE/EFRAIM Study)

Percentages of specific cells relative to the lymphocyte population were used as Treg cell markers. As the distribution of Treg cells (%) was partly not normally distributed, all markers for Treg cells were log10-transformed, and 0 values in the percentages were set to a minimal value of 0.005 before log-transformation.

Data analyses for Treg cell markers on the surface were performed only in the German subgroup while intracellular analyses were performed in both, German and French children. Intracellular data were also analyzed stratified by center.

Association between Treg markers and farm-related variables were analyzed by regression analyses of the log-transformed variables. Geometric mean ratios and 95%-confidence intervals (CI) are reported. Additionally, non-parametric Wilcoxon or Kruskal-Wallis tests were used (to confirm the results).

Associations between atopic sensitization and Treg markers were analyzed by logistic regression. Odds Ratios and 95%-CI are given on the log 10-scale. All analyses for intracellular staining were

adjusted for center. Additional relevant covariates included in some of the models are mentioned in the respective results section.

Statistical significance was defined by $p \leq 0.05$, and all analyses were performed using SAS 9.2.

2.2.13.3 Th17 cells in cord blood of a farming population (the PAULCHEN Study)

Gene expression of Th17-lineage related markers were analyzed in cord blood samples of a farming population. Non-detectable samples were set to 38 CT to be included in the analyses. In the statistical analysis (Ballenberger *et al.* Manuscript submitted) these values were considered to be right censored as being ">38". The data were not normally distributed and could not be transformed to normality. Hence, non-parametric tests and models were applied. Summary statistics such as the median and its confidence interval was conducted by the Kaplan-Meier method (Kaplan *et al.* 1958), which is a non-parametric procedure taking right censoring at multiple detection levels into account.

Testing on group differences without adjusting for covariates was performed by the generalized Wilcoxon test (Prentice 1978), which tests whether the survival functions of two or more groups statistically differ. Its application to environmental settings with censored data is recommended by NADA (Helsel 2005). In order to adjust for covariates the Tobit model (Tobin 1958) was applied to the ranks of the original data. Rank transforming was conducted as the assumption of normality was violated. According to Iman and Conover (Iman *et al.* 1979) regression on rank transformed data is a distribution free procedure. Tobit regression (on ranks) takes both left and right censoring with multiple detection levels into account.

Additionally, an interaction term was added to the model which was defined as the product of a dichotomous and a continuous variable, meaning that the influence of one variable on the other is dependent of the level of a third variable. An interaction effect with $p \leq 0.05$ was considered to be significant.

Statistical analysis was performed with SAS 9.2. Statistical significance was defined by $p \leq 0.05$.

2.2.13.4 Retinoic acid stimulation experiments

Gene expression of Treg and Th17 markers was analyzed in cord blood before and upon stimulation with retinoic acid (RA) at different doses. Non-detectable samples were set to 38 CT to be included in the analyses.

Summary statistics such as the median and quartiles was conducted by the Kaplan-Meier method (Kaplan *et al.* 1958). For the comparison of paired censored observations the paired Prentice-Wilcoxon test was performed (O'Brien *et al.* 1987). A p-value ≤ 0.05 was considered to be significant. Statistical analysis was performed with SAS 9.2.

2.2.13.5 Single nucleotide polymorphisms in Th17-lineage genes

For Th17-lineage SNPs, Th17 and Treg cell markers were analyzed on a protein and mRNA level. Th1 and Th2 cytokines were assessed at protein level.

For PCR analysis, non-detectable samples were set to 38 CT and after cytokine measurements, non-detectable concentrations were set to 0.01pg/mL to be included in the analyses. In the statistical analyses (Ballenberger *et al.* Manuscript submitted) these values were considered to be right and left censored as being ">38" and "<0.01" respectively.

Summary statistics such as the median and quartiles was conducted by the Kaplan-Meier method (Kaplan *et al.* 1958).

Testing on group differences was performed by the generalized Wilcoxon test (Prentice 1978) using SAS 9.2. Statistical significance was assigned to values $p \leq 0.05$.

A recessive model (recessive on the rare allele) was used comparing carriers of the rare allele in comparison to the two other putative allelic states together. A dominant model was applied to compare the homozygous wildtype (WT) carriers vs. the heterozygous and homozygous SNP carriers. The models increased the statistical power and avoided the heterozygous effect observed in the linear model (Lluis *et al.* Manuscript submitted).

2.2.13.6 Single nucleotide polymorphisms in 17q21 region

As described in Lluis *et al.* (Lluis *et al.* 2011), all SNPs were tested for deviation from Hardy-Weinberg equilibrium by χ^2 test. Linkage disequilibrium (LD) between all SNPs was calculated by using R^2. The R^2 threshold was set to 0.85 to determine tagging SNPs, each representing one tagging bin. Bins correspond to LD blocks. LD patterns in our population were highly comparable to the data reported for the HapMap CEU (Utah residents with Northern and Western European ancestry from the Centre d'Etude du Polymorphisme Humain collection) population (http://hapmap.ncbi.nlm.nih.gov). For the combination of tagging SNPs, haplotype frequencies were estimated by using the expectation-maximization algorithm. Because allele frequencies for most of the 17q21 SNPs were close to 50%, the respective risk allele (instead of the minor allele) was reported. The risk allele was defined as the allele increasing the risk for the development of childhood asthma as described in the GWAS publication (Moffatt *et al.* 2007).

Risk and non-risk haplotypes were defined. The non-risk haplotype contains non-risk alleles only of the 4 tagging SNPs, whereas the risk haplotype contains of all respective risk alleles. An allele-counting model was assumed for the genotypes (0, homozygous non-risk; 1, heterozygous; 2, homozygous risk).

For RT-PCR analysis, the Wilcoxon test was used for the comparison of ΔCT values because some ΔCT values were not normally distributed. A Bonferroni-based correction was used to adjust for multiple testing. As we tested gene expression in relation to 4 tagging SNPs after no stimulation and innate and adaptive stimulation (N=3), we used a significance threshold of 0.05/12=0.004 for SNP analyses. Data below the multiple testing significance threshold of $p \leq 0.004$ were marked (*) in all figures.

Multiple regression analysis and Kruskal-Wallis tests were performed to test for differences of cytokine concentrations for different genotypes. Non-detectable concentrations were set to 0.01 pg/mL to include the subjects in the analysis. Because the distribution of cytokine levels was skewed, variables were log-transformed, and geometric mean ratios (GMRs) and 95% CIs were computed.

Validating the results by methods that take censoring into account (generalized Wilcoxon test and Tobit model) resulted in comparable data. All GMRs were reported in an unadjusted model (crude) or in an adjusted model for maternal atopy. Statistical significance was defined by $p \leq 0.05$.

3 Results

3.1 The role of regulatory T cells during immune maturation

Regulatory T (Treg) cells are a population of the immune system capable to suppress the immune responses of other cells and to maintain tolerance to self antigens. In the last years they have been related to the pathogenesis of atopic diseases (ADs). However their role during immune maturation in the development of ADs in childhood remains unclear.

In this study, Treg cells were examined during early immune maturation at different levels. First, the reliability of a good quantitative marker for Treg cells in adults, *FOXP3* demethylation at Treg-specific demethylated region (TSDR), was assessed in cord blood. Second, Treg cells were analyzed in 4.5 year-old children in relation to farming exposure and their contribution in the allergy-protective effect observed in farming children.

3.1.1 *FOXP3* demethylation in cord blood as a reliable quantitative marker of regulatory T cells

The transcription factor FOXP3 is the most specific marker used to quantify Treg cells to date. *FOXP3* demethylation at the TSDR has been suggested as a novel and specific marker for natural Treg (nTreg) cells in adults (Baron *et al.* 2007). However, little is known about TSDR demethylation in nTreg cells early in life. Thus, a comparison between *FOXP3* demethylation to previously described Treg markers such as $CD4^{+}CD25^{high}$, *FOXP3* mRNA levels and Treg suppressive capacity was analyzed in cord blood mononuclear cells (CBMCs) (Liu *et al.* 2010).

CBMCs from 70 healthy neonates were isolated. Cells were either unstimulated or stimulated for 3 days with the microbial stimulus Lipid A (LpA). Treg cells were quantified by flow cytometry ($CD4^{+}CD25^{high}$), mRNA expression (*FOXP3*) and suppressive Treg function was analyzed. *FOXP3* demethylation at TSDR was investigated in whole EDTA-blood. Cord blood $CD4^{+}CD25^{-}$ and

$CD4^+CD25^{high}$ cells were sorted by FACS and FOXP3 protein expression or demethylation at TSDR was assessed (Liu *et al.* 2010).

3.1.1.1 FOXP3 *demethylation was specific for isolated regulatory T cells in cord blood*

To confirm the specificity of *FOXP3* demethylation in cord blood Treg cells, unstimulated $CD4^+CD25^-$ and $CD4^+CD25^{high}$ cells were FACS-sorted and *FOXP3* demethylation at the TSDR was analyzed. *FOXP3* demethylation was hardly detectable in $CD4^+CD25^-$ cells (<1%) while *FOXP3* was 81% demethylated in $CD4^+CD25^{high}$ T cells (Figure 2 A/B). FOXP3 expression was also measured by flow cytometry in the $CD4^+CD25^{high}$ population, being expressed with a mean of 90%; one representative sample is depicted in Figure 2 C (Liu *et al.* 2010).

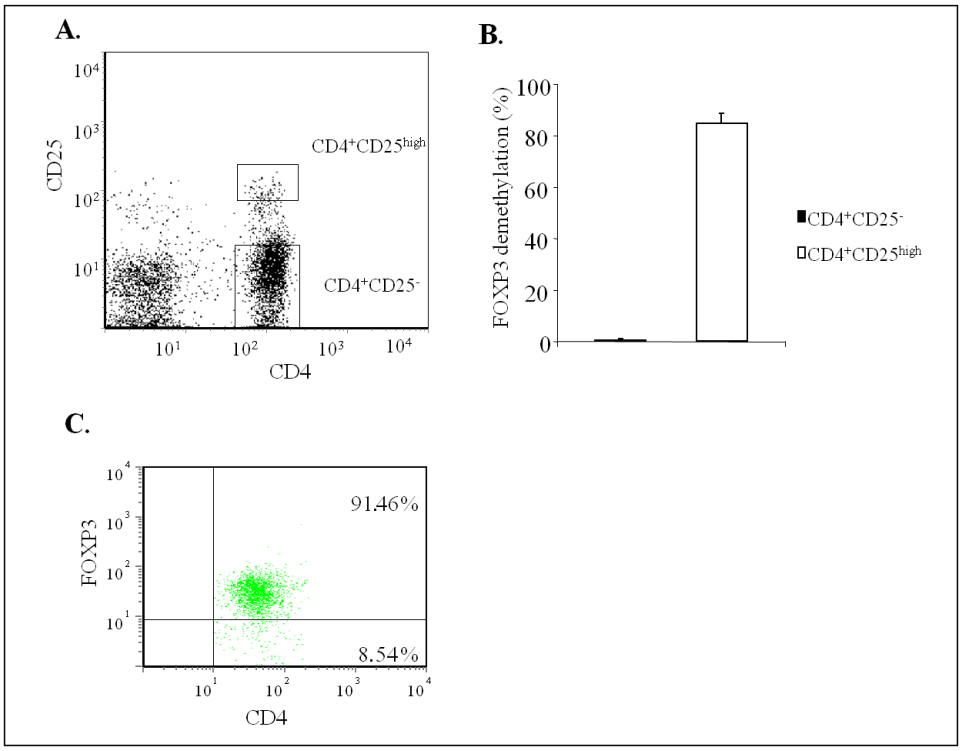


Figure 2. *FOXP3* demethylation in isolated $CD4^+CD25^-$ and $CD4^+CD25^{high}$ cells. **A/B.** $CD4^+CD25^-$ and $CD4^+CD25^{high}$ cells were isolated with Dako MoFlow. DNA was extracted and demethylation (%) of *FOXP3* was measured by real-time PCR. **C.** Intracellular FOXP3 protein was measured in $CD4^+CD25^{high}$ cells as described in methods. N=5, mean±SEM were shown in B, one representative sample for A/C.

3.1.1.2 Demethylation of **FOXP3** *correlated with* **FOXP3** *gene expression in cord blood*

Gene expression of *FOXP3* was determined in unstimulated and stimulated cord blood samples and correlated to *FOXP3* demethylation in whole cord blood. Figure 3 shows *FOXP3* mRNA expression as Δct values, axis vice versa. A lower Δct resembles higher *FOXP3* gene expression. *FOXP3* mRNA expression in unstimulated (U) and LpA stimulated cells was positively correlated with demethylation (%) of *FOXP3* in whole blood, though with low correlation coefficient (r=0.32, p=0.02; r=0.29, p=0.05, respectively; Figure 3 A/B) (Liu *et al.* 2010).

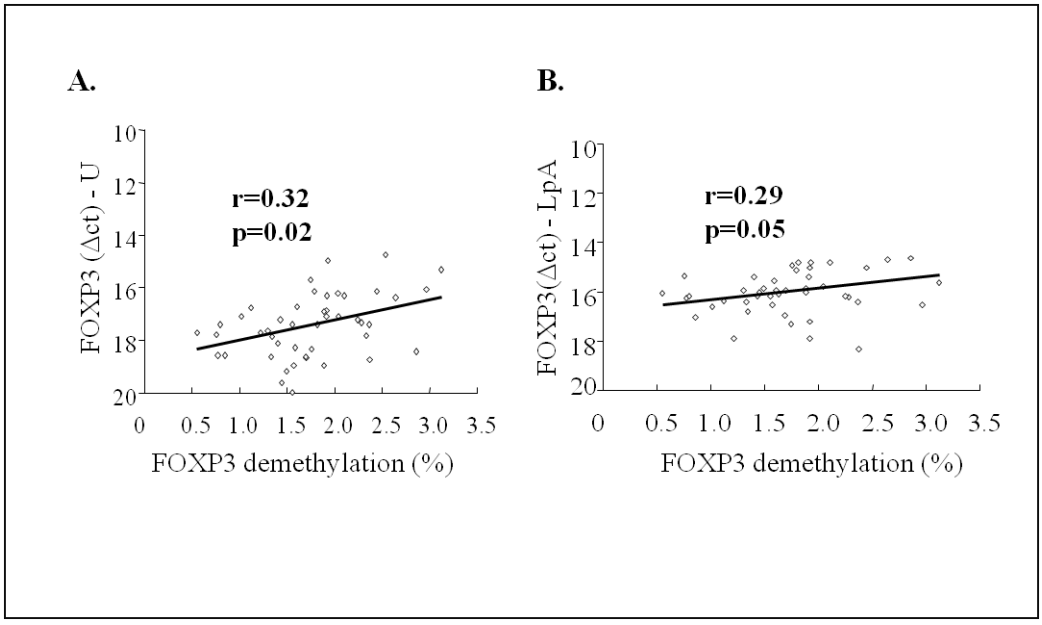


Figure 3. Correlation between demethylation of *FOXP3* and unstimulated and LpA-stimulated *FOXP3* mRNA expression. Demethylation (%) of *FOXP3* in whole cord blood was measured by real-time PCR. *FOXP3* gene expression was measured by real-time RT-PCR in **A.** unstimulated and **B.** LpA-stimulated CBMCs, shown as Δct to *18SrRNA*. Lower Δct represents higher mRNA expression. N=48; correlation was analyzed with Pearson's correlation coefficient.

3.1.1.3 Demethylation of **FOXP3** *correlated with $CD4^+CD25^{high}$ T cells in cord blood*

Treg cells express high amounts of the IL-2 receptor (CD25). $CD4^+CD25^{high}$ is a common marker used to define Treg cells by flow cytometry. *FOXP3* demethylation in whole blood (%) was positively correlated with the amount of unstimulated and LpA-stimulated $CD4^+CD25^{high}$ T cells in cord blood ($r=0.49$, $p<0.05$) (Figure 4 A/B) (Liu *et al.* 2010).

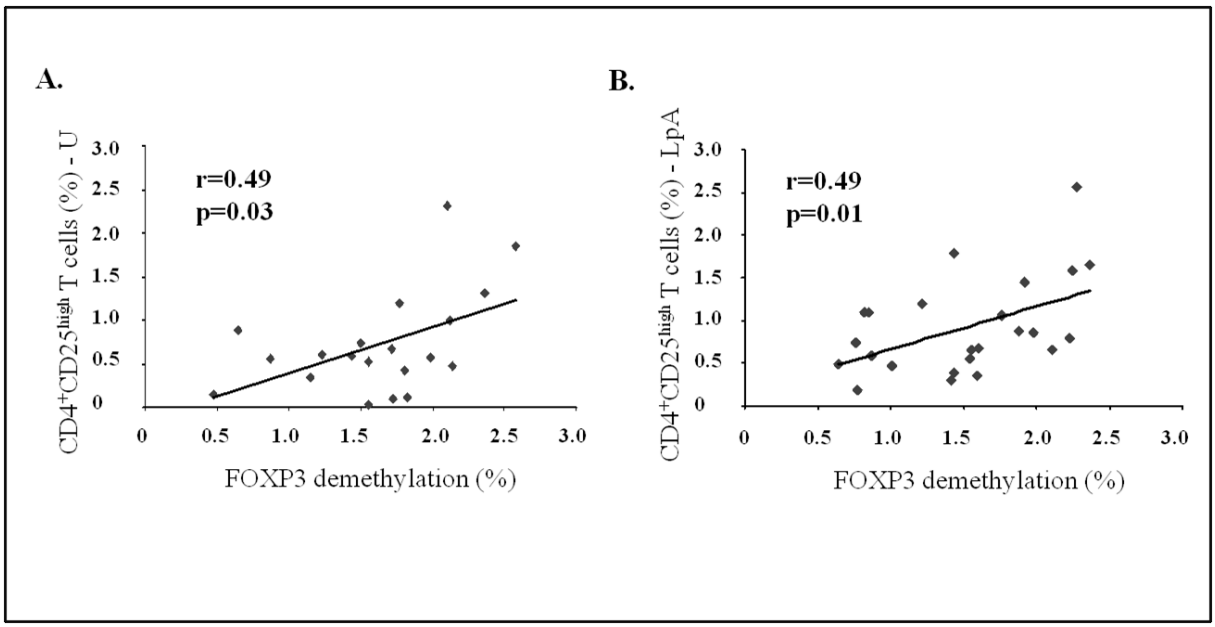


Figure 4. Correlation between demethylation of *FOXP3* and $CD4^+CD25^{high}$ T cells (%). Demethylation (%) of *FOXP3* was measured by real-time PCR. $CD4^+CD25^{high}$ T cells (% in lymphocytes) were measured by flow cytometry. **A.** Correlation between *FOXP3* demethylation and unstimulated $CD4^+CD25^{high}$ T cells (%, N=20). **B.** Correlation between *FOXP3* demethylation and LpA-stimulated $CD4^+CD25^{high}$ T cells (%, N=24); correlation was analyzed with Pearson's correlation coefficient.

3.1.1.4 Demethylation of FOXP3 correlated with the suppressive capacity of regulatory T cells in cord blood

As the quantification of Treg cells is not indicative of Treg function, the suppressive capacity of $CD4^+CD25^{high}$ T cells on the division and proliferation of $CD4^+CD25^-$ T effector cells was additionally assessed in a subgroup of cord blood samples (N=13). Cell division was measured with CFSE (carboxyfluorescein diacetate succinimidyl ester) labeling of effector cells. In addition, proliferation was measured by 3 [H]-thymidin incorporation reflecting the overall capacity of effector cell proliferation.

The suppressive capacity of Treg cells on cell division was positively correlated with demethylation of *FOXP3* (r=0.72, p=0.005, Figure 5 A), while the suppressive capacity of Treg cells on proliferation showed a trend correlated with demethylation (r=0.4, p=0.2, Figure 5 B). When dividing *FOXP3*

demethylation according to the median (0.70%) into groups with relatively higher or lower demethylation, the suppressive capacity of Treg cells on effector cells (division and proliferation) was significantly more effective in the group with higher compared to the lower demethylation (p=0.005, p=0.03; Figure 5 C) (Liu *et al.* 2010).

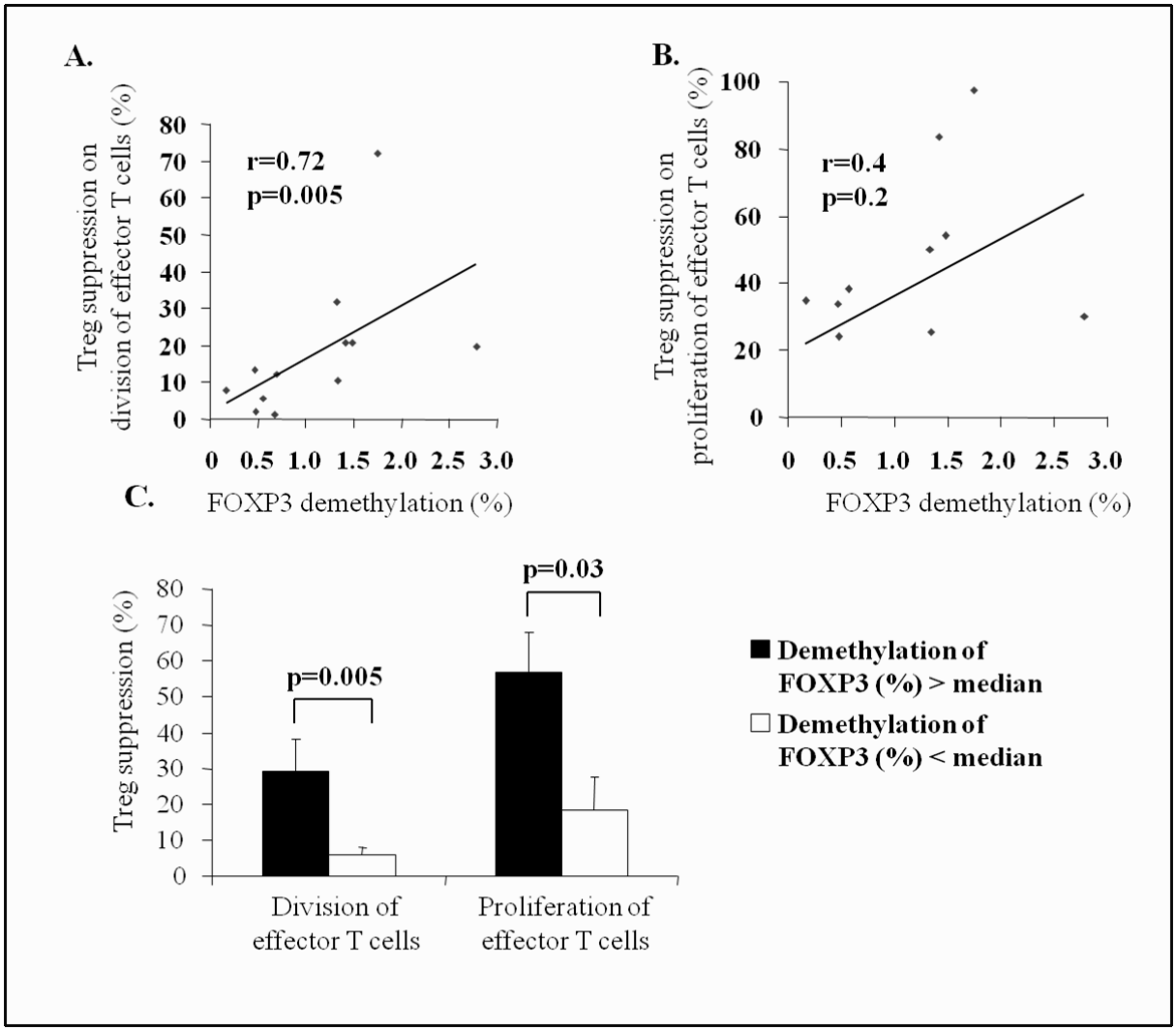


Figure 5. Relationship between demethylation (%) of *FOXP3* and suppressive capacity of Treg cells. Demethylation (%) of *FOXP3* was measured by real-time PCR. **A.** Correlation of demethylation of *FOXP3* and suppressive capacity of $CD4^{+}CD25^{high}$ T cells on the division of CFSE-labelled $CD4^{+}CD25^{-}$ T effector cells assessed by flow cytometry. **B.** Correlation of demethylation of *FOXP3* and suppressive capacity of $CD4^{+}CD25^{high}$ T cells on the proliferation of $CD4^{+}CD25^{-}$ T effector cells, measured with 3[H]-thymidin incorporation. **C.** The suppressive capacity of Treg cells was more effective in cord blood with higher *FOXP3* demethylation (%) (>median of 0.70). A–B: N=13/12; Pearson's correlation coefficient. C: N=13, t-test, data were shown as mean±SEM.

3.1.2 Regulatory T cells in a farming population in early childhood (the PASTURE/EFRAIM Study)

In the PAULCHEN birth cohort study higher Treg cell numbers and more efficient Treg cell function were observed in cord blood from farm exposed mothers (Schaub *et al.* 2009). Along with these findings *FOXP3* demethylation was increased in offspring of mothers with farm milk exposure (Schaub *et al.* 2009). Indeed, several cross-sectional studies demonstrated that farm exposure is a protective factor against the development of atopic diseases (ADs) in childhood (Riedler *et al.* 2001; Braun-Fahrlander *et al.* 2002; Ege *et al.* 2006), a phenomenon known as the "farm effect". In this context it was shown that early life exposure is relevant, which suggest an influence on immune maturation (Ege *et al.* 2006). However, little is known whether these previously reported differences observed in Treg cells in cord blood from farming children persist over time in childhood and whether they may be relevant for the development of atopic diseases in childhood. To investigate this hypothesis, Treg cells were studied in children at age 4.5 years in the context of farming exposure and their role in the allergy-protective "farm effect" was determined.

For this purpose 325 German or French children from the PASTURE/EFRAIM study (von Mutius *et al.* 2006) were followed until the age of 4.5 years. Treg cells were analyzed in PBMCs from these subjects in unstimulated (U) and stimulated (PI=PMA/Ionomycin, LPS=Lipopolysaccharide) conditions (Lluis, Depner *et al.* Manuscript submitted).

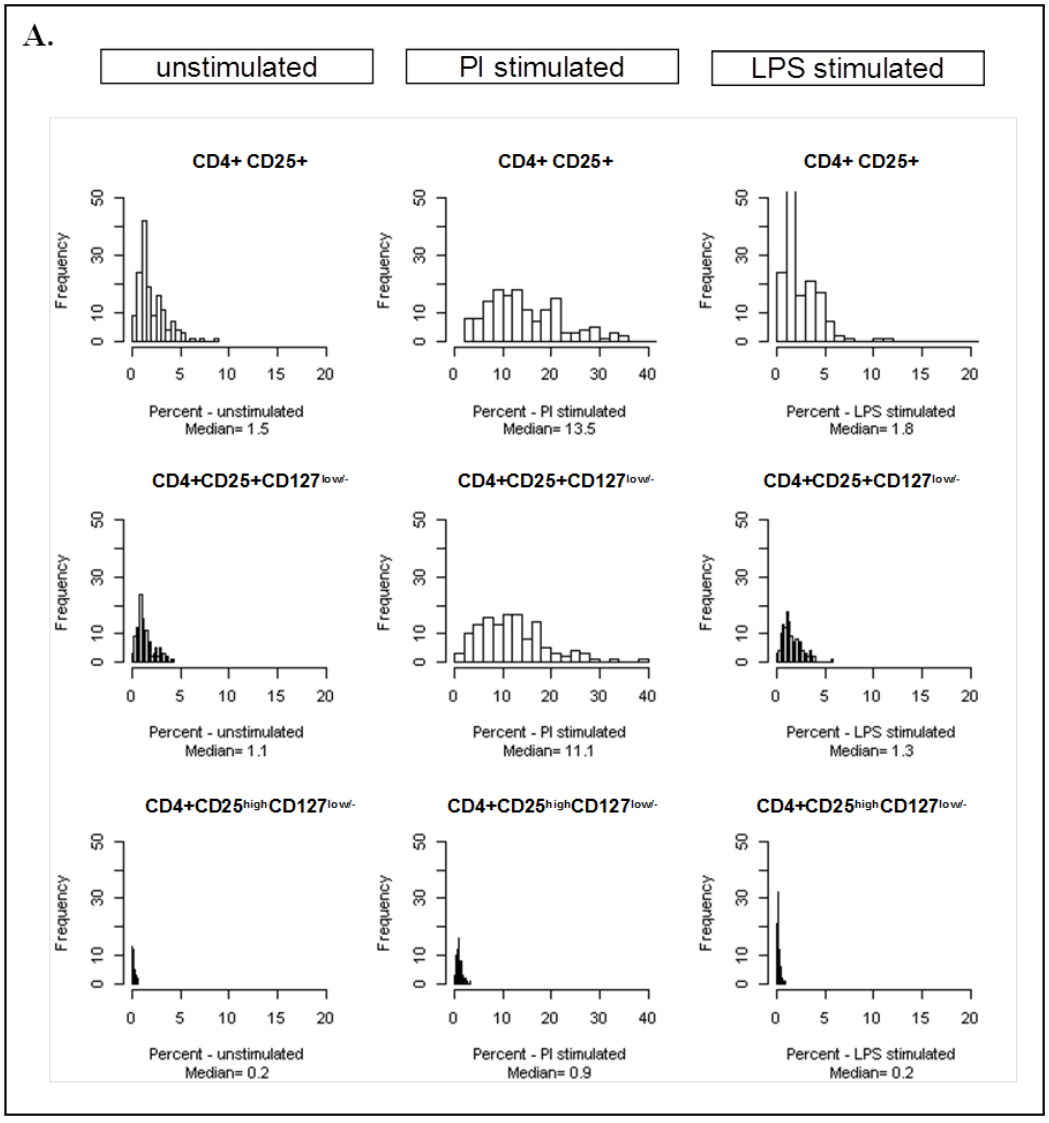
Population characteristics are shown in Table I and are described in detail in Material and Methods section 2.2.1.1.3.

3.1.2.1 Regulatory T cells in 4.5 year-old children

Treg cells relative to the lymphocyte population (in %) were assessed by measuring the expression of $CD4^{+}CD25^{high}$ in combination with low surface expression of CD127 or with intracellular expression of FOXP3 before and following stimulation with PI and LPS. The distribution of the respective cells

(%) and the medians are shown in Figure 6. The median of typical Treg cell markers $CD4^{+}25^{high}FOXP3^{+}$, defined here as Upper20%$CD4^{+}CD25^{+}$, $FOXP3^{+}$; and $CD4^{+}25^{high}CD127^{low/-}$ under unstimulated conditions was 0.2 % (Figure 6 A/B). These results indicate that the percentage of Treg cells in 4.5 year-old children was generally low.

After LPS stimulation a slight increase in Treg cells was observed (median of Upper20%$CD4^{+}CD25^{+}$, $FOXP3^{+}$, $CD4^{+}25^{high}CD127^{low/-}$; 0.3%, 0.2%, respectively Figure 6 A/B). PI stimulation increased the activated T cell populations and to a lower extent the Treg cell population (median Upper20%$CD4^{+}CD25^{+}$, $FOXP3^{+}$, $CD4^{+}25^{high}CD127^{low/-}$; 0.4%, 0.9%, respectively) (Lluis, Depner *et al.* Manuscript submitted).



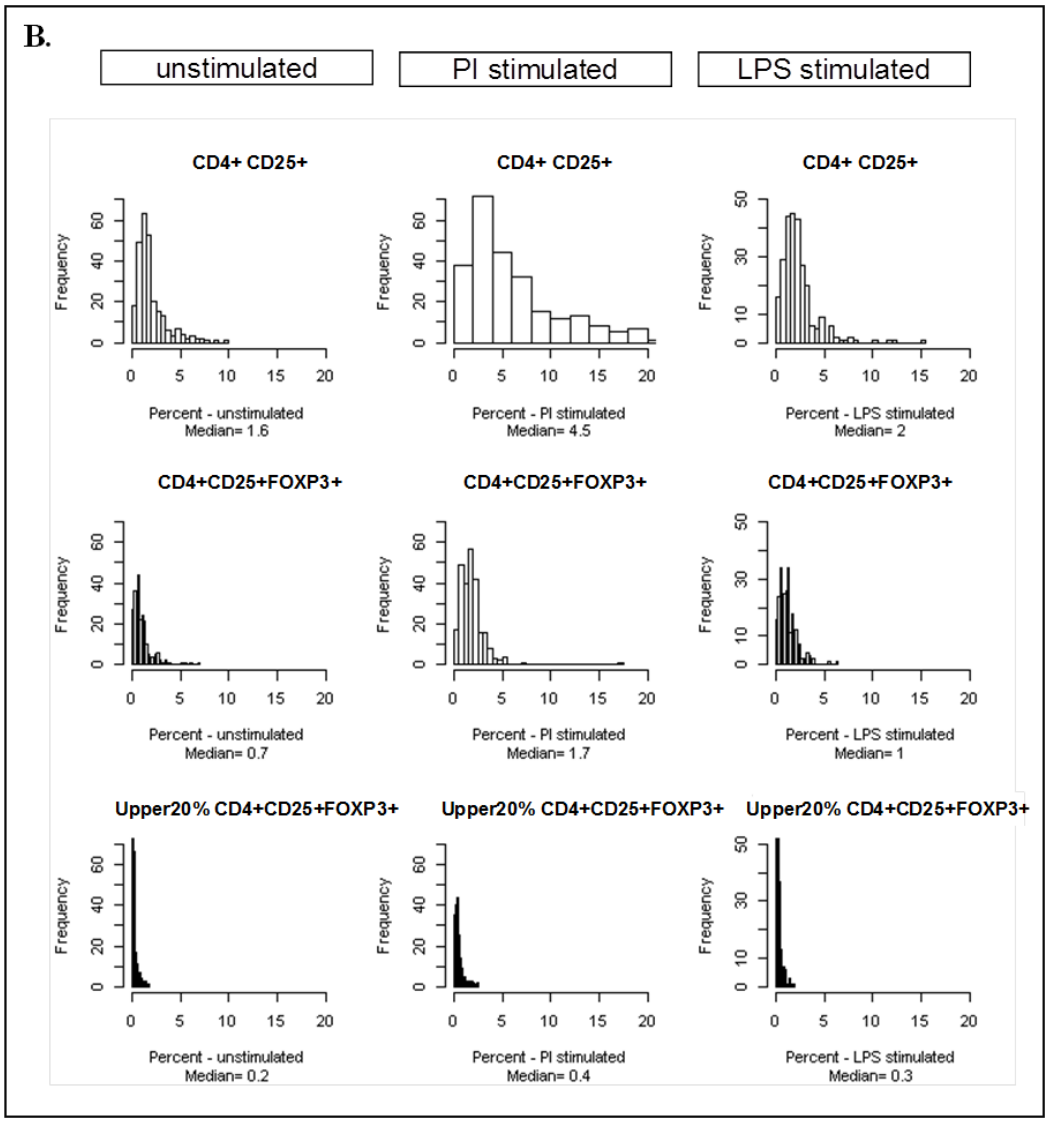


Figure 6. Distribution of activated T cells and Treg cells relative to lymphocytes (%). **A.** By surface staining using the markers CD4, CD25, CD127 (N=157, Germany) **B.** By intracellular staining using the markers CD4, CD25, FOXP3 (N=298, Germany and France).

3.1.2.2 Regulatory T cells were quantitatively increased in blood from farming children

Next, it was investigated whether Treg cells (%) were consistently increased in 4.5 year old children exposed to a farming environment compared to non-farming children living in the same rural area (Table III). This effect was significant for Treg cells determined by intracellular staining (Upper20%$CD4^+CD25^+$, $FOXP3^+$) upon stimulation (PI and LPS), being more pronounced upon LPS stimulation (GMR and 95%-C.I. for Upper20%$CD4^+CD25^+$, $FOXP3^+$, PI, LPS; 1.23 [1.04 - 1.46];

p=0.01; 1.44 [1.15 - 1.79]; p=0.001, respectively) (Lluis, Depner *et al.* Manuscript submitted). Moreover, activated $CD4^+CD25^+$ T cells were also significantly increased upon stimulated conditions (PI, LPS) in the intracellular staining (Table III). Interestingly, Treg cells (%) (surface staining $CD4^+CD25^{high}CD127^{low/-}$) in the German farming population were also increased in unstimulated samples (GMR and 95%-C.I. 1.31 [1.04 - 1.66]; p=0.02).

Table III. Association of activated T cells and Treg cells with farming lifestyle.

			Farm vs. reference	
Staining	**Stimulus and Marker [2]**		**GMR [95%-C.I.] [3]**	**p**
Surface Germany	U	$CD4^+CD25^+$	0.96 [0.76 - 1.22]	0.76
		$CD4^+25^+CD127^{low/-}$	1.12 [0.88 - 1.42]	0.36
		$CD4^+25^{high}CD127^{low/-}$	**1.31** [1.04 - 1.66]	**0.02**
	PI	$CD4^+CD25^+$	0.97 [0.80 - 1.18]	0.78
		$CD4^+25^+CD127^{low/-}$	1.06 [0.84 - 1.34]	0.63
		$CD4^+25^{high}CD127^{low/-}$	1.10 [0.88 - 1.38]	0.41
	LPS	$CD4^+CD25^+$	0.87 [0.68 - 1.12]	0.29
		$CD4^+25^+CD127^{low/-}$	1.03 [0.79 - 1.36]	0.81
		$CD4^+25^{high}CD127^{low/-}$	1.10 [0.83 - 1.46]	0.49
Intracellular [1] Germany + France	U	$CD4^+CD25^+$	1.15 [0.97 - 1.36]	0.12
		$CD4^+CD25^+$ $FOXP3^+$	1.04 [0.82 - 1.33]	0.72
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.15 [0.91 - 1.44]	0.24
	PI	$CD4^+CD25^+$	**1.30** [1.11 - 1.54]	**0.002**
		$CD4^+CD25^+$ $FOXP3^+$	**1.30** [1.10 - 1.53]	**0.002**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	**1.23** [1.04 - 1.46]	**0.01**
	LPS	$CD4^+CD25^+$	**1.32** [1.10 - 1.57]	**0.003**
		$CD4^+CD25^+$ $FOXP3^+$	**1.27** [1.04 - 1.56]	**0.02**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	**1.44** [1.15 - 1.79]	**0.001**

[1] Intracellular staining adjusted for center.
[2] U, unstimulated; PI, PMA/Ionomycin; LPS, Lipopolysaccharide.
[3] GMR= geometric mean ratio, 95%.-C.I. =95%-confidence intervals, p=p-value of regression analyses in the log-transformed data.
P values in bold letters reached significance with $p \leq 0.05$.

3.1.2.3 Farm milk consumption was associated with increased regulatory T cells in early childhood

To further dissect specific farm-related factors potentially influencing Treg cell expression, we studied the effect of several farm exposures (being in stables, farm milk consumption, regular contact to hay, type of farm and number of animal species) on Treg cells.

Both farm milk consumption and staying in stables were associated with increased Treg cells (%) upon PI and LPS stimulation, but only current farm milk consumption remained significant upon adjustment for farming (Table IV, GMR and 95%-C.I for Upper20%$CD4^{+}CD25^{+}$, $FOXP3^{+}$; 1.36 [1.08 - 1.70]; p=0.008; 1.46 [1.10 - 1.94]; p=0.01, respectively). Additionally, an increase in Treg cells ($CD4^{+}25^{high}CD127^{low/-}$) measured by surface staining was seen upon PI and LPS stimulation ($p<0.05$). All other farm-related exposures analyzed including regular contact to hay, type of farm and number of exposed animal species at age 4 did not show any association (Table IV) (Lluis, Depner *et al.* Manuscript submitted).

The influence of farm milk consumption on Treg cells was further investigated in the subgroups of farm and reference children. Although there was an overlap between farming and farm exposures, some farm children did not consume farm milk while some reference children drank farm milk (Figure 7).

Table IV. Association of activated T cells and Treg cells with typical farm-related exposures.

			Contact to stable [1]		Farm milk consumption [1]		Regular contact to hay [1]	
Staining	**Stimulus and Marker [2]**		**GMR [95%-C.I.] [3]**	**p**	**GMR [95%-C.I.] [3]**	**p**	**GMR [95%-C.I.] [3]**	**p**
Surface Germany	U	$CD4^+CD25^+$	1.07 [0.76 - 1.51]	0.69	1.05 [0.77 - 1.44]	0.75	0.97 [0.73 - 1.28]	0.82
		$CD4^+25^+CD127^{low/-}$	0.93 [0.66 - 1.31]	0.69	1.01 [0.74 - 1.37]	0.95	0.95 [0.72 - 1.25]	0.71
		$CD4^+25^{high}CD127^{low/-}$	0.98 [0.70 - 1.37]	0.91	1.13 [0.83 - 1.53]	0.44	0.96 [0.73 - 1.26]	0.76
	PI	$CD4^+CD25^+$	1.01 [0.76 - 1.35]	0.93	1.23 [0.95 - 1.59]	0.11	1.11 [0.88 - 1.40]	0.39
		$CD4^+25^+CD127^{low/-}$	0.94 [0.67 - 1.32]	0.72	1.16 [0.86 - 1.55]	0.34	1.09 [0.82 - 1.44]	0.56
		$CD4^+25^{high}CD127^{low/-}$	0.92 [0.67 - 1.28]	0.64	**1.35** [1.03 - 1.79]	**0.03**	1.05 [0.81 - 1.37]	0.71
	LPS	$CD4^+CD25^+$	1.03 [0.71 - 1.49]	0.88	1.03 [0.75 - 1.43]	0.84	0.92 [0.69 - 1.23]	0.58
		$CD4^+25^+CD127^{low/-}$	0.95 [0.64 - 1.42]	0.81	0.98 [0.69 - 1.39]	0.91	1.01 [0.73 - 1.39]	0.97
		$CD4^+25^{high}CD127^{low/-}$	0.97 [0.65 - 1.47]	0.90	**1.60** [1.13 - 2.26]	**0.009**	0.94 [0.67 - 1.30]	0.69
Intracellular [1] Germany + France	U	$CD4^+CD25^+$	0.97 [0.75 - 1.27]	0.84	**1.31** [1.05 - 1.65]	**0.02**	0.93 [0.75 - 1.16]	0.52
		$CD4^+CD25^+$ $FOXP3^+$	0.84 [0.59 - 1.21]	0.36	1.23 [0.90 - 1.69]	0.20	0.79 [0.59 - 1.07]	0.14
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	0.80 [0.57 - 1.14]	0.22	1.24 [0.91 - 1.67]	0.17	0.83 [0.62 - 1.10]	0.20
	PI	$CD4^+CD25^+$	1.09 [0.85 - 1.42]	0.49	**1.29** [1.03 - 1.60]	**0.03**	1.05 [0.85 - 1.29]	0.66
		$CD4^+CD25^+$ $FOXP3^+$	1.09 [0.84 - 1.41]	0.52	**1.31** [1.05 - 1.64]	**0.02**	0.99 [0.81 - 1.22]	0.96
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.16 [0.89 - 1.50]	0.28	**1.36** [1.08 - 1.70]	**0.008**	1.01 [0.82 - 1.25]	0.91
	LPS	$CD4^+CD25^+$	1.07 [0.81 - 1.40]	0.64	**1.33** [1.06 - 1.69]	**0.02**	0.84 [0.67 - 1.05]	0.13
		$CD4^+CD25^+$ $FOXP3^+$	0.93 [0.68 - 1.27]	0.64	**1.30** [1.00 - 1.70]	**0.05**	0.81 [0.63 - 1.05]	0.11
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.06 [0.76 - 1.48]	0.73	**1.46** [1.10 - 1.94]	**0.01**	0.83 [0.63 - 1.09]	0.18

[1] All analyses are adjusted for farming, analyses for intracellular staining additionally adjusted for center.
[2] U, unstimulated; PI, PMA/Ionomycin; LPS, Lipopolysaccharide.
[3] GMR= geometric mean ratio, 95%.-C.I. =95%-confidence intervals, p=p-value of regression analysis in the log-transformed data. *P* values in bold letters reached significance with $p \leq 0.05$.

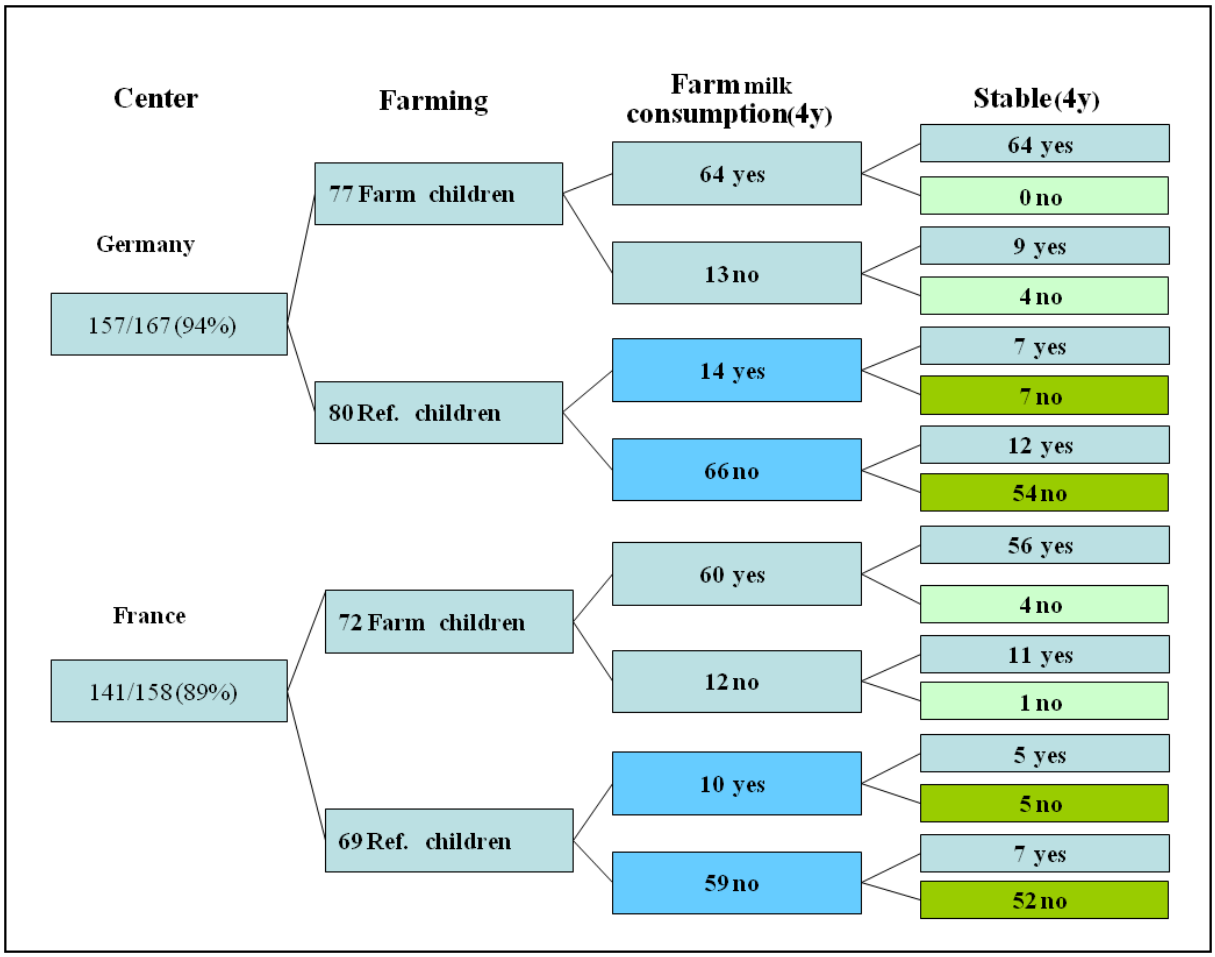


Figure 7. Farm milk consumption in farm and reference children

A consistent positive association between Treg cells and farm milk consumption was observed after PI and LPS stimulation in farm and reference children, reaching significance only within the reference group, particularly without contact to stable (Table V) (Lluis, Depner *et al.* Manuscript submitted).

Table V. Association of activated T cells and Treg cells with farm milk consumption at age 4 years stratified by different subgroups.

			Farm children (N=149)		Reference children (N=149)		Reference children with contact to stable (N=31)		Reference children without contact to stable (N=118)	
Staining	**Stimulus and Marker[2]**		**GMR [95%-C.I.)] [3]**	**p**	**GMR [95%-C.I.] [3]**	**p**	**GMR [95%-CI] [3]**	**p**	**GMR [95%-CI] [3]**	**p**
Surface Germany	U	$CD4^+CD25^+$	1.02 [0.66 - 1.58]	0.93	1.08 [0.69 - 1.70]	0.73	1.06 [0.60 - 1.89]	0.84	1.03 [0.53 - 1.99]	0.93
		$CD4^+25^+CD127^{low/-}$	0.96 [0.63 - 1.45]	0.84	1.06 [0.67 - 1.68]	0.81	0.96 [0.41 - 2.25]	0.93	1.17 [0.62 - 2.19]	0.63
		$CD4^+25^{high}CD127^{low/-}$	0.95 [0.63 - 1.42]	0.80	1.32 [0.84 - 2.08]	0.23	1.39 [0.73 - 2.65]	0.34	1.23 [0.63 - 2.39]	0.55
	PI	$CD4^+CD25^+$	1.21 [0.83 - 1.76]	0.32	1.25 [0.88 - 1.77]	0.22	1.20 [0.62 - 2.30]	0.59	1.33 [0.83 - 2.12]	0.24
		$CD4^+25^+CD127^{low/-}$	1.13 [0.74 - 1.73]	0.57	1.18 [0.78 - 1.79]	0.44	1.23 [0.58 - 2.60]	0.60	1.26 [0.71 - 2.25]	0.43
		$CD4^+25^{high}CD127^{low/-}$	1.26 [0.83 - 1.90]	0.28	**1.46** [1.01 - 2.12]	**0.05**	1.93 [0.96 - 3.87]	0.09	1.43 [0.88 - 2.32]	0.16
	LPS	$CD4^+CD25^+$	1.32 [0.88 - 1.97]	0.18	0.82 [0.50 - 1.35]	0.45	0.85 [0.42 - 1.75]	0.67	0.76 [0.37 - 1.54]	0.44
		$CD4^+25^+CD127^{low/-}$	1.09 [0.71 - 1.70]	0.69	0.88 [0.51 - 1.51]	0.63	0.73 [0.25 - 2.13]	0.58	1.00 [0.49 - 2.06]	0.99
		$CD4^+25^{high}CD127^{low/-}$	**2.06** [1.28 - 3.32]	**0.004**	1.24 [0.75 - 2.03]	0.41	1.37 [0.54 - 3.50]	0.52	1.31 [0.67 - 2.57]	0.43
Intracellular [1] Germany + France	U	$CD4^+CD25^+$	1.33 [0.99 - 1.77]	0.06	1.32 [0.93 - 1.89]	0.12	1.62 [0.89 - 2.94]	0.13	1.37 [0.85 - 2.21]	0.20
		$CD4^+CD25^+$ $FOXP3^+$	1.08 [0.69 - 1.69]	0.72	1.44 [0.91 - 2.27]	0.12	1.57 [0.69 - 3.56]	0.29	1.78 [0.97 - 3.24]	0.06
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.16 [0.76 - 1.78]	0.49	1.33 [0.87 - 2.06]	0.19	1.78 [0.85 - 3.74]	0.14	1.54 [0.87 - 2.71]	0.14
	PI	$CD4^+CD25^+$	1.10 [0.82 - 1.48]	0.53	**1.54** [1.11 - 2.13]	**0.01**	1.74 [1.00 - 3.03]	0.07	1.54 [1.00 - 2.38]	0.05
		$CD4^+CD25^+$ $FOXP3^+$	1.14 [0.83 - 1.57]	0.42	**1.54** [1.13 - 2.10]	**0.007**	1.48 [0.77 - 2.83]	0.25	**1.78 [1.20 - 2.64]**	**0.005**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.14 [0.83 - 1.55]	0.42	**1.66** [1.21 - 2.29]	**0.002**	1.68 [0.90 - 3.14]	0.12	**1.79 [1.19 - 2.71]**	**0.007**
	LPS	$CD4^+CD25^+$	1.29 [0.93 - 1.79]	0.12	**1.44** [1.04 - 2.00]	**0.03**	1.26 [0.69 - 2.30]	0.46	**1.82 [1.17 - 2.82]**	**0.009**
		$CD4^+CD25^+$ $FOXP3^+$	1.21 [0.85 - 1.73]	0.30	1.45 [0.98 - 2.16]	0.07	1.27 [0.54 - 2.96]	0.59	**2.06 [1.25 - 3.41]**	**0.006**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.40 [0.98 - 2.02]	0.07	**1.60** [1.04 - 2.47]	**0.04**	1.21 [0.53 - 2.78]	0.66	**2.32 [1.31 - 4.12]**	**0.005**

[1] Analyses for intracellular staining are adjusted for center.
[2] U, unstimulated; PI, PMA/Ionomycin; LPS, Lipopolysaccharide.
[3] GMR= geometric mean ratio, 95%.-C.I. =95%-confidence intervals, p=p-value of regression analyses in the log-transformed data. *P* values in bold letters reached significance with $p \leq 0.05$.

In a next step, specific patterns of farm milk consumption (exclusively drinking farm milk or a mixed consumption of farm and shop milk) were analyzed. Children only drinking shop milk were defined as the reference group. These data indicated that the observed effect on Treg cells was independent of the pattern of farm milk consumption (Table VI, A). Consumption of boiled and unboiled farm milk also revealed a similar effect on Treg cells (Table VI, B) (Lluis, Depner *et al.* Manuscript submitted).

Table VI. Association of activated T cells and Treg cells with specific milk consumption. A. Farm and shop milk consumption vs. exclusively farm milk consumption. B. Boiled vs. unboiled farm milk consumption.

A.

			Mixed milk vs. shop milk (N=48) [1]		Farm milk vs. shop milk (N=100) [1]	
Staining	**Stimulus and Marker [2]**		**GMR [95%-C.I.] [3]**	**p**	**GMR [95%-C.I.] [3]**	**p**
Surface Germany	U	$CD4^+CD25^+$	0.94 [0.67 - 1.31]	0.71	1.06 [0.81 - 1.39]	0.68
		$CD4^+25^+CD127^{low/-}$	1.07 [0.76 - 1.49]	0.71	1.06 [0.81 - 1.38]	0.70
		$CD4^+25^{high}CD127^{low/-}$	1.30 [0.94 - 1.80]	0.11	1.18 [0.91 - 1.53]	0.21
	PI	$CD4^+CD25^+$	1.10 [0.84 - 1.44]	0.49	1.10 [0.89 - 1.38]	0.38
		$CD4^+25^+CD127^{low/-}$	1.12 [0.81 - 1.56]	0.49	1.10 [0.84 - 1.43]	0.50
		$CD4^+25^{high}CD127^{low/-}$	1.26 [0.94 - 1.70]	0.12	1.21 [0.95 - 1.54]	0.12
	LPS	$CD4^+CD25^+$	0.85 [0.60 - 1.19]	0.34	1.02 [0.77 - 1.34]	0.90
		$CD4^+25^+CD127^{low/-}$	1.05 [0.71 - 1.56]	0.80	0.97 [0.71 - 1.32]	0.85
		$CD4^+25^{high}CD127^{low/-}$	**1.51** [1.02 - 2.24]	**0.04**	**1.36** [1.00 - 1.86]	**0.05**
Intracellular [1] Germany + France	U	$CD4^+CD25^+$	1.24 [0.97 - 1.57]	0.09	**1.30** [1.06 - 1.58]	**0.01**
		$CD4^+CD25^+$ $FOXP3^+$	1.13 [0.80 - 1.58]	0.49	1.15 [0.88 - 1.52]	0.31
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.11 [0.81 - 1.53]	0.52	**1.30** [1.00 - 1.68]	**0.05**
	PI	$CD4^+CD25^+$	**1.50** [1.18 - 1.90]	**0.0008**	**1.33** [1.10 - 1.60]	**0.003**
		$CD4^+CD25^+$ $FOXP3^+$	**1.47** [1.16 - 1.86]	**0.002**	**1.37** [1.13 - 1.65]	**0.001**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	**1.46** [1.14 - 1.85]	**0.002**	**1.35** [1.12 - 1.63]	**0.002**
	LPS	$CD4^+CD25^+$	**1.28** [1.00 - 1.65]	**0.05**	**1.48** [1.21 - 1.81]	**0.0001**
		$CD4^+CD25^+$ $FOXP3^+$	1.20 [0.91 - 1.60]	0.20	**1.41** [1.12 - 1.78]	**0.003**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	**1.37** [1.01 - 1.85]	**0.04**	**1.67** [1.31 - 2.13]	**<.0001**

B.

Staining	Stimulus and Marker [2]		Boiled farm milk vs. shop milk (N=51) [1] GMR [95%-C.I.] [3]	p	Unboiled farm milk vs. shop milk (N=82) [1] GMR [95%-C.I.] [3]	p
Surface Germany	U	$CD4^+CD25^+$	1.05 [0.71 - 1.55]	0.82	0.98 [0.75 - 1.27]	0.85
		$CD4^+25^+CD127^{low/-}$	0.98 [0.65 - 1.47]	0.92	1.04 [0.80 - 1.36]	0.75
		$CD4^+25^{high}CD127^{low/-}$	**1.57** [1.07 - 2.32]	**0.02**	1.09 [0.84 - 1.40]	0.52
	PI	$CD4^+CD25^+$	**1.58** [1.15 - 2.16]	**0.005**	1.01 [0.82 - 1.25]	0.92
		$CD4^+25^+CD127^{low/-}$	1.40 [0.95 - 2.08]	0.09	1.08 [0.84 - 1.39]	0.55
		$CD4^+25^{high}CD127^{low/-}$	**1.69** [1.19 - 2.40]	**0.003**	1.21 [0.97 - 1.52]	0.09
	LPS	$CD4^+CD25^+$	1.05 [0.69 - 1.61]	0.82	0.90 [0.68 - 1.19]	0.45
		$CD4^+25^+CD127^{low/-}$	1.04 [0.63 - 1.73]	0.88	0.94 [0.69 - 1.27]	0.67
		$CD4^+25^{high}CD127^{low/-}$	**1.72** [1.03 - 2.86]	**0.04**	1.32 [0.97 - 1.80]	0.08
Intracellular [1] Germany + France	U	$CD4^+CD25^+$	**1.33** [1.04 - 1.70]	**0.03**	1.19 [0.96 - 1.46]	0.11
		$CD4^+CD25^+FOXP3^+$	1.04 [0.74 - 1.48]	0.81	1.12 [0.84 - 1.50]	0.45
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.20 [0.86 - 1.67]	0.27	1.14 [0.86 - 1.50]	0.36
	PI	$CD4^+CD25^+$	**1.27** [1.00 - 1.60]	**0.05**	**1.43** [1.17 - 1.74]	**0.0004**
		$CD4^+CD25^+FOXP3^+$	**1.30** [1.02 - 1.65]	**0.03**	**1.45** [1.19 - 1.77]	**0.0003**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	**1.28** [1.01 - 1.63]	**0.04**	**1.40** [1.14 - 1.71]	**0.001**
	LPS	$CD4^+CD25^+$	1.24 [0.97 - 1.60]	0.09	**1.39** [1.13 - 1.71]	**0.002**
		$CD4^+CD25^+FOXP3^+$	1.23 [0.92 - 1.64]	0.17	1.26 [0.99 - 1.59]	0.06
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	**1.41** [1.04 - 1.91]	**0.03**	**1.49** [1.16 - 1.92]	**0.002**

[1] Reference group are 135 children who only drink shop milk; analyses for intracellular staining adjusted for center.
[2] U, unstimulated; PI, PMA/Ionomycin; LPS, Lipopolysaccharide.
[3] GMR= geometric mean ratio, 95%.-C.I. =95%-confidence intervals, p=p-value of regression analyses in the log-transformed data. *P* values in bold letters reached significance with $p \leq 0.05$.

To assess whether farm milk consumption earlier in life may already lead to increased Treg cells at age 4.5 years, the role of farm milk consumption at different time points was studied.

Because consumption at different time points was highly correlated, a categorical variable (combining farm milk consumption in pregnancy and childhood) was analyzed. In this study population 18.2% of children drank farm milk although the mother did not consume any farm milk during pregnancy. It was observed that only very few children (3.4%) were not exposed to farm milk consumption in their childhood when mothers drank farm milk during pregnancy. In each group under study consuming farm milk led to increased Treg cells (%). This effect was more pronounced in children exposed to farm milk already prenatally and in childhood (Table VII) (Lluis, Depner *et al.* Manuscript submitted). Due to low numbers it was not possible to disentangle this effect during pregnancy only (Table VII).

Table VII. Association of activated T cells and Treg cells with patterns of milk consumption over time.

			in pregnancy YES; in childhood NO (N=10) [1]		in pregnancy NO; in childhood YES ((N=54) [1]		in pregnancy YES in childhood YES (N=122) [1]	
Staining	**Stimulus and Marker [2]**		**GMR [95%-C.I.] [3]**	**p**	**GMR [95%-C.I.] [3]**	**p**	**GMR [95%-C.I.] [3]**	**p**
Surface Germany	U	$CD4^+CD25^+$	1.55 [0.83 - 2.87]	0.17	1.04 [0.73 - 1.49]	0.82	1.07 [0.82 - 1.39]	0.62
		$CD4^+25^+CD127^{low/-}$	1.62 [0.85 - 3.08]	0.14	1.22 [0.85 - 1.74]	0.28	1.17 [0.89 - 1.52]	0.26
		$CD4^+25^{high}CD127^{low/-}$	1.70 [0.90 - 3.20]	0.10	1.37 [0.96 - 1.95]	0.08	**1.37** [1.06 - 1.78]	**0.02**
	PI	$CD4^+CD25^+$	1.67 [0.97 - 2.89]	0.07	**1.37** [1.03 - 1.82]	**0.03**	1.16 [0.93 - 1.44]	0.18
		$CD4^+25^+CD127^{low/-}$	1.60 [0.80 - 3.18]	0.18	1.33 [0.94 - 1.88]	0.11	1.17 [0.90 - 1.52]	0.23
		$CD4^+25^{high}CD127^{low/-}$	1.39 [0.73 - 2.65]	0.32	**1.53** [1.10 - 2.11]	**0.01**	1.23 [0.97 - 1.57]	0.09
	LPS	$CD4^+CD25^+$	1.89 [0.94 - 3.78]	0.07	0.99 [0.68 - 1.43]	0.95	0.99 [0.76 - 1.31]	0.97
		$CD4^+25^+CD127^{low/-}$	1.79 [0.80 - 4.01]	0.16	1.14 [0.76 - 1.72]	0.52	1.09 [0.81 - 1.48]	0.57
		$CD4^+25^{high}CD127^{low/-}$	1.26 [0.56 - 2.83]	0.57	0.72 [0.48 - 1.09]	0.12	1.33 [0.98 - 1.80]	0.06
Intracellular [1] Germany + France	U	$CD4^+CD25^+$	1.03 [0.62 - 1.71]	0.92	1.04 [0.82 - 1.33]	0.75	**1.21** [1.00 - 1.47]	**0.05**
		$CD4^+CD25^+$ $FOXP3^+$	1.21 [0.60 - 2.43]	0.59	0.81 [0.58 - 1.13]	0.21	1.23 [0.94 - 1.61]	0.12
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.12 [0.57 - 2.19]	0.74	0.96 [0.70 - 1.33]	0.82	1.20 [0.93 - 1.55]	0.16
	PI	$CD4^+CD25^+$	1.46 [0.87 - 2.43]	0.15	**1.33** [1.05 - 1.68]	**0.02**	**1.34** [1.12 - 1.61]	**0.002**
		$CD4^+CD25^+$ $FOXP3^+$	1.29 [0.77 - 2.16]	0.32	**1.28** [1.01 - 1.62]	**0.04**	**1.43** [1.19 - 1.72]	**0.0001**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.40 [0.83 - 2.35]	0.20	**1.37** [1.08 - 1.74]	**0.009**	**1.37** [1.14 - 1.65]	**0.0008**
	LPS	$CD4^+CD25^+$	1.46 [0.80 - 2.66]	0.22	1.11 [0.87 - 1.43]	0.40	**1.36** [1.11 - 1.66]	**0.003**
		$CD4^+CD25^+$ $FOXP3^+$	1.74 [0.89 - 3.43]	0.11	1.02 [0.77 - 1.36]	0.89	**1.43** [1.14 - 1.79]	**0.002**
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	1.46 [0.70 - 3.02]	0.31	1.14 [0.84 - 1.55]	0.40	**1.57** [1.23 - 2.00]	**0.0003**

[1] Reference group are 111 children who don't drink farm milk; analyses for intracellular staining adjusted for center.
[2] U, unstimulated; PI, PMA/Ionomycin; LPS, Lipopolysaccharide.
[3] GMR= geometric mean ratio, 95%.-C.I. =95%-confidence intervals, p=p-value of regression analyses in the log-transformed data. *P* values in bold letters reached significance with $p \leq 0.05$.

3.1.2.4 Regulatory T cells were inversely associated with atopic sensitization at age 4 years

It has been demonstrated that farming lifestyle is a protective factor for the development of atopic diseases in childhood. In this study, Treg cells were increased (%) in 4.5-year-old farming children and in children drinking farm milk. Thus, as a next step it was investigated whether Treg cells were inversely associated with atopic sensitization at age 4 years.

Treg cells (Upper20%$CD4^+CD25^+$, $FOXP3^+$) were negatively associated with food and inhalant allergens. Unstimulated Treg cells were significantly associated with lower sensitization to inhalant allergens (≥3.5IU/mL) (Upper20%$CD4^+CD25^+$, $FOXP3^+$, adjusted odds ratio (OR=0.38 [0.16-0.86]; p=0.02) (Table XVIII) (Lluis, Depner *et al.* Manuscript submitted).

Table VIII. Association of Treg cells and atopic sensitization (IgE ≥3.5IU/mL).

			IgE≥3.5 IU/mL [1]			
			Inhalant IgE		Food IgE	
Staining	**Stimulus and Marker[2]**		**OR [95%-C.I.] [3]**	**p**	**OR [95%-C.I.] [3]**	**p**
Surface Germany	U	$CD4^+CD25^+$	0.75 [0.20-2.88]	0.68	0.30 [0.04-1.99]	0.21
		$CD4^+25^+CD127^{low/-}$	0.59 [0.13-2.72]	0.50	0.19 [0.02-1.59]	0.12
		$CD4^+25^{high}CD127^{low/-}$	0.99 [0.20-4.80]	0.99	0.63 [0.06-6.36]	0.70
	PI	$CD4^+CD25^+$	0.46 [0.09-2.33]	0.35	0.17 [0.01-2.67]	0.21
		$CD4^+25^+CD127^{low/-}$	1.14 [0.22-5.97]	0.88	0.43 [0.03-6.07]	0.53
		$CD4^+25^{high}CD127^{low/-}$	0.75 [0.14-4.02]	0.73	0.84 [0.04-18.56]	0.91
	LPS	$CD4^+CD25^+$	**0.24** [0.06-0.94]	**0.04**	0.14 [0.02-1.09]	0.06
		$CD4^+25^+CD127^{low/-}$	0.53 [0.16-1.81]	0.31	**0.14** [0.03-0.66]	**0.01**
		$CD4^+25^{high}CD127^{low/-}$	0.63 [0.18-2.18]	0.47	1.00 [0.13-7.73]	1.00
Intracellular [1] Germany + France	U	$CD4^+CD25^+$	0.56 [0.18-1.77]	0.32	0.93 [0.15-5.94]	0.94
		$CD4^+CD25^+$ $FOXP3^+$	0.48 [0.22-1.06]	0.07	0.69 [0.18-2.72]	0.60
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	**0.38** [0.16-0.86]	**0.02**	0.88 [0.20-3.75]	0.86
	PI	$CD4^+CD25^+$	1.18 [0.35-4.01]	0.79	0.17 [0.02-1.09]	0.06
		$CD4^+CD25^+$ $FOXP3^+$	0.52 [0.16-1.67]	0.27	0.22 [0.03-1.43]	0.11
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	0.50 [0.16-1.61]	0.25	0.16 [0.03-1.05]	0.06
	LPS	$CD4^+CD25^+$	0.82 [0.28-2.42]	0.72	1.57 [0.26-9.45]	0.62
		$CD4^+CD25^+$ $FOXP3^+$	0.41 [0.16-1.05]	0.06	0.52 [0.12-2.25]	0.38
		Upper20%$CD4^+CD25^+$, $FOXP3^+$	0.51 [0.22-1.20]	0.12	0.70 [0.18-2.66]	0.60

[1] All analyses are adjusted for farming, analyses for intracellular staining additionally adjusted for center.
[2] U, unstimulated; PI, PMA/Ionomycin; LPS, Lipopolysaccharide.
[3] OR= Odds ratio, 95%.-C.I. =95%-confidence intervals, p=p-value of logistic regression analyses in the log-transformed data. *P* values in bold letters reached significance with p≤0.05.

In summary, *FOXP3* demethylation was a reliable marker to assess Treg cells in cord blood. Besides, Treg cells were increased (%) in a farming population at age 4.5 years. In addition, farm milk consumption in early childhood led to an increase of Treg cells (%) and an inverse association of Treg cells with inhalant IgE was observed at age 4 years. These data suggests a potential protective influence for the development of atopic diseases in childhood.

3.2 The role of Th17 cells during immune maturation

3.2.1 The relevance of Th17 cells in cord blood

Early life contact and particularly prenatal exposure to a farm environment is a protective factor for the development of ADs, potentially through an effect on immune maturation (Ege *et al.* 2006; von Mutius *et al.* 2010). Previous studies suggest that Treg and Th17 cells are involved in the inflammatory process and/or immunopathogenesis of ADs (Bullens *et al.* 2006; Koga *et al.* 2008; Ryanna *et al.* 2009; Strickland *et al.* 2011). Indeed, Treg cells were increased and functionally more suppressive in cord blood from neonates born in a faming environment compared to reference neonates from the same rural areas (Schaub *et al.* 2009). However, little is known whether Th17 cells, a cell subset generally increased in ADs (Bullens *et al.* 2006; Koga *et al.* 2008), may be altered in children growing up on a farm. To date, analyses of IL-17 expression in cord blood of farming compared to non-farming mothers did not reveal any significant difference (Schaub *et al.* 2009). However, more analyses are required to address this complex question in more detail.

Hence, gene and protein expression of Th17-lineage related markers including the transcription factors *RORC* and *RORA,* the cytokines IL-17, *IL-17F* and *IL-22* and the transmembrane protein *IL-23R* were analyzed unstimulated and after innate (Lipid A, LpA; peptidoglycan, Ppg) and mitogen (phytohemagglutinin, PHA) stimulation in neonatal cord blood from 22 farming and 60 non-farming mothers (Lluis *et al.* Manuscript submitted).

3.2.1.1 Th17-lineage markers were expressed in cord blood

Gene expression of Th17-lineage markers was assessed in unstimulated and stimulated (PHA; LpA; Ppg) cord blood samples (Table II). For the main transcription factor of Th17 cells, *RORC*, two isoforms are described. Two different primer sets were used. RORC3 primers amplified isoform b; RORC4 primers amplified both isoforms a and isoform b (Figure 8). Delta CT (Δct) values are depicted. A higher Δct resembles lower mRNA expression. An increase of gene expression levels of

RORC (RORC3: isoform b; RORC4: isoforms a+b), *IL-17, IL-17F, IL-22* and *IL-23R* was observed upon stimulation ($p<0.05$, Figure 9 A-B, D-G) while *RORA* was downregulated ($p<0.001$, Figure 9 C) (Lluis *et al.* Manuscript submitted). Paired Prentice-Wilcoxon test was used for analyses (Ballenberger *et al.* Manuscript submitted).

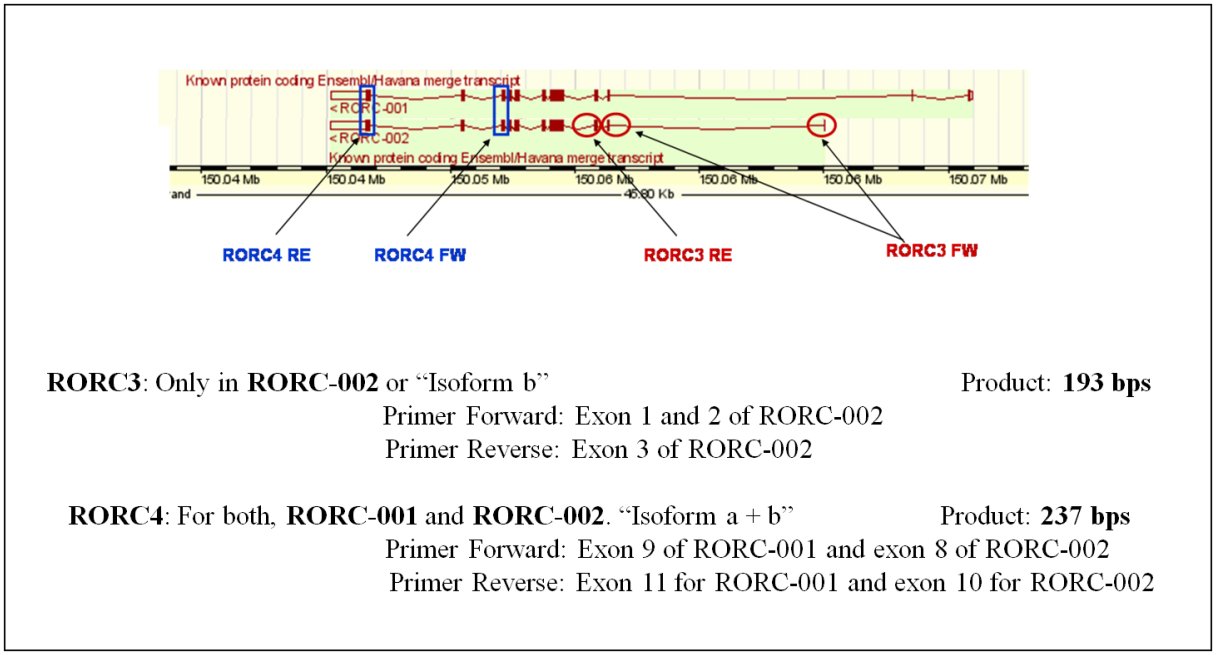


Figure 8. Primer design for *RORC* amplification. 2 transcripts described: "*RORC* isoform a", also known as *RORC1*; and "*RORC* isoform b", also known as *RORC2*. The primers named RORC3, in red, amplify for *RORC* isoform b; and the primers pair RORC4, in blue, amplifies both *RORC* isoforms a and b.

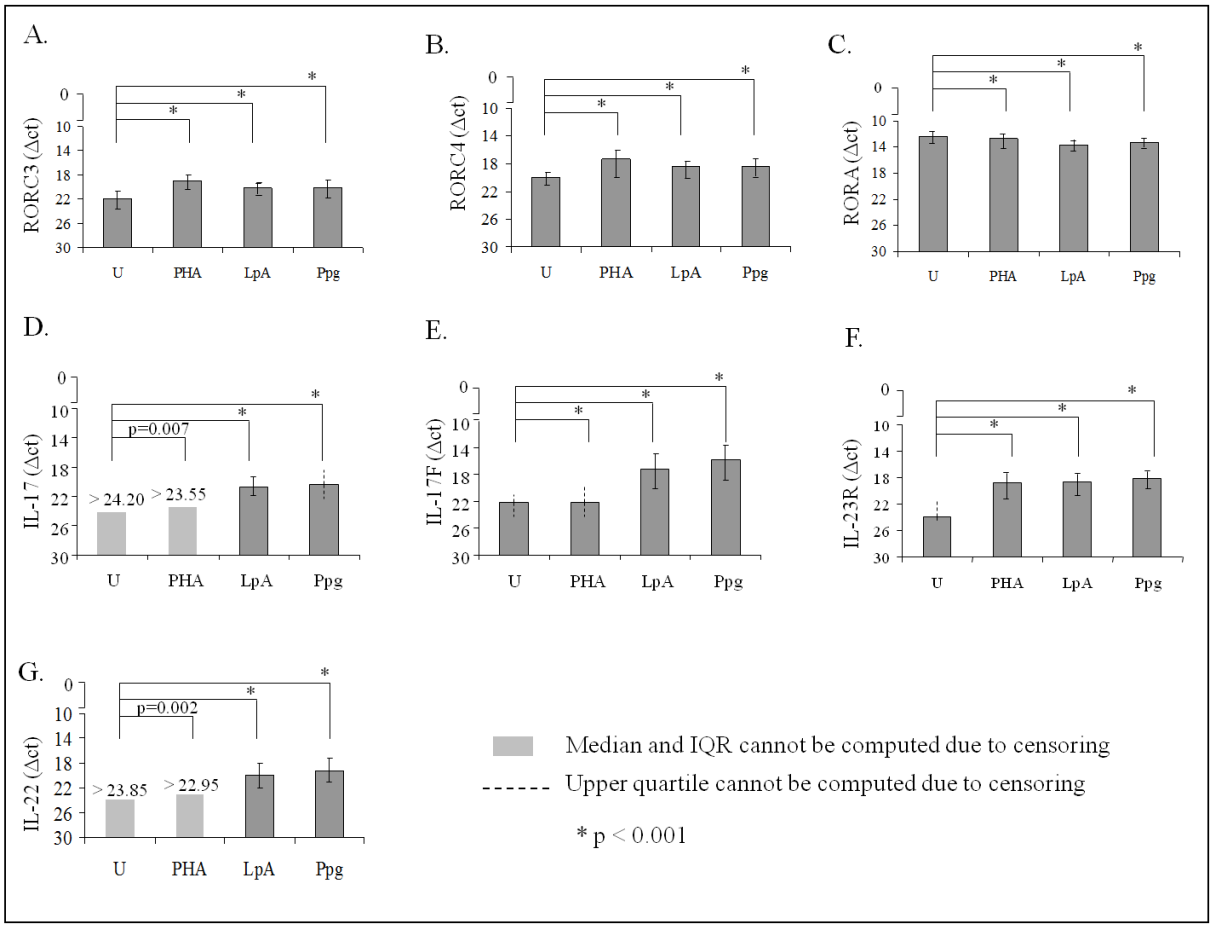


Figure 9. Gene expression of Th17-lineage related markers in CBMCs. Δct are shown; axes vice versa; lower Δct values between groups correspond to increased mRNA expression. Data are shown as median (interquartile range, IQR). Paired Prentice-Wilcoxon test. U, unstimulated; PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan. N=67. *p<0.001.

3.2.1.2 Th17 cells were positively correlated with gene expression of regulatory T cell markers in cord blood

To study putative reciprocal differentiation of Th17 and Treg cells in cord blood, a correlation between these two cell populations under different stimulation conditions was assessed. Hence, gene expression of Th17 makers *RORC* with respective isoforms, *RORA, IL-23R, IL-17, IL-17F*, *IL-22* and Treg markers *FOXP3, LAG3* and *GITR* was analyzed. Gene expression of Th17 transcription factors *RORC* (primers RORC4: isoform a+b,) and *RORA* were highly positively correlated with *FOXP3* mRNA levels following all conditions under study (PHA; LpA; Ppg; Table IX). The expression of

isoform b of *RORC* (primers RORC3) was strongly positively correlated with levels of *FOXP3* upon PHA stimulation (Table IX). In contrast, *IL-17, IL-17F* and *IL-22* cytokines were positively correlated upon innate stimulation (LpA and Ppg) (Table IX). No correlations were found in unstimulated conditions (data not shown). Similarly, significant positive correlations between Th17 mRNA markers and other Treg-related markers such as *LAG3* and *GITR* were observed (data not shown) (Lluis *et al.* Manuscript submitted).

Table IX. Correlation between *FOXP3* mRNA (Treg cells) and Th17-lineage genes mRNA expression. Spearman correlation coefficient test.

Marker	Stimulus	N	$r^{\#}$	p
RORC3	PHA	54	0.70	**$<1x10^{-5}$**
	LpA	44	0.26	0.09
	Ppg	44	0.41	0.005
RORC4	PHA	53	**0.70**	**$<1x10^{-5}$**
	LpA	43	**0.55**	**$2x10^{-4}$**
	Ppg	43	**0.70**	**$<1x10^{-5}$**
RORA	PHA	54	**0.55**	**$2x10^{-5}$**
	LpA	44	**0.58**	**$4x10^{-5}$**
	Ppg	44	**0.45**	**$2x10^{-3}$**
IL-23R	PHA	54	**0.69**	**$<1x10^{-5}$**
	LpA	44	**0.45**	**$2x10^{-3}$**
	Ppg	44	**0.37**	**0.01**
IL-17	PHA	54	0.07	0.62
	LpA	44	**0.48**	**$9x10^{-4}$**
	Ppg	44	**0.77**	**$<1x10^{-5}$**
IL-17F	PHA	53	0.20	0.16
	LpA	44	**0.54**	**$1x10^{-4}$**
	Ppg	44	**0.71**	**$<1x10^{-5}$**
IL-22	PHA	54	0.20	0.15
	LpA	44	**0.47**	**$1x10^{-3}$**
	Ppg	44	**0.63**	**$<1x10^{-5}$**

PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan.
RORC3: *RORC* isoform b; RORC4: *RORC* isoform a+b
p= p-value. P values in bold letters reached significance with $p \leq 0.05$.
[#] Spearman's correlation coefficient *r*.

3.2.1.3 Th17 mRNA markers in cord blood were independent of maternal farm exposure

Th17 cells were increased in patients with ADs compared to healthy donors (Toda *et al.* 2003; Bullens *et al.* 2006; Ciprandi *et al.* 2009; Cosmi *et al.* 2011). Nevertheless, little is known about Th17 cells in early life in a protective environment for development of ADs such as farming exposure. Thus, gene expression of Th17 markers was studied in cord blood from a farming population in comparison to a reference group.

IL-23R mRNA expression was significantly lower in unstimulated samples from cord blood of farming compared to non-farming mothers (Table X, p=0.02). No other significant differences in gene expression of Th17-lineage markers were found between the two groups (Table X) (Lluis *et al.* Manuscript submitted).

Since Th17 and Treg mRNA markers were correlated in cord blood (Table IX), the data was adjusted for *FOXP3* mRNA expression. An interaction effect was observed between Treg (*FOXP3* mRNA) and farming on the Th17 related markers *IL-22* (p=0.038) and *IL-23R* (p=0.052) upon LpA stimulation. No significant differences on Th17 markers depending on farming status were observed (data not shown) (Lluis *et al.* Manuscript submitted).

Neonatal CBMCs from farm exposed mothers (including farming mothers and mothers exposed to stable, barn, farm animals and/or farm milk consumption) compared to non-farm exposed mothers showed similar effects (data not shown) (Lluis *et al.* Manuscript submitted).

Table X. Gene expression of Th17 markers in CBMCs from neonates from farming mothers compared to non-farming mothers. A positive estimate resembles lower gene expression. Tobit regression on rank transformed data is depicted. N=67.

Stimulus	Marker	Estimate	p	Stimulus	Marker	Estimate	p
U	RORC3	12.04	0.08	LpA	RORC3	6.45	0.21
	RORC4	-3.94	0.51		RORC4	-0.59	0.92
	RORA	-1.42	0.80		RORA	-2.65	0.60
	IL23R	23.54	**0.02**		IL23R	1.70	0.76
	IL17	448.72	1.00		IL17	-0.24	0.97
	IL17F	-9.85	0.27		IL17F	-2.81	0.66
	IL22	17.50	0.39		IL22	-3.98	0.48
PHA	RORC3	0.36	0.95	Ppg	RORC3	1.13	0.83
	RORC4	-4.82	0.39		RORC4	1.22	0.82
	RORA	-3.25	0.54		RORA	3.30	0.51
	IL23R	-8.88	0.15		IL23R	-2.99	0.59
	IL17	5.23	0.74		IL17	-1.20	0.87
	IL17F	-12.98	0.11		IL17F	-2.70	0.62
	IL22	-13.22	0.17		IL22	-3.08	0.61

U, unstimulated; PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan.
RORC3: *RORC* isoform b; RORC4: *RORC* isoform a+b
p= p-value. P values in bold letters reached significance with $p \leq 0.05$.

3.2.1.4 Maternal farm exposure influenced the Th17 and regulatory T cell correlation

A significant interaction effect was found in CBMCs between Treg cells and maternal farming status on Th17 markers. Consequently, the correlation between gene expression of *FOXP3* (Treg cells) and Th17-lineage genes was determined in CBMCs stratified by maternal farming status in order to assess whether the Th17/Treg correlation was different in these two groups (Table XI).

A positive correlation was observed upon stimulation (PHA; LpA; Ppg) within CBMCs of non-farming children (Table XI). In the farming group, gene expression of Th17 and Treg markers was positively correlated upon PHA and Ppg stimulation, but not in LpA-stimulated samples (Table XI)

(Lluis *et al.* Manuscript submitted). The data indicate a stimulus-specific effect on Th17/Treg regulation in neonates with low maternal exposure to LpA, the main active component of endotoxin, compared to neonates with maternal farm exposure, where no effect was observed in CMBCs.

Table XI. Correlation between *FOXP3* mRNA (Treg cells) and Th17-lineage genes mRNA expression in CBMCs stratified by maternal farming status. Spearman correlation coefficient.

		Maternal farming			Maternal non-farming		
Stimulus	**Marker**	**N**	***r***	**p**	**N**	***r***	**p**
U	RORC3	17	0.17	0.51	41	0.05	0.74
	RORC4	17	0.43	0.09	40	0.24	0.14
	RORA	17	0.28	0.27	41	**0.44**	**0.004**
	IL-23R	17	0.25	0.33	41	**0.56**	**$2x10^{-4}$**
	IL-17	17	0.16	0.54	41	0.13	0.41
	IL-17F	17	0.004	0.99	41	0.05	0.75
	IL-22	17	0.34	0.18	41	0.14	0.37
PHA	RORC3	17	**0.62**	**0.008**	37	**0.70**	**$<1x10^{-5}$**
	RORC4	17	**0.79**	**$2x10^{-4}$**	36	**0.65**	**$2x10^{-5}$**
	RORA	17	**0.65**	**0.005**	37	**0.50**	**0.002**
	IL-23R	17	**0.66**	**0.004**	37	**0.73**	**$<1x10^{-5}$**
	IL-17	17	-0.09	0.74	37	0.14	0.42
	IL-17F	17	0.17	0.52	36	0.22	0.20
	IL-22	17	0.11	0.67	37	0.26	0.12
LpA	RORC3	12	0.52	0.08	32	0.19	0.30
	RORC4	12	0.56	0.06	31	**0.54**	**0.002**
	RORA	12	0.35	0.27	32	**0.66**	**$3x10^{-5}$**
	IL-23R	12	0.20	0.53	32	**0.68**	**$2x10^{-5}$**
	IL-17	12	0.32	0.31	32	**0.57**	**$7x10^{-4}$**
	IL-17F	12	0.38	0.22	32	**0.64**	**$7x10^{-5}$**
	IL-22	12	0.20	0.53	32	**0.63**	**$1x10^{-4}$**
Ppg	RORC3	12	**0.65**	**0.02**	32	0.30	0.10
	RORC4	12	**0.86**	**$4x10^{-4}$**	31	**0.60**	**$4x10^{-4}$**
	RORA	12	0.26	0.42	32	**0.47**	**0.007**
	IL-23R	12	0.34	0.27	32	0.31	0.08
	IL-17	12	**0.77**	**0.003**	32	**0.73**	**$<1x10^{-5}$**
	IL-17F	12	**0.62**	**0.03**	32	**0.67**	**$2x10^{-5}$**
	IL-22	12	**0.66**	**0.02**	32	**0.61**	**$2x10^{-4}$**

U, unstimulated; PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan.
RORC3: *RORC* isoform b; RORC4: *RORC* isoform a+b
p=p-value. *P* values in bold letters reached significance with $p \leq 0.05$.

3.2.1.5 Effect of retinoic acid on mRNA expression of Th17 and regulatory T cells

Retinoic acid (RA), a derivate of vitamin A, is a key regulator of the Th17/Treg cell regulation (Mucida *et al.* 2007; Bai *et al.* 2009). As data suggest an interaction between Th17 and Treg cells in cord blood, a potential hypothesis was that RA may already influence Th17/Treg regulation early in life. Hence, the effect of different doses of RA *in vitro* on unstimulated or PHA stimulated CBMCs of healthy controls was analyzed and its influence on gene expression of both cell populations was measured.

CBMCs were stimulated with or without PHA and different doses of RA for 48h. Increasing doses of RA led to downregulation of Treg and Th17 mRNA markers (Figure 10). *RORC* (primers RORC4: isoform a+b,) was downregulated upon PHA stimulation with 50nM and 100nM RA, while *IL-23R* was downregulated under both conditions, unstimulated and after PHA stimulation with all doses of RA (Figure 10, $p \leq 0.05$). In addition, lower levels of *FOXP3* were detectable in both unstimulated and PHA stimulated cells with 50nM and 100 nM retinoic acid (Figure 10, $p < 0.05$) (Lluis *et al.* Manuscript submitted).

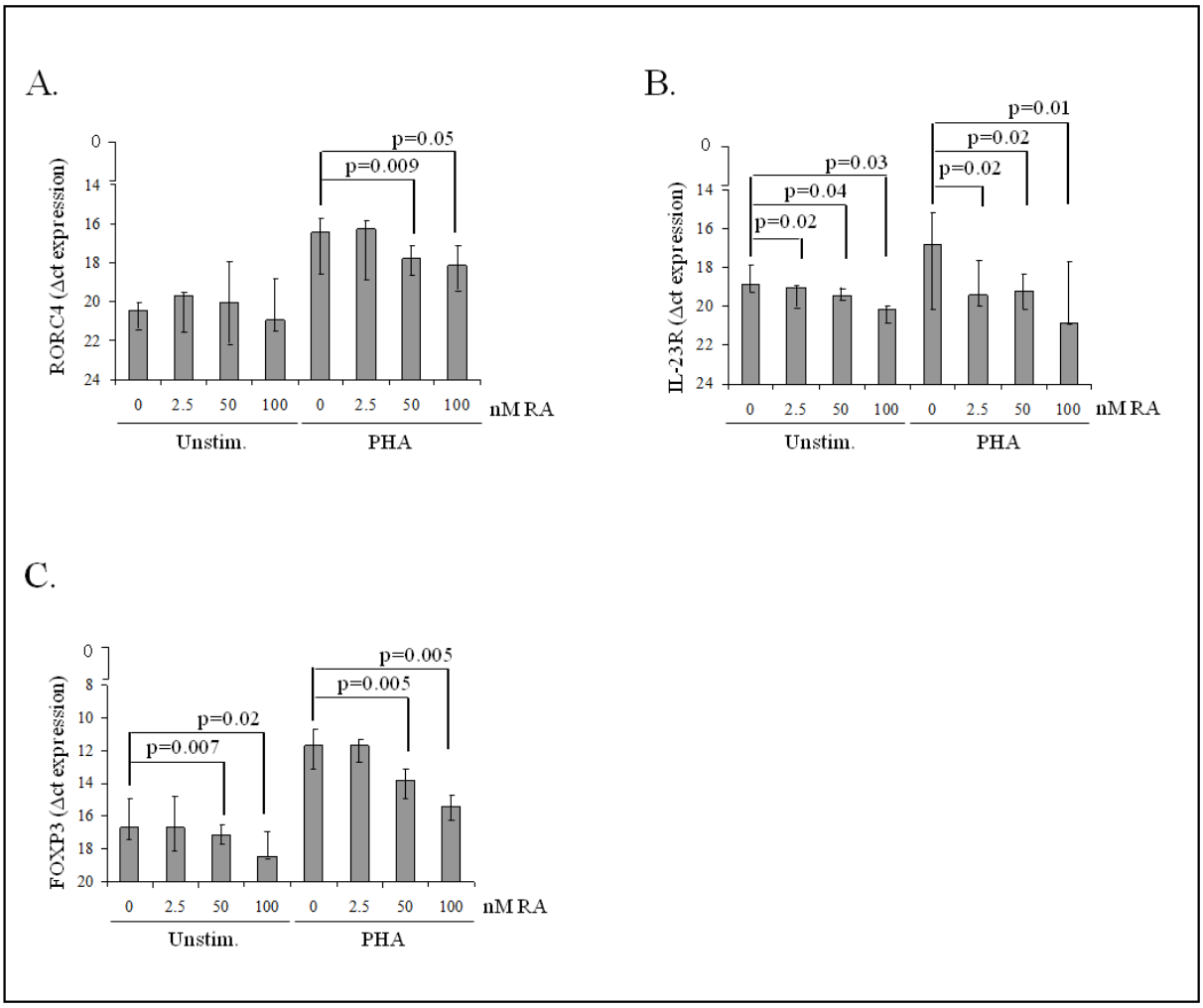


Figure 10. Effect of retinoic acid on Th17 and Treg cells in CBMCs. Δct are shown; axes vice versa; lower Δct values between groups correspond to increased mRNA expression. Data are shown as median (interquartile range, IQR). Paired Prentice-Wilcoxon test. U, unstimulated; PHA, phytohemagglutinin, N=7.

In summary, Th17 cells were present in cord blood at low levels. Maternal farm exposure did not influence gene expression of Th17-lineage markers at birth. Furthermore, cord blood Th17 and Treg cells were positively correlated upon stimulation (PHA; Ppg) in both farming and non-farming children. Upon LpA stimulation, only the non-farming group showed positive correlation between Th17 and Treg cells, suggesting an influence of maternal farming at this early stage of immune maturation.

3.3 The role of genetic polymorphisms on T cells and other markers at birth

In addition to environmental factors, genetic polymorphisms are known to influence the immune system with a subsequent effect on the risk of developing ADs (Cameron *et al.* 2006; Schedel *et al.* 2008; Schedel *et al.* 2009).

In the present study, genetic variants located within genes of the Th17 pathway, a T cell subset that play a role in the pathogenesis of ADs (Toda *et al.* 2003; Bullens *et al.* 2006; Ciprandi *et al.* 2009; Cosmi *et al.* 2011), were examined in cord blood. The aim was to assess whether these polymorphisms may already influence Th17 and other T cell subsets including Treg, Th1 and Th2 cells at this early stage of immune maturation (Lluis *et al.* Manuscript submitted).

In addition it was analyzed if genetic variants within the chromosomal region 17q2,1 previously associated with childhood asthma (Moffatt *et al.* 2007; Moffatt *et al.* 2010) influencing *ORMDL3* and *GSDMB* gene expression (Moffatt *et al.* 2007; Verlaan *et al.* 2009; Halapi *et al.* 2010), may already influence the immune system in cord blood. Thus, genetic variants within 17q21 region were examined in relation to the expression levels of Th17, Treg, Th1 and Th2 cell markers at birth. In a subsample, it was investigated if 17q21 polymorphic alleles influenced expression levels of the genes located within the 17q21 region (*ORMDL3, GSDMA, GSDMB, ZNFN1A3, ZPBP2* and *PSMD3*) in cord blood (Lluis *et al.* 2011).

3.3.1.1 The role of polymorphisms within genes of the Th17-lineage in cord blood

The impact of polymorphisms within Th17-lineage genes on mRNA expression of Th17- and Treg-related genes and protein cytokine secretion was studied in cord blood (the PAULCHEN study).

3.3.1.1.1 SNP characteristics

Seven SNPs located within the *IL-17*, *IL23R* and *RORC* genes and previously known to be relevant for childhood asthma (Michael Kabesch, personal communication) were genotyped (Table XII). The genotyping success rate was at least 96%. None of the polymorphisms significantly deviated from Hardy-Weinberg equilibrium.

Analyses were performed using the recessive and dominant model. The best fitting model was applied to avoid heterozygous effects observed in the linear model and to receive enough statistical power (Lluis *et al.* Manuscript submitted).

Table XII. Description of analyzed SNPs in the Th17 pathway genes and their respective rs number, position in the gene structure and allele frequency.

Gene	rs no.	Position to first ATG*	Position in the Gene Structure	Major/Minor Allele [CEU]#	MAF [CEU]#	MAF [our cohort]
RORC	rs949969	11427 (transcript variant 1) 5599 (transcript variant 2)	intron	C/T	0.25	0.28
	rs7540530	13383 (transcript variant 1) 7555 (transcript variant 2)	intron	A/G	0.45	0.53
IL-23R	rs790631	43119	intron	T/C	0.25	0.21
	rs7517847	47866	intron	T/G	0.45	0.60
	rs10889675	88413	intron	C/A	0.15	0.14
IL-17	rs9395766	-5215	5' upstream	T/G	0.39	0.37
	rs2275913	-197	promoter region	G/A	0.36	0.38

* Based on the genes sequences obtained from Ensembl (http://www.ensembl.org; NCBI36 Release 54, May2009)
# CEU: Utah residents with Northern and Western European ancestry from the CEPH collection. Data obtained from SNPPER database (http://snpper.chip.org/)

3.3.1.1.2 Polymorphisms within the Th17-lineage genes influenced T cell subsets and other immune-related markers in cord blood

In a recessive model, homozygous carriers of the polymorphic allele of rs7517847 (*IL-23R)* showed a significant downregulation of Th17-lineage markers for *IL-23R (*LpA, Ppg), *IL-17 (*LpA), *IL-17F* (U, LpA, Ppg) and *IL-22* (LpA) mRNA expression; and of IL-17 (LpA, Ppg) protein secretion ($p \leq 0.04$) (Table XIII, A-Table I in Appendix). A similar trend was observed for homozygous carriers of the polymorphic allele of rs2275913 located in the *IL-17* gene. In homozygous carriers of the polymorphic allele of rs790631 (*IL23R)* an upregulation of Th17-lineage markers *RORC* (isoform b; Ppg) and *IL-17F* (U) gene expression was detectable. In contrast, lower expression levels of *IL23R* (PHA) and *FOXP3* (Ppg) mRNA levels ($p \leq 0.05$) (Table XIII, A-Table I in Appendix) were observed in the presence of the polymorphic allele of rs790631 (Lluis *et al.* Manuscript submitted).

As rs10889675 in *IL-23R* has a minor allele frequency (MAF) of 0.14 (Table XII) only one subject in the PAULCHEN study population was found to be homozygous for the polymorphic allele. Thus, the dominant model was applied for rs10889675. A downregulation of Th17-lineage markers (U: *RORC* isoform b, *RORA, IL-22*) and increased gene expression levels for *FOXP3* (LpA) were observed (Table XIII, A-Table I in Appendix). In carriers of the polymorphic allele of rs10889675 downregulation of the respective cytokines IL-5 (PHA, Ppg), IL-13 (PHA), IL-6 (PHA), and IL-15 (PHA, LpA) ($p \leq 0.05$) was present after stimulation (Table XIII, A-Table I in Appendix).

None of the other SNPs under study significantly influenced gene or protein expression levels of Treg and Th17 markers (data not shown) (Lluis *et al.* Manuscript submitted).

Table XIII. Summary of the findings for polymorphisms located within Th17-related genes. A. Recessive model. B. Dominant model.

A.

Gene	rs no.	Th17							Treg
		mRNA						protein	mRNA
		RORC	RORA	IL-23R	IL-17	IL-17F	IL-22	IL-17	FOXP3
IL-23R	rs7517847 GG	n.d	↓ LpA	↓ LpA* Ppg*	↓ LpA*	↓ U* LpA* Ppg*	↓ LpA*	↓ LpA* Ppg*	n.d.
	rs790631 CC	↑ Ppg*	n.d.	↓ PHA*	n.d.	↑ U*	n.d.	n.d.	↓ PHA Ppg*
IL-17	rs2275913 AA	n.d.	↓ LpA Ppg	↓ PHA	↓ LpA	↓ Ppg	↓ Ppg	n.d.	n.d.

B.

Gene	rs no.	Th17							Treg
		mRNA						protein	mRNA
		RORC	RORA	IL-23R	IL-17	IL-17F	IL-22	IL-17	FOXP3
IL-23R	rs10889675 AA	↓ U*	↓ U*	n.d.	n.d.	↓ U	↓ U*	↓ Ppg	↑ LpA* Ppg

Gene	rs no.	protein						
		IFNγ	IL-5	IL-13	IL-6	IL-15	TNF-α	GM-CSF
IL-23R	rs10889675 AA	n.d.	↓ PHA* LpA Ppg*	↓ PHA*	↓ PHA*	↓ PHA* LpA*	↓ PHA	↓ LpA

↑: expression upregulated. ↓: expression downregulated. U, unstimulated; PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan. *RORC,* primers RORC3: *RORC* isoform b,. *n.d.*, no difference.
* p-value <0.05.

3.3.1.2 The role of asthma-associated polymorphisms within the 17q21 region in cord blood

It has previously been demonstrated that genetic variants in a determined pathway may influence the expression of other cell subsets not directly related to the pathway (Liu *et al.* 2011; Casaca *et al.* 2012). Indeed, in this study, polymorphisms within the Th17-lineage genes influenced gene expression of *FOXP3*, the main transcription factor of Treg cells, in cord blood.

In two Genome Wide Association Studies (GWAS), genetic variants of the 17q21 locus were strongly associated with childhood asthma (Moffatt *et al.* 2007; Moffatt *et al.* 2010) and influenced transcription levels of *ORMDL3* and *GSDMB* (Moffatt *et al.* 2007; Verlaan *et al.* 2009). However, little is known about the role of 17q21 polymorphisms in an early stage of immune maturation.

For this purpose, 200 neonates including the PAULINA and the PAULCHEN datasets were recruited. Gene expression and cytokine secretion of T cell subsets and mRNA expression of genes located within 17q21 region were assessed before and following microbial (LpA; Ppg); PHA or Derp1 stimulation and related to genetic variants within the 17q21 locus (Lluis *et al.* 2011).

3.3.1.2.1 SNP characteristics

Genotyping of 10 SNPs within the 17q21 locus previously shown to be relevant for childhood asthma was performed (Moffatt *et al.* 2007) (Figure 11; Table XIV). The genotyping success rate was at least 94%, whereas rs2290400 (block 1) was excluded from all analyses because genotyping was not successful. None of the polymorphisms significantly deviated from Hardy-Weinberg equilibrium (HWE) (Table XIV). As allele frequencies for most of the 17q21 SNPs were close to 50%, the respective risk allele (instead of the minor allele) was reported (Table XIV). The respective risk allele was defined as the allele increasing the risk for development of childhood asthma corresponding to GWAS (Moffatt *et al.* 2007).

Linkage disequilibrium (LD) analyses identified 4 major LD blocks (Figure 11), each represented by a tagging SNP (rs7216389, rs4795405, rs8079416, rs3859192) (Lluis *et al.* 2011).

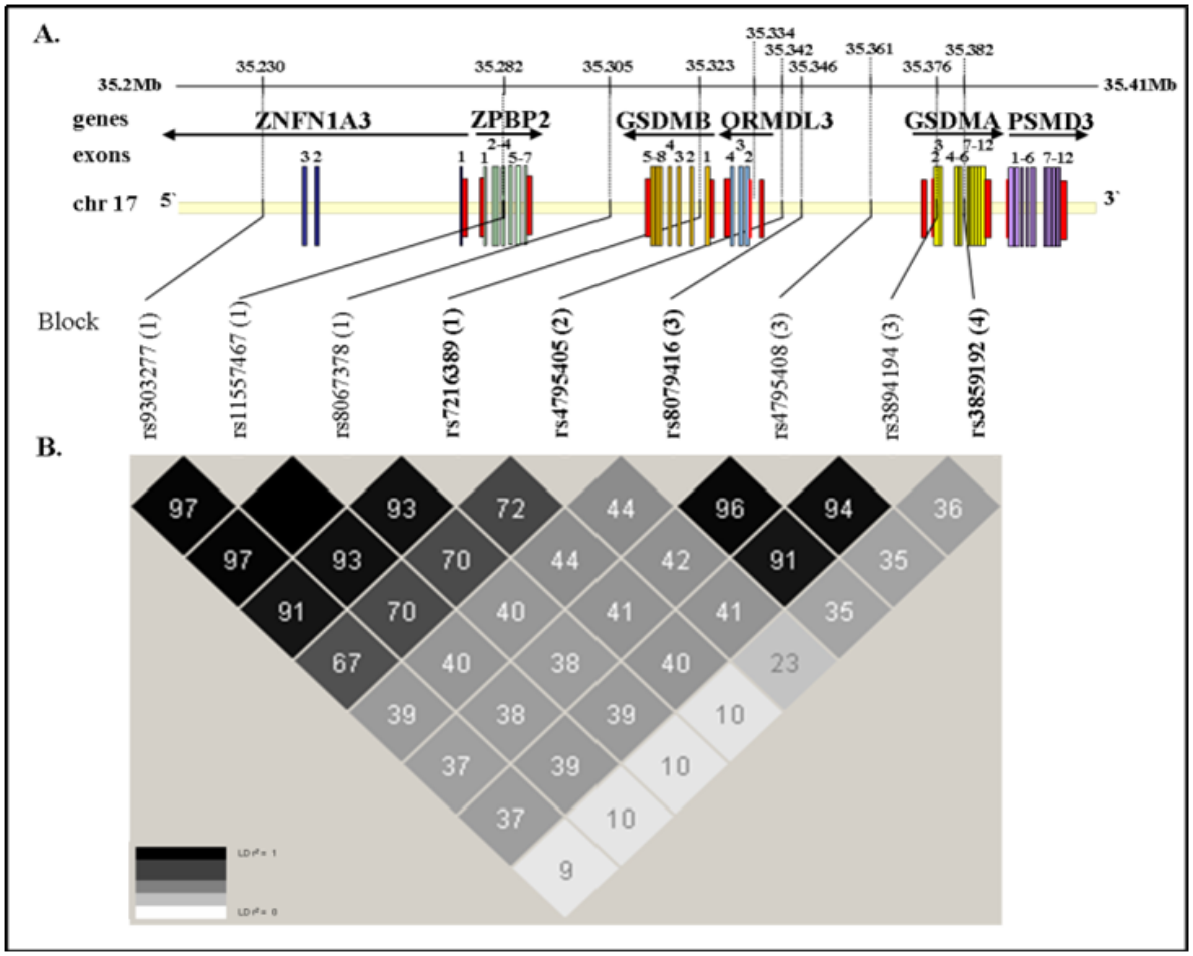


Figure 11. Genomic structure, localization, and linkage disequilibrium (R^2 plot) of the genotyped polymorphisms in the 17q21 locus. *Arrows* indicate transcriptional direction. Exons with exon numbers are displayed in color codes. Smaller red boxes represent 5' and 3' untranslated regions. Tagging SNPs (R^2>0.85) for each linkage disequilibrium block (brackets) are depicted in boldface. Chr, Chromosome; Mb, Megabase.

Table XIV. Genotyping information for polymorphisms within the Chr17q21 locus (N=200).

Genotyped SNPs	Non-risk allele	Risk allele	AF[1] risk allele	Call rate (%)	LD[2] blocks for 17q21	p (HWE[3])
rs9303277	T	C	0.49	95.0	block 1	0.99
rs11557467	T	G	0.48	94.5	block 1	0.96
rs8067378	G	A	0.48	94.0	block 1	0.90
rs2290400[4]					block 1	
rs7216389	C	T	0.48	95.5	block 1	0.70
rs4795405	T	C	0.55	94.0	block 2	0.58
rs8079416	T	C	0.45	95.0	block 3	0.99
rs4795408	G	A	0.44	95.5	block 3	0.88
rs3894194	G	A	0.44	95.5	block 3	0.99
rs3859192	C	T	0.44	95.5	block 4	0.19

AF=allele frequency. p=p-value.
Bold letters represent the identified tagging SNPs for the 17q21 locus based on R^2>0.85.
[1] Because AFs for most of the 17q21 SNPs were close to 50%, the respective risk allele (instead of the minor allele) is depicted. This was defined as the allele increasing the risk for the development of asthma as shown in previous publications (Moffatt *et al.* 2007).
[2] LD=linkage disequilibrium.
[3], p-value of χ^2 test for deviation of Hardy-Weinberg Equilibrium.
[4] Genotyping failed for rs2290400.

3.3.1.2.2 The asthma-associated risk diplotypes within the 17q21 region influenced ORMDL3 *and* GSDMA *gene expression*

As previously described, polymorphisms within the 17q21 region influenced the gene expression of *ORMDL3* and *GSDMB*, two genes located within the locus (Moffatt *et al.* 2007; Verlaan *et al.* 2009; Halapi *et al.* 2010). However, little is known about the influence of these polymorphisms on mRNA levels of genes located within this region in early life.

For these analyses, haplotype frequencies were estimated on the basis of the four 17q21 tagging SNPs. A haplotype is a set of alleles of polymorphisms that are transmitted together to the next generation. Unlike genotype, these polymorphisms are located on a single chromatide and are not detectable on both strands of a chromosome. Two haplotypes were observed that contained either only non-risk alleles of 17q21 (H1 frequency, 0.36, Table XV) or the combination of the respective risk alleles (H2 frequency, 0.29; Table XV). Because linkage disequilibrium (LD) is strong in the 17q21 region and

risk and non-risk alleles were almost equally distributed, it was possible to select a number of children that were either homozygous for the risk alleles at all 4 SNP loci (N=9) and to compare these with homozygous carriers of the non-risk alleles (N=8) at the respective positions (diplotypes).

Table XV. Estimated haplotypes based on the four tagging SNPs within the chromosome 17q21 region and their frequencies (N=187 with complete phase information).

Haplotype	rs7216389	rs4795405	rs8079416	rs3859192	Estimated frequency
H1	C	T	T	C	0.36
H2	T	C	C	T	0.29
H3	T	C	T	C	0.09
H5	T	C	C	C	0.09
H5	C	T	T	T	0.05
H6	C	C	T	T	0.05
H7	C	T	C	T	0.03
H8	C	C	C	T	0.02
H9	C	C	C	C	0.007
H10	T	T	T	C	0.007
H11	C	T	C	C	0.004
H12	T	T	T	T	0.002

H1 represents the non-risk and H2 the risk haplotype.

Children homozygous for all 4 risk alleles for 17q21 tagging SNPs showed increased expression for *ORMDL3* (Derp1) and *GSDMA* (PHA /Derp1); both remained significant after adjustment for multiple testing (*; Figure 12, A and B).

Furthermore, it was assessed whether the combination of 17q21 risk diplotypes also influenced mRNA expression of other genes located within the 17q21 cluster including *GSDMB*, *ZNFN1A3*, *ZPBP2*, and *PSMD3*. When homozygous carriers of the four 17q21 risk alleles and carriers of non-risk alleles were compared, a trend to decreased expression of *ZNFN1A3* after peptidoglycan stimulation was detectable (Figure 12, D). No significant effect on gene expression for the additionally analyzed genes of 17q21 (*GSDMB, ZPBP2, PSMD3*) was observed (Figure 12, C, E, F) (Lluis *et al.* 2011).

The data on *ORMDL3* and *GSDMA* was in accordance with the effects observed for individual risk alleles of each tagging SNPs (data not shown).

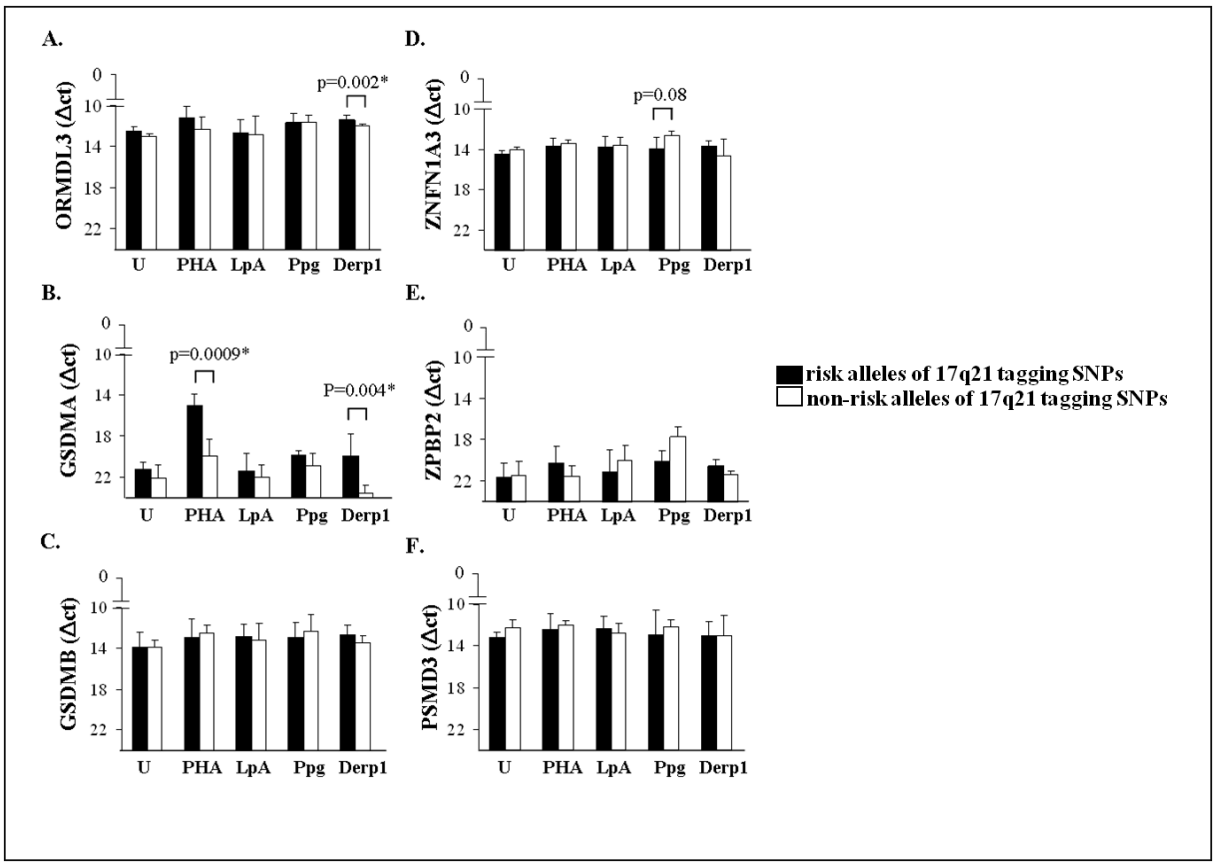


Figure 12. *ORMDL3* and *GSDMA* gene expression was increased in homozygous carriers of the risk haplotype (black bars) in comparison with the non-risk haplotype (white bars) after Derp1 and/or PHA stimulation in CBMCs. mRNA expression of genes located in 17q21 locus (N=9/8). ΔCT values are shown; axes are vice versa; lower ΔCT values between groups correspond to increased mRNA expression. Data are shown as median (interquartile range, IQR), analyzed applying the Wilcoxon test. mRNA expression was assessed in homozygous subjects for the risk or the non-risk haplotype. U, unstimulated; PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan; Derp1, house dust mite. *Significance of $p \leq 0.004$ (below the multiple testing significance threshold).

3.3.1.2.3 Polymorphisms in the 17q21 cluster influenced IL-17 expression in cord blood

As previously described, polymorphisms within a specific pathway may affect the transcription and protein levels of other genes not directly related to their respective downstream pathways (Liu *et al.* 2011; Casaca *et al.* 2012). Hence, it was hypothesized that polymorphisms in the 17q21 cluster may influence T cell cytokine secretion. In this study, none of the presented tagging SNPs influenced Th1 (IFN-γ) or Th2 (IL-13, IL-5) cytokine secretion, or Treg cell markers (*FOXP3*, *GITR*, *LAG3* mRNA expression; data not shown).

However, homozygous carriers of the risk allele of each tagging SNP individually within the 17q21 locus (rs7216389, rs4795405, rs8079416, rs3859192) showed increased IL-17 secretion in unstimulated (U) and PHA (Table XVI) but not innate (LpA, Ppg) stimulated cord blood samples (data not shown). When polymorphisms within one LD block were analyzed separately, similar results were observed for each SNP (A-Table II, A and B). IL-17 secretion was detectable but low. Both parametric and non-parametric tests showed significance for the same associations. To address the question of whether polymorphic alleles on the 17q21 locus may have an effect on IL-17 secretion primarily for the children with risk for atopy, geometric mean rations (GMRs) were adjusted for maternal atopy. Yet no significant influence of maternal atopy on the association of IL-17 and the respective genotype was observed in cord blood (Table XVI, A-Table II, A and B) (Lluis *et al.* 2011).

In summary, polymorphic alleles within the Th17-lineage genes showed an effect on cord blood T cells and cytokine secretion while genetic variants within the asthma-associated region 17q21 influenced IL-17 secretion and *ORMDL3* and *GSDMA* gene expression already in cord blood. Thus, genetic polymorphisms may play an important role on immune maturation and potentially impact the development of atopic diseases in childhood.

Table XVI. Risk alleles of 17q21 tagging SNPs increased IL-17 secretion in unstimulated and PHA stimulated cord blood (max. N=200).

Tagging SNP	Stimulation condition of IL-17	Genotype	N	Geometric mean (95% CI)[2]	GMR (95% CI)[2] p[3]	
					Crude	Adjusted for maternal atopy
rs7216389 (block 1)	U	TT risk allele[1]	43	0.19 (0.11-0.35)	1.83 (1.22 - 2.75) p=0.004	1.83 (1.22 - 2.76) p=0.004
		CT	96	0.10 (0.07-0.15)		
		CC	48	0.06 (0.03-0.10)		
	PHA	TT risk allele[1]	40	0.26 (0.12-0.56)	1.67 (1.04 - 2.66) p=0.03	1.67 (1.04 - 2.67) p=0.03
		CT	84	0.10 (0.06-0.16)		
		CC	45	0.09 (0.05-0.18)		
rs4795405 (block 2)	U	CC risk allele[1]	54	0.15 (0.09-0.26)	1.73 (1.14 - 2.62) p=0.01	1.73 (1.14 - 2.62) p=0.01
		CT	96	0.11 (0.08-0.17)		
		TT	35	0.05 (0.02-0.10)		
	PHA	CC risk allele[1]	48	0.25 (0.12-0.49)	1.79 (1.11 - 2.90) p=0.02	1.79 (1.11 - 2.91) p=0.02
		CT	86	0.11 (0.07-0.17)		
		TT	33	0.08 (0.04-0.18)		
rs8079416 (block 3)	U	CC risk allele[1]	38	0.23 (0.12-0.44)	1.73 (1.15 - 2.59) p=0.009	1.72 (1.15 - 2.59) p=0.009
		CT	92	0.09 (0.06-0.14)		
		TT	56	0.07 (0.04-0.13)		
	PHA	CC risk allele[1]	35	0.25 (0.11-0.55)	1.66 (1.04 - 2.64) p=0.04	1.66 (1.04 - 2.64) p=0.04
		CT	81	0.12 (0.08-0.20)		
		TT	52	0.09 (0.05-0.16)		
rs3859192 (block 4)	U	TT risk allele[1]	41	0.20 (0.11-0.37)	1.52 (1.03 - 2.25) p=0.04	1.53 (1.03 - 2.26) p=0.04
		CT	84	0.09 (0.06-0.13)		
		CC	62	0.08 (0.05-0.13)		
	PHA	TT risk allele[1]	35	0.23 (0.11-0.51)	1.42 (0.90 - 2.25) p=0.14	1.42 (0.90 - 2.26) p=0.14
		CT	77	0.10 (0.06-0.17)		
		CC	57	0.11 (0.06-0.19)		

U, unstimulated; PHA, phytohemagglutinin. p=p-value.

[1] the respective risk allele was defined as the allele increasing the risk for the development of asthma as shown in the original publication (Moffatt *et al.* 2007).

[2] GMR = Geometric mean ratio, 95% CI = 95% confidence interval.

[3] p-value of geometric mean ratio by regression models using an allele-counting model (0, 1, 2) for coding of the respective SNP.

4 Discussion

Early childhood is a time period which plays a key role on immune maturation. During this time frame both environmental exposures and genetic predisposition are thought to modulate the immune system, influencing the risk of developing atopic diseases (ADs). In this context, children being exposed to farming environment revealed a lower prevalence of ADs (von Mutius *et al.* 2010; Lluis *et al.* 2012). Besides Th1 and Th2 cells, other T cell subsets including regulatory T (Treg) and Th17 cells have been identified to be involved in the manifestation of ADs. Nevertheless, little is known about the mechanistic contribution of Treg and Th17 cells during immune maturation influenced by environmental and genetic factors for the development of ADs in childhood.

The present data demonstrates that *FOXP3* demethylation at the Treg-specific demethylated region (TSDR) is a valuable and reliable marker to assess Treg cells in cord blood (Liu *et al.* 2010). In neonates, it has previously been shown that *FOXP3* demethylation at the TSDR was associated with maternal farm exposures. In the same study, Treg cells counts were increased in cord blood from farming mothers (Schaub *et al.* 2008). Here is shown that Treg cells (%) were increased in 4.5-year-old children living in a farm and/or drinking farm milk while they were negatively associated with inhalant IgE (Lluis, Depner *et al.* Manuscript submitted). In contrast, maternal farming did not influence Th17 cells in cord blood; yet, in this population Treg and Th17 cells were positively correlated upon stimulation, influenced by maternal farming (Lluis *et al.* Manuscript submitted). In addition, the present study indicates an effect of genetic variants within the Th17-lineage genes (Lluis *et al.* Manuscript submitted) and the asthma-associated region 17q21 (Lluis *et al.* 2011) on gene expression and cytokine secretion already in neonates.

In this study it was demonstrated that *FOXP3* demethylation at the TSDR is a suitable and reliable marker to assess Treg cells in cord blood. Quantification of Treg cells isolated from cord blood may be not feasible, especially in large field studies due to methodological challenges. However, the present study indicates that *FOXP3* demethylation levels in EDTA-blood directly corresponded to a higher suppressive capacity of Treg cells and with standard Treg cell markers such as *FOXP3* mRNA

expression and $CD4^+CD25^{high}$ cells in unstimulated and Lipid A (LpA)-stimulated samples (Liu *et al.* 2010). Recent studies demonstrated that demethylation of *FOXP3* at the TSDR region was a reliable marker to quantify Treg cells in adults and mice (Baron *et al.* 2007; Floess *et al.* 2007). TSDR, a conserved region within the *FOXP3* locus, was demethylated in CpG motifs in natural Treg (nTreg) but not in $CD4^+CD25^-$ T, *in vitro* induced $FOXP3^+$ Treg cells or transiently activated $FOXP3^+$ T cells (Baron *et al.* 2007; Floess *et al.* 2007). So far, the markers used to define nTreg cells quantitatively included FOXP3 expression - the Treg transcription factor (Fontenot *et al.* 2005); $CD4^+CD25^{high}$, $CD4^+CD25^{high}FOXP3^+$ or $CD4^+CD25^{high}CD127^{low/-}$ (Shevach 2006); or functional examination of Treg cells through assessment of their suppressive capacity. However, these techniques require large amounts of cells and may therefore not always be feasible.

Some major advantages of determining Treg cells through *FOXP3* demethylation compared to other Treg parameters is the stability of DNA methylation compared to mRNA expression or protein synthesis (Baron *et al.* 2006). In contrast to other techniques such as suppression assays or flow cytometry, methylation analysis can be performed at any time point independent of sample withdrawal. Nonetheless, present studies have only been performed in adults (Baron *et al.* 2007; Floess *et al.* 2007), and the potential of TSDR demethylation as a marker for cord blood Treg cells remained unclear.

In this study it was demonstrated that *FOXP3* demethylation at the TSDR was restricted to $CD4^+CD25^{high}$ cells in cord blood, while it was generally very low in $CD4^+CD25^-$ cells. Thus, *FOXP3* demethylation was restricted to Treg cells. Furthermore, *FOXP3* demethylation in EDTA-blood clearly correlated with $CD4^+CD25^{high}$ expression but to a lower extent with *FOXP3* mRNA expression in cord blood mononuclear cells (CBMCs). The latter maybe explained by the fact that the analyses were performed in bulk culture and measured *FOXP3* gene expression may also encompass its expression of non-Treg cells. In addition, *FOXP3* demethylation was indicated as a dependable marker to determine Treg cell functional capacity in cord blood since it positively correlated with Treg suppression activity on divison of effector T cells and trendwise with Treg suppression on proliferation of effector T cells. Since TSDR demethylation was assessed in EDTA-blood, the

percentage was low; yet, methylation status was analyzed in whole blood, which contains a mixture of cell subsets. From those, only Treg cells were demethylated at the TSDR region in *FOXP3* locus. The data suggest that the methylation status of the TSDR in *FOXP3* using whole cord blood is a valid method to determine Treg cells instead of studying them in mononuclear cells or isolated Treg cells. In conclusion, *FOXP3* demethylation is a reliable marker to assess quantitatively and qualitatively Treg cells in early life (Liu *et al.* 2010).

Interestingly, these effects were observed not only in unstimulated samples but also after stimulation with LpA. The TLR4-ligand LpA, part of the endotoxin of the gram-negative bacteria, was used to quantify Treg activity using a stimulus which mimics microbial exposure as a "protective stimulus" against allergy development. The data on LpA-stimulated samples suggest that *FOXP3* demethylation is also a valuable marker to assess the activity of Treg cells in children exposed to rich microbial burden such as children living on a farm (Liu *et al.* 2010).

Growing up on a farm indeed decreases the risk to develop ADs in childhood (Von Ehrenstein *et al.* 2000; Riedler *et al.* 2001; Alfven *et al.* 2006). Notwithstanding that the underlying mechanisms are not clear to date. Immune regulatory mechanisms including upregulation of innate immune receptors are suggested to be involved, with the strongest effects upon prenatal farming exposure (Lauener *et al.* 2002; Ege *et al.* 2006). In this context, Treg cells have been shown to be increased and functionally more efficient in neonatal cord blood of farming families (Schaub *et al.* 2009), supporting the hypothesis that the "farm effect" starts already *in utero*. Here it is demonstrated in another birth cohort (the PASTURE/EFRAIM Study) that Treg cells were increased in farming compared to reference children at age 4.5 years. The data suggest a long-lasting effect of the farming environment on increased Treg cells that may influence the development of ADs in childhood. Indeed, Treg as well as activated T cells were increased, suggesting that both may be relevant for the protective "farm effect". Only further investigations will provide additional knowledge to understand the functional relevance of these findings (Lluis, Depner *et al.* Manuscript submitted).

Of note, one need to consider to additionally take into account the best markers to determine Treg cells, the identification of the specific type or types of exposure mediating similar or potentially

different regulatory mechanisms, the timing and duration of exposure and the effect on clinical atopic phenotypes such as atopic sensitization (Lluis, Depner *et al.* Manuscript submitted).

Several markers are currently used to determine Treg cell numbers including high expression of the IL-2 receptor (CD25), low surface expression of CD127 (Liu *et al.* 2006), or intracellular expression of FOXP3 (Shevach 2006). Nevertheless, the expression of the main transcription factor, FOXP3, is still considered the most specific marker to define Treg cells (Fontenot *et al.* 2005) (Lluis, Depner *et al.* Manuscript submitted).

When studying the effect of certain farm exposures detailed analysis revealed an influence of farm milk consumption on Treg cells upon stimulation (PI=PMA/Ionomycin; LPS= Lipopolysaccharide). Several studies reported the relation between farm milk consumption and lower risk of ADs (Braun-Fahrlander *et al.* 2011). Although the specific underlying mechanisms are not known, the latter findings suggest that it maybe partially explained through altered regulation of Treg cells. In the ALEX study, farm milk consumption during the first year of life was associated with a reduced risk to suffer from asthma, hay fever or atopic sensitization during school-age (Riedler *et al.* 2001). In contrast, farm milk consumption ever in life in the PARSIFAL study was associated with lower prevalence of asthma, rhinoconjunctivitis, sensitization to pollen and food allergens (Waser *et al.* 2007). Analyses regarding raw milk consumption in the GABRIELA study showed an inverse association with asthma, atopy and hay fever (Loss *et al.* 2011). Other farm-related exposures analyzed such as staying in stables (Riedler *et al.* 2001; Ege *et al.* 2006) or contact to hay (Ege *et al.* 2007) have been associated with the "farm effect". However, no correlation with increased Treg cells was observed. Based on these findings one may speculate that different mechanisms are involved in the protective effect driven by atopy including environmental exposures linked to farming. While Treg cells may evoke a protective effect through farm milk consumption, additional or different mechanisms seem to be involved after the exposure to other farm-related factors (Lluis, Depner *et al.* Manuscript submitted).

Further analysis on the different types of milk revealed that both, the consumption of farm milk alone or in combination with shop milk, were associated with increased Treg cells at age 4.5 years upon

stimulation (PI, LPS). Drinking boiled or unboiled farm milk had similar effects on Treg cells. Farm milk and specifically raw milk consumption is one of the most discussed farm exposures in relation to the allergy-protective "farm effect" (von Mutius *et al.* 2010; Braun-Fahrlander *et al.* 2011). Other studies observed a negative association of raw farm milk consumption with asthma, hay fever (Loss *et al.* 2011), eczema (Wickens *et al.* 2002; Perkin *et al.* 2006) and atopy (Barnes *et al.* 2001; Radon *et al.* 2004; Perkin *et al.* 2006; Loss *et al.* 2011). Raw milk may contain more bacteria, including pathogenic strains than boiled milk (Perkin 2007). Milk treatments such as heating and homogenization may affect heat-sensitive milk components or the physical structure of fat milk, respectively, and subsequently abrogate the farm milk effect on allergy protection (Braun-Fahrlander *et al.* 2011). However, this study did not reveal major differences depending on farm milk boiling status. One may therefore speculate that Treg cells are rather modulated by farm heat-resistant milk components synergistically through alternative mechanisms involving other farm milk components (Lluis, Depner *et al.* Manuscript submitted).

Of note, the effect of farm milk consumption on Treg cells in the present study was even stronger within the non-farming compared to the farming children which was even more pronounced in subjects without any contact to stables. These data suggest a potential "saturation" effect for farming children drinking farm milk as farming children are continuously in contact with several other farm-related exposures possibly affecting Treg cell numbers. In contrast, the reference group drinking farm milk has few or even no contact to farming environment, in particular those without any contact to stables. In line with this observation Barnes *et al*. (Barnes *et al.* 2001) described a lower prevalence of atopy among children that consumed unpasteurized milk products early in life. However, when stratified analysis were applied, the association was only discernible among the urban children but not among the rural children (Barnes *et al.* 2001) (Lluis, Depner *et al.* Manuscript submitted).

The strongest effect of farm milk exposure on Treg cells was consistently observed upon LPS stimulation and to a lower extent after PI stimulation while solely some effects were detected in unstimulated conditions. PI is a potent polyclonal stimulus that strongly activates proliferation of T cells, while LPS, an endotoxin found in the outer membrane of the gram-negative bacteria, is a TLR4

ligand present in high amounts in the farming environment (Braun-Fahrlander *et al.* 2002). The data suggest that previous continuous exposure to LPS promote a stronger Treg cell response in childhood. Interestingly, among farm milk drinkers' stronger responses were observed in the non-farming group, in particular without any contact to stable. This may lead to the hypothesis of an additive effect existing for non-farming children who consume farm milk. The finding is of special relevance as a larger percentage of the population is affected by this outcome. Yet, this effect and the underlying immunological mechanisms controlled by exposures present in stables need to be further investigated (Lluis, Depner *et al.* Manuscript submitted).

Regarding time and length of exposure, farm milk consumption during pregnancy and early childhood revealed a stronger positive association with Treg cells than farm milk consumption only in childhood. Although no effect was observed when exposure occurred only during pregnancy, the data need to be interpreted carefully as the subgroup was rather small and the time points are highly correlated. The early influence of *in utero* exposure to farming or farm milk consumption may contribute to the neonatal immune system has previously been reported. In the PARSIFAL study, prenatal farm exposure was associated with increased levels of pattern recognition receptors in childhood (Ege *et al.* 2006) and previous data in the same study population revealed an association between maternal farm milk consumption and higher IFN-γ levels in cord blood (Pfefferle *et al.* 2010). IFN-γ presence in cord blood has been associated with lower risk of atopic diseases in childhood (Macaubas *et al.* 2003) (Lluis, Depner *et al.* Manuscript submitted).

Treg cells were additionally correlated with clinical atopic outcomes including lower inhalant IgE in unstimulated conditions, suggesting a protective role for Treg cells on atopic sensitization. Yet, a more detailed assessment of atopic sensitization and asthma in relation to Treg cells later in childhood is required (Lluis, Depner *et al.* Manuscript submitted).

In parallel to the in depth analyses of Treg cells, the aim of this study was to assess the role of Th17 cells in a farming population early in life. Th17 cells have been shown to be increased in ADs (Tesmer *et al.* 2008). Growing up on a farm was reported as a protective factor for the development of ADs

(Riedler *et al.* 2001; Alfven *et al.* 2006; Ege *et al.* 2011), specially when exposures occur already *in utero*. Thus, time of exposure to farming is crucial for this protective effect (Ege *et al.* 2006). Based on these previous findings, it maybe suggested that Th17 cells are downregulated in cord blood from farming children. From this study two specific questions were addressed. First, it was investigated whether Th17 cells were influenced by maternal farm exposure in cord blood. Second, it was studied whether Th17 and Treg cells were reciprocally regulated at birth (Lluis *et al.* Manuscript submitted).

Thus, Th17 cells- determined by gene expression of Th17-lineage genes- were already detectable in cord blood. Characteristic Th17-lineage genes including the transcription factor *RORC*, the transmembrane receptor *IL-23R* and several cytokines such as *IL-17*, *IL-17F* and *IL-22* were increased upon specific stimulation. For *RORA* mRNA expression a slightly downregulated was observed. Of note, this study was performed in unfractionated cord blood mononuclear cells. Therefore, the cell source of gene expression cannot be specified to a certain sub-cell population. Although Th17-lineage markers are characteristic of this cell subset, they are not exclusively expressed in Th17 cells (Dzhagalov *et al.* 2004) (Lluis *et al.* Manuscript submitted).

In a next step, the role of maternal farm exposure on cord blood Th17 cells –comprehensively assessed by gene expression of Th17-lineage genes- was determined. Maternal farming did not reveal any effect on gene expression of Th17 markers in cord blood *per se* (Lluis *et al.* Manuscript submitted). These data are consistent with previous results on IL-17 protein secretion in the same birth cohort, where no differences were observed between farming and non-farming CBMCs (Schaub *et al.* 2009) (Lluis *et al.* Manuscript submitted).

One of the main findings of this study demonstrated that gene expression of Th17-lineage markers was positively correlated with Treg cells upon stimulation. Several studies reported a close relationship between these two subsets. For instance, Treg cells are known to limit the immunopathology of Th17-driven diseases such as inflammatory bowel disease (IBD) (Sakaguchi *et al.* 2007). A higher ratio of Th17/Treg cells has been related to graft versus host disease (Ratajczak *et al.* 2010). Later studies in a mouse model demonstrated that $FOXP3^+$ Treg cells are required for development of Th17 cells *in vivo*

in a IL-2 dependent manner (Chen *et al.* 2011) (Lluis *et al.* Manuscript submitted). Interestingly, the positive correlation upon stimulation was not observed within the farming group after LpA stimualtion. LpA is the main active component of endotoxin, abundant in the farming environment. The data suggest a stimulus-specific effect between these two subsets in neonates with low exposures to endotoxin (non-farming group), or no effect in previously exposed neonates (farming group). This is consistent with the interaction effect observed between Treg cells and farming on Th17-lineage genes upon LpA stimulation (Lluis *et al.* Manuscript submitted).

The positive correlation between Th17 and Treg cells in cord blood points out a parallel regulation of both subsets at this early stage of immune maturation. In concordance, Chen *et al.* recently described in a murine model that Treg and Th17 cells were positively regulated in early stages of immune development through IL-2. While Treg cells required IL-2 for their expansion, Th17 development was inhibited by IL-2. However, the effect was only present in early stages of Th17 development. Putatively, the parallel regulation only occurs in the first phases of differentiation and Treg cells may inhibit Th17 cell responses in a later phase of inflammation (Chen *et al.* 2011). Thus, the positive correlations observed in the PAULCHEN population in cord blood may be different later in childhood (Lluis *et al.* Manuscript submitted). On this account, Th17 and Treg cells demonstrate certain plasticity (Voo *et al.* 2009) and several factors may contribute to their lineage fate including external factors like the cytokine milieu (Kimura *et al.* 2010) or the presence of regulatory metabolites (Mucida *et al.* 2007). In addition, genetic regulation may also play a role on the fate of both cell subsets, e.g. through direct or indirect interaction of FOXP3 and RORC transcription factors that may regulate the cell fate (Ichiyama *et al.* 2008; Zhang *et al.* 2008) (Lluis *et al.* Manuscript submitted).

Indeed, an important regulatory metabolite of Th17/Treg regulation is retinoic acid (RA), a derivate of vitamin A. Hence, the influence of Th17/Treg cell fate upon RA stimulation early in life was assessed in this study. RA-treated cord blood samples showed a downregulation of Th17 (*RORC, IL-23R*) and Treg (*FOXP3*) markers. A dose-response effect, especially upon PHA stimulation was observed. RA is relevant for immune cell differentiation and maintenance of immune homeostasis (Bai *et al.* 2009) and can enhance iTreg (induced Treg cells) cells (Mucida *et al.* 2007; Nolting *et al.* 2009) while

suppressing Th17 differentiation in mice (Mucida *et al.* 2007). One may speculate that RA regulates both populations in opposite directions during an inflammatory process. In cord blood, the parallel regulation of both Th17 and Treg cells upon RA treatment is in line with the overall regulation detectable in the study. The findings may be related to a dose-dependent response to RA, although the doses used were comparable to other studies; or the results may reflect a specific strong parallel regulation of Th17 and Treg cells at birth. Further investigations are required, including functional characterization of the cell subsets. This specific question was not addressed in this study due to too low cell numbers and logistic issues (Lluis *et al.* Manuscript submitted).

Overall, a parallel regulation of both Th17 and Treg cells was observed at birth upon different stimulation conditions. While exposure to RA downregulated T cell markers of both populations, PHA and Ppg stimulation increased the expression of both Th17- and Treg- lineage markers independent of maternal farm exposures. Upon LpA stimulation, a positive Th17/Treg correlation was only observed within the non-farming group, but not in farming children. Differences between both groups suggest a potential role of prenatal farm exposure on the interaction between these two T cell subsets. A follow-up of the cohort, including a functional characterization of the T cell populations, is required to enlighten whether these findings might influence the development of ADs later in childhood.

In addition to environmental factors influencing Th17 and Treg cells, genetic relevance of polymorphisms within the Th17-lineage pathway and the asthma-associated 17q21 region on mRNA expression of the related genes and on T cell markers were also examined. Genetic polymorphisms are important modulators of the immune system with potential consequences on the susceptibility for ADs (Schedel *et al.* 2008; Suttner *et al.* 2009).

Polymorphisms within the Th17-lineage pathway influenced Th17 and other T cells – comprehensively assessed by cytokine secretion and gene expression of related markers- already at birth. The influence of the polymorphism in the *IL-23R* rs7517847 on Th17 cell marker expression is consistent with findings from a genome-wide association study (GWAS) where this genetic variant was a protective factor for IBD, potentially a Th17 driven disease (Duerr *et al.* 2006). In contrast to our results, in a Chinese study of school-age children the polymorphism rs2275913 did not show any

difference in IL-17 secretion. However, rs2275913 was associated with childhood asthma and within the asthmatic group the SNP was associated with abnormal lung function and higher total IgE in serum (Chen *et al.* 2010). Out of the seven investigated polymorphisms two genetic variants (rs790631, rs10889675) revealed an opposite regulation of Th17 and Treg cells. Homozygous carriers of the polymorphic allele of rs790631 showed an increase of Th17 markers but a decrease in *FOXP3* expression. Inversely, homozygous carriers of the polymorphic allele of rs10889675 showed higher *FOXP3* mRNA but lower Th17 and Th2 expression. Of note, since only one child was homozygous carrier for this SNP, a dominant model was used. As this was an explorative study, no adjustment for multiple testing was applied; instead further study replications are required (Lluis *et al.* Manuscript submitted).

Hence, SNPs within Th17-lineage pathway affected T cell gene expression already in cord blood. Whether this may have an impact later in childhood for the development of atopic diseases requires a follow up of the cohort.

As observed for polymorphisms within the *IL-17* and *IL-23R* genes, the influence of genetic variants on gene expression of respective pathway gene is already detectable in early stages of immune maturation, with potential influences later in childhood. As little is known about the influence of genetic variants of the highly asthma-associated 17q21 locus (Moffatt *et al.* 2007; Moffatt *et al.* 2010) early in life, the effect of these SNPs in cord blood was investigated.

Previous studies showed an association between SNPs within 17q21 region with *ORMDL3* (Dixon *et al.* 2007; Verlaan *et al.* 2009; Halapi *et al.* 2010), *GSDMB* (Dixon *et al.* 2007; Verlaan *et al.* 2009; Verlaan *et al.* 2009; Halapi *et al.* 2010) and *ZPBP2* gene expression (Verlaan *et al.* 2009) potentially due to allele-specific chromatin remodeling within the 17q12-q21 region (Verlaan *et al.* 2009). In the present data not only the previously analyzed candidate genes (*IKZF3, ZPBP2, GSDMB* and *ORMDL3*) (Verlaan *et al.* 2009) were analyzed but also *GSDMA* and *PSMD3*, additionally located within this asthma-associated locus (Lluis *et al.* 2011). Besides of the previously described increase in *ORMDL3* expression (Verlaan *et al.* 2009), higher levels of *GSDMA* within the risk alleles carriers was observed in cord blood. Previous findings on *GSDMB* and *ZPBP2* (Verlaan *et al.* 2009; Halapi *et*

al. 2010) could not be reproduced in our study population. Differences between studies might be due to different cell populations or to the different status of cell immune maturation, since in this study gene expression was analyzed in cord blood (Lluis *et al.* 2011).

The function of these genes is not fully understood yet. *GSDMA* was described to induce apoptosis in the gastric epithelium and has been suggested as a tumor suppressor (Saeki *et al.* 2009) while Orosomucoid family proteins are involved in the metabolism of sphingolipids in *Saccharomyces cerevisiae* (Breslow *et al.* 2010). Furthermore, *ORMDL3* seems to play a role in Ca^{2+} homeostasis and unfolded protein response (Lewis 2001; Gallo *et al.* 2006; Cantero-Recasens *et al.* 2010), which itself is involved in downstream events such as effector T cell regulation (Lewis 2001; Gallo *et al.* 2006; Lluis *et al.* 2011).

In addition to altered *ORMDL3* and *GSDMA* mRNA expression, an increase in IL-17 secretion was observed in carriers of the asthma risk alleles within the 17q21 locus in unstimulated and PHA-stimulated samples. In parallel, increased levels of IL-17, the main secreted cytokine by Th17 cells, have been associated with asthma (Molet *et al.* 2001; Wong *et al.* 2001; Barczyk *et al.* 2003; Bullens *et al.* 2006), atopic eczema (Toda *et al.* 2003; Koga *et al.* 2008) and allergic rhinitis (Ciprandi *et al.* 2008; Ciprandi *et al.* 2009). Thus, one may hypothesize that the risk alleles of 17q21 may induce a Th17 polarized response early in life that may prone to develop asthma later in childhood. This may be in concert with other mechanisms including *ORMDL3* and *GSDMA/GSDMB* expression, as they were also influenced by these polymorphic risk alleles. A follow up of this or other studies is very important to elucidate the role of these findings and to analyze their contribution on the development of childhood asthma (Lluis *et al.* 2011).

In conclusion, Treg and Th17 cells are influenced by environmental as well as genetic factors early in life with a potential contribution to the development of atopic diseases later in childhood. The influence on Treg cells observed in young farming children is of particular interest as farming is associated with an allergy-protective effect. Interestingly, farm milk consumption was the most relevant farm-related exposure influencing Treg cells, particularly in children not exposed to farming environment. Presented data lead to the hypothesis that components in farm milk may contribute to the

modulation of the immune system driving a non-allergic phenotype partially mediated through Treg cells. Identifying these components is of high interest for the general population since the protective effect could then be directly translated.

In parallel, the close relationship between Treg and Th17 cells at birth is particularly intriguing as their gene expression markers were similarly correlated. However, further studies are needed at later phases of immune maturation to gain a broader understanding of the reciprocal regulation of both subsets and their influence on their cell fate. In addition, both subsets were affected by genetic predisposition already early in life. In general, Th17 cells have been shown to be increased in atopic diseases. Based on the data presented here, one may speculate that asthma-risk alleles may lead to a Th17 driven immune response early in life which may subsequently prone to developing atopic diseases.

Indisputably, other mechanisms may contribute to the development of atopic diseases in concert with Treg and Th17 cells. Comprehensive functional analyses are therefore required to further characterize their unique contribution to the pathogenesis of atopic diseases during immune maturation in childhood.

5 Summary

Early childhood is a biological window where the immune maturation is thought to be modulated by environmental and genetic factors, subsequently influencing the development of atopic diseases (ADs). Within the past decade it was clearly shown that not only Th1 and Th2 cells are involved in its manifestation but also Treg and Th17 cell subsets play a crucial role. In AD patients Th17 cells were indicated to be increased whereas data on Treg cells is rather contradictory. Yet, little is known about the role of these T cell subsets in the development of ADs during immune maturation. In this context, growing up on a farm is a protective factor for the development of ADs in childhood. Increased numbers and more efficient Treg cells were observed in cord blood from farming compared to non-farming mothers.

The present work indicates that the demethylation of *FOXP3* gene– the transcription factor of Treg cells – at the Treg-specific demethylated region (TSDR) can be used as a reliable marker to assess the number and functional activity of Treg cells in cord blood. *FOXP3* demethylation has also previously been shown to be associated with maternal farming exposures in cord blood. In the present study, children at the age 4.5 years exposed to farming environment and/or farm milk consumption revealed an increase in Treg cell numbers. In contrast, inhalant IgE was associated with lower Treg cell numbers. A positive correlation between Th17 and Treg cell numbers upon stimulation was influenced by maternal farming in cord blood. However, maternal farm exposure did not alter Th17-lineage gene expression *per se*.

In addition to environmental factors, genetic polymorphisms within the Th17-lineage pathway influenced T cell expression in cord blood. The secretion of IL-17 – the major cytokine of Th17 cells – was elevated in cord blood samples carrying certain genetic variants of the asthma-associated 17q21 region. These polymorphisms also influenced the gene expression of two genes (*ORMDL3* and *GSDMA)* located within the 17q21 susceptibility locus selective for childhood asthma.

These data contribute to a better understanding of Treg and Th17 cell regulation during immune maturation influenced by environmental as well as genetic factors. Hence, new insights into the

potential mechanisms regulating the development of ADs in childhood are provided. A follow up of these birth cohorts is currently conducted to assess the correlation of the observed effects on the development of AD later in life.

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9 Abbreviations

AD	Atopic disease
AE	Atopic eczema
AHR	Aryl Hydrocarbon Receptor
ALEX	Allergy and Endotoxin study population
AMICS	Asthma Multicentre Infants Cohort Study
APC	Antigen presenting cell
ATS	American Thoracic Society
bp	Basepair
BALF	Bronchoalveolar lavage fluids
BSA	Bovine serum albumin
CBMC	Cord blood mononuclear cell
CD	Cluster designation
cDNA	Coding DNA
cpm	Counts per minute
CT	Threshold cycle
Derp1	*Dermatophagoides pteronyssinus*; house dust mite
DNA	Deoxyribonucleic acid
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal Calve Serum
FOXP3	Forkhead box P3
FW	Forward
GABRIELA	GABRIEL Advanced Surveys Study
GITR	Glucocorticoid-induced TNF receptor
GMC-SF	Granulocyte-macrophage colony-stimulating factor
GMR	Geometric mean ratio
GSDMA	Gasdermin A, alias GSDM1
GSDMB	Gasdermin B, alias GSDML
GWAS	Genome-wide association study
H	Haplotype
HWE	Hardy-Weinberg equilibrium
IFN-γ	Interferon gamma
IgE	Immunglobulin E
IL	Interleukin
IL-23R	Interleukin-23 receptor
IQR	Interquartile Range
ISAAC	International Study of Asthma and Allergies in Children
LAG3	Lymphocyte activation gene-3
LD	Linkage disequilibrium
LpA	Lipid A
LPS	Lipopolysaccharide
MAF	Minor Allele Frequency
MALDI-TOF MS	Matrix Assisted Laser Desorption-Time of Flight Mass Spectrometry
mRNA	Messenger RNA
OR	Odds Ratio
ORMDL3	Orosomucoid 1–like 3
p	P-value
PI	PMA/Ionomycin
PAULCHEN	Prospective Cord Blood Study in Rural Southern Germany
PAULINA	Paediatric Alliance for Unselected Longitudinal Investigation of Neonates for Allergies
PASTURE/EFRAIM	Protection against allergy: Study of Rural Environments

9. ABBREVIATIONS

PARSIFAL	Prevention of Allergy-Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle
PBMC	Peripheral blood monocytes
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
Ppg	Peptidoglycan
PRR	Pattern recognition receptors
PSMD3	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
r	Correlation coefficient
Real-time RT-PCR	Real-time Reverse-Transcriptase-PCR
RE	Reverse
RAST	Radioallergosorbent test
RNA	Ribonucleic acid
RORA	Retinoic acid receptor-related orphan receptor alpha
RORC	Retinoic acid receptor-related orphan receptor C
rs	Reference SNP
SEM	Standard error of the mean
SD	Standard deviation
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Teff	Effector T cell
TF	Transcription Factor
Th cell	T helper cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell
TSDR	Treg Specific Demethylated Region
t-test	Statistical test: Student's t distribution
U	Unstimulated
USA	United States of America
UTR	Untranslated region
WT	Wildtype allele
ZNFN1A3	Ikaros family zinc finger 3, alias IKZF3
ZPBP2	Zona pellucida binding protein 2

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11 Appendix

A-Table I. SNPs within the Th17-lineage genes in relation to Th17 and Treg mRNA markers and protein secretion of immune factors including T cell cytokines. General Wilcoxon test.

A. Recessive model

IL23R RS7517847 SNP: G

Marker	Stimulus	N	SNP	Minimum	1st quartile	Median	3rd quartile	Maximum	p
RORA	LpA	28	GG	11.15	13.30	14.15	15.20	17.60	0.10
		48	GT/TT	10.90	12.70	13.45	14.20	16.50	
IL-23R	LpA	28	GG	14.45	17.95	19.70	21.45	23.10	**0.005**
		48	GT/TT	14.20	17.20	18.13	19.55	21.45	
IL-23R	Ppg	28	GG	16.60	17.90	19.10	20.43	23.95	**0.004**
		48	GT/TT	15.20	16.25	17.53	19.50	24.20	
IL-17	LpA	28	GG	14.45	19.70	20.65	23.10	25.05	**0.006**
		48	GT/TT	14.60	18.65	19.80	20.80	25.60	
IL-17F	U	28	GG	15.40	21.25	22.60	24.45	25.30	**0.04**
		48	GT/TT	17.85	20.90	21.63	23.60	25.40	
IL-17F	LpA	28	GG	13.25	16.05	17.45	19.70	25.05	**0.01**
		48	GT/TT	9.65	14.00	15.25	18.00	22.65	
IL-17F	Ppg	28	GG	10.10	14.83	17.70	19.68	22.45	**0.02**
		48	GT/TT	9.95	13.15	15.00	16.45	20.50	
IL-22	LpA	28	GG	14.45	18.45	19.70	21.15	25.05	**0.04**
		48	GT/TT	14.25	17.05	18.70	20.55	23.70	

Marker protein	Stimulus	N	SNP	Minimum	1st quartile	Median	3rd quartile	Maximum	p
IL-17	LpA	28	GG	0.01	0.01	0.56	2.44	10.79	**0.006**
		48	TT/GT	0.01	0.46	2.63	6.81	33.55	
IL-17	Ppg	28	GG	0.01	0.01	1.44	3.93	14.96	**0.003**
		48	TT/GT	0.01	1.53	4.18	11.95	21.45	

IL23R RS790631 SNP: C

Marker	Stimulus	N	SNP	Minimum	1st quartile	Median	3rd quartile	Maximum	p
RORC	Ppg	6	CC	17.25	17.55	18.08	20.30	22.30	**0.05**
		71	CT/TT	17.00	18.80	19.98	21.30	23.95	
IL-23R	PHA	6	CC	18.05	19.90	21.60	22.00	24.20	**0.05**
		71	CT/TT	13.50	16.95	18.45	20.00	23.45	
IL-17F	U	6	CC	18.35	19.95	20.45	21.10	22.30	**0.002**
		71	CT/TT	15.40	20.90	22.00	24.30	25.40	
FOXP3	PHA	6	CC	11.90	14.45	17.03	18.63	20.20	0.08
		71	TT/CT	10.10	12.35	13.05	14.70	16.95	
FOXP3	Ppg	6	CC	15.80	16.40	17.03	17.23	17.40	**0.05**
		71	TT/CT	13.20	14.90	15.60	16.40	18.50	

IL17 RS2275913 SNP: A

Marker	Stimulus	N	SNP	Minimum	1st quartile	Median	3rd quartile	Maximum	p
RORA	LpA	13	AA	12.85	14.00	14.55	15.10	16.50	0.06
		63	GA/GG	10.90	12.70	13.55	14.20	17.60	
RORA	Ppg	13	AA	12.25	13.60	13.93	15.30	17.90	0.09
		63	GA/GG	10.85	12.70	13.18	14.05	18.75	
IL-23R	PHA	13	AA	15.10	17.40	20.55	22.70	23.45	0.11
		63	GA/GG	13.50	17.15	18.48	19.90	24.20	
IL-17	LpA	13	AA	14.45	19.53	21.33	22.90	25.60	0.09
		63	GA/GG	14.60	18.90	20.05	20.90	25.05	
IL-17F	Ppg	13	AA	14.05	15.18	17.18	20.23	20.80	0.10
		63	GA/GG	9.95	13.30	15.55	17.50	22.45	
IL-22	Ppg	13	AA	17.90	18.73	20.23	20.93	24.20	0.07
		63	GA/GG	13.90	17.00	18.58	20.60	24.85	

B. Dominant model

IL23R RS10889675 SNP: A

Marker	Stimulus	N	SNP	Minimum	1st quartile	Median	3rd quartile	Maximum	p
RORC	U	20	AA/CA	15.40	21.35	21.95	23.85	26.00	**0.03**
		57	CC	16.90	20.25	21.75	22.40	25.30	
RORA	U	20	AA/CA	11.30	12.75	13.15	14.15	20.65	**0.007**
		57	CC	9.85	11.35	11.95	13.00	14.60	
IL-17F	U	20	AA/CA	15.40	21.15	22.30	23.60	25.35	0.11
		57	CC	17.85	20.90	21.60	24.40	25.40	
IL-22	U	20	AA/CA	15.40	23.55	23.85	24.55	26.00	**0.04**
		57	CC	20.20	22.35	23.53	24.80	26.30	
FOXP3	LpA	20	AA/CA	14.00	14.80	15.70	16.05	17.90	**0.05**
		57	CC	14.25	15.40	16.15	16.80	18.30	
FOXP3	Ppg	20	AA/CA	14.05	14.40	15.00	15.75	17.50	0.08
		57	CC	13.20	15.10	15.85	16.65	18.50	

Marker protein	Stimulus	N	SNP	Minimum	1st quartile	Median	3rd quartile	Maximum	p
IL-17	Ppg	20	AA/CA	0.01	0.12	1.95	3.21	14.96	0.07
		57	CC	0.01	1.19	3.98	11.20	21.45	
IL-5	PHA	20	AA/CA	0.01	1.45	3.35	12.03	145.08	**0.005**
		57	CC	0.01	7.17	26.58	37.28	168.94	
IL-5	LpA	20	AA/CA	0.01	1.46	2.61	9.47	89.95	0.07
		57	CC	0.13	2.74	5.77	13.20	68.04	
IL-5	Ppg	20	AA/CA	4.59	11.02	21.48	48.73	135.99	**0.05**
		57	CC	5.21	21.89	42.58	67.65	255.90	
IL-6	PHA	20	AA/CA	4575.97	10291.60	19606.03	24943.53	109257.72	**0.003**
		57	CC	1853.29	21692.64	34000.00	38447.17	70881.62	
IL-13	PHA	20	AA/CA	0.23	1.90	32.71	76.12	14219.30	**0.02**
		57	CC	0.08	12.74	139.36	1284.73	19150.01	
IL-15	PHA	20	AA/CA	0.01	0.68	1.00	1.97	2.73	**0.05**
		57	CC	0.69	1.51	1.72	2.39	3.95	
IL-5	LpA	20	AA/CA	0.25	1.25	1.57	1.75	2.41	**0.03**
		57	CC	1.03	1.60	1.85	2.77	3.71	
TNF-α	PHA	20	AA/CA	1.40	14.21	43.07	198.30	3535.47	0.07
		57	CC	2.16	54.17	184.88	528.11	2366.21	

U, unstimulated; PHA, phytohemagglutinin; LpA, Lipid A; Ppg, peptidoglycan.
RORC, primers RORC3: *RORC* isoform b.
p=p-value. Data in *boldface* are significant.

A-Table II. Risk alleles of 17q21 SNPs increased IL-17 secretion in unstimulated and PHA stimulated cord blood (N=200).

A. Block 1

Cytokine	Genotype	N	Geometric mean (95% CI)[2]	GMR (95% CI)[2] p[3]	
				Crude	Adjusted for maternal atopy
rs9303277					
IL-17 U	CC risk allele[1]	45	0.18 (0.10-0.33)	1.64 (1.09 - 2.46) p=0.018	1.65 (1.10 - 2.47) p=0.017
	CT	93	0.10 (0.07-0.15)		
	TT	48	0.07 (0.04-0.12)		
IL-17 PHA	CC risk allele[1]	41	0.26 (0.12-0.55)	1.61 (1.01 - 2.57) p=0.048	1.61(1.01 - 2.59) p=0.048
	CT	83	0.10 (0.07-0.16)		
	TT	44	0.10 (0.05-0.19)		
rs11557467					
IL-17 U	GG risk allele[1]	44	0.19 (0.11-0.35)	1.73 (1.15 - 2.59) p=0.009	1.74 (1.16 - 2.60) p=0.008
	GT	92	0.10 (0.07-0.15)		
	TT	49	0.06 (0.04-0.12)		
IL-17 PHA	GG risk allele[1]	40	0.28 (0.13-0.59)	1.71 (1.07 - 2.73) p=0.026	1.72 (1.07 - 2.75) p=0.025
	GT	82	0.11 (0.07-0.17)		
	TT	45	0.09 (0.05-0.18)		
rs8067378					
IL-17 U	AA risk allele[1]	44	0.19 (0.11-0.35)	1.73 (1.15 - 2.59) p=0.009	1.74 (1.16 - 2.60) p=0.008
	AG	92	0.10 (0.07-0.15)		
	GG	49	0.06 (0.04-0.12)		
IL-17 PHA	AA risk allele[1]	40	0.28 (0.13-0.59)	1.71 (1.07 - 2.73) p=0.026	1.72 (1.07 - 2.75) p=0.025
	AG	82	0.11 (0.07-0.17)		
	GG	45	0.09 (0.05-0.18)		

B. Block 3

Cytokine	Genotype	N	Geometric mean (95% CI)[2]	GMR (95% CI)[2] p[3]	
				Crude	Adjusted for maternal atopy
rs4795408					
IL-17 U	AA risk allele[1]	36	0.22 (0.11-0.43)	1.67 (1.11 - 2.51) p=0.016	1.67 (1.11 - 2.51) p=0.016
	AG	93	0.09 (0.06-0.14)		
	GG	58	0.07 (0.04-0.13)		
IL-17 PHA	AA risk allele[1]	33	0.27 (0.12-0.61)	1.60 (1.00 - 2.56) p=0.052	1.60 (1.00 - 2.57) p=0.053
	AG	82	0.11 (0.07-0.17)		
	GG	54	0.10 (0.05-0.17)		
rs3894194					
IL-17 U	AA risk allele[1]	37	0.23 (0.12-0.43)	1.61 (1.07 - 2.42) p=0.023	1.62 (1.07 - 2.43) p=0.023
	AG	92	0.08 (0.06-0.13)		
	GG	58	0.08 (0.05-0.13)		
IL-17 PHA	AA risk allele[1]	34	0.24 (0.11-0.56)	1.51 (0.94 - 2.41) p=0.091	1.51 (0.94 - 2.42) p=0.091
	AG	82	0.11 (0.07-0.17)		
	GG	53	0.10 (0.05-0.18)		

U, unstimulated, PHA, PHA stimulated. p=p-value.

[1] the respective risk allele was defined as the allele increasing the risk for the development of asthma as shown in previous publications (Moffatt *et al.* 2007).

[2] GMR = Geometric mean ratio, 95% CI = 95% Confidence Interval.

[3] p-value of geometric mean ratio by regression models using an Allele-counting model (0,1,2) for coding of the respective SNP.

12 Curriculum Vitae

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Academic Degree

Nov 07 - Present	PhD student of Biology, Dept. of Allergy and Pulmonary of LMU Munich University Children's Hospital, Munich, Germany. Project: Role of regulatory T cells and Th17 cells during immune maturation for the development of atopic diseases in childhood influenced by genetic and environmental factors. Advisors: Prof. Dr. Thomas Illig, PD. Dr. Bianca Schaub
Feb 12 – Present	Research associate at the Pediatrics Department of National Jewish Health, Denver, CO, USA. Advisor: Prof. Dr. Erwin Gelfand
Sept 02/ June 07	Degree in Biology, Pompeu Fabra University, Barcelona, Spain

Further education during Biology degree

July 06- June 07	Diploma thesis in the Molecular Virology Unit, Pompeu Fabra University, Barcelona, Spain. Project: Functional characterization of host factors in viral replication of positive strand RNA viruses. Advisor: Dr. Juana Diez
January- April 07	Institute of Virology, University of Saarland, Germany. Collaboration with Prof. Dr. A. Meyerhans group as part of the project developed in Dr. J. Diez group in Barcelona. Advisor: Dr. Juana Diez
July 06- Aug 07	Pharmacology Research Unit, Institut Municipal d'Investigació Mèdica (IMIM), Barcelona, Spain. Study of ABC (ATP Binding Cassette) transporters in microgravity. Advisor: Prof. Dr. Rafael de la Torre
July - Aug 05	Microbiology & Immunology Department, Faculty of Medicine, McGill University, Montreal, Canada. Identification and characterization of new detection methods and therapeutical targets for invasive aspergilosis treatment, caused by *Aspergillus fumigatus*. Advisor: Assoc. Prof. Dr. Donald Sheppard
July 04	Evolutionary Biology Research Unit, Department of Experimental and Health Sciences, Pompeu Fabra University, Barcelona, Spain. Analysis of genetic differences among African populations using markers of the type STR / SNP in chromosome Y. Advisor: Dr. David Comas

Honors

2007-2010 Marie Curie Early Stage Research Training (EST) Fellowship as part of the European GALTRAIN consortium

2007 Summer School in Alpbach (Austria): Workshop on "Astrobiology: Life Detection in and from Space", organized by ESA (European Space Agency)

2006 Research Fellowship from Ministerio de Educación y Ciencia, Spain, in order to collaborate with the Department of Microbiology in the Universitat Pompeu Fabra, Barcelona, Spain

2002 Grant from Ministerio de Educación y Ciencia, Spain, intensive English course, Cork, Ireland

2002 Grant from Orator Project, Generalitat de Catalunya, Spain, intensive English course, Oxford, United Kingdom

2001 Grant from Ministerio de Educación y Ciencia, Spain, intensive English course, Harrogate, United Kingdom

Techniques

- Fundamental techniques in molecular biology
- Cell line and primary cell cultures
- Cell sorting and isolation
- Flow cytometry
- Luminex technology
- Regulatory T cell suppression assay
- Western blotting
- DNA- and RNA- extraction
- Real-time RT-PCR
- Fundamental techniques in virology, including growth and manipulation of HCV replicons
- Molecular cloning
- Knockdown of proteins by siRNA techniques
- Basic yeast culture techniques
- GC/MS analysis

Skills

- Highly dedicated with a positive attitude
- Language skills: Spanish (native speaker), Catalan (native speaker), English (fluent, both spoken and written), German (intermediate level), French (basic level)
- Computer skills: proficiency in common computer programs, specific flow cytometry software applications and SPSS (statistical analyses)
- Data management of large study populations
- Optimization of new protocols
- Coordinating, training and supervising lab technicians and medical students

12. CURRICULUM VITAE

Publications

Lluis A et al. The role of Th17 cells in early immune development in the context of maternal farm exposure. *Manuscript submitted.*

Lluis A, Depner M et al. Farm exposure and particularly farm milk exposure increases T regulatory cells in childhood: the PASTURE/EFRAIM Study. *Manuscript submitted.*

Ballenberger N, **Lluis A** et al. Novel statistical approaches for non-normal censored immunological data: analysis of cytokine and gene expression data. *Manuscript submitted.*

Lluis A, Schaub B. Lessons from the farm-environment. Curr Opin Allergy Clin Immunol. Curr Opin Allergy Clin Immunol. 2012 Apr;12(2):158-63.

Lluis A, Schedel M et al. Asthma-associated polymorphisms in 17q21 influence cord blood *ORMDL3* and *GSDMA* gene expression and IL-17 secretion. J Allergy Clin Immunol. 2011 Jun;127(6):1587-1594.e6.

Liu J, **Lluis A** et al. T regulatory cells in cord blood - *FOXP3* demethylation as reliable quantitative marker. PloS One. 2010 Oct 12;5(10):e13267.

Schaub B, Liu J, Höppler S, Haug S, Sattler C, **Lluis A** et al. Impairment of T-regulatory cells in cord blood of atopic mothers. J Allergy Clin Immunol. 2008 Jun; 121(6):1491-9.

Presentations at international scientific meetings

- **Poster discussion: Respiratory Society Annual Congress**, 2011, Amsterdam, The Netherlands
  Protection against atopic diseases through farm exposure is partially mediated by regulatory T cells

- **Poster presentation: World Immune Regulation Meeting V**, 2011, Davos, Switzerland
  Asthma-associated genetic variants in 17q21 influence *ORMDL3* and *GSDMA* gene expression and IL-17 secretion in cord blood

- **Oral presentation: Annual GALTRAIN meeting,** 2010, Nottingham, United Kingdom
  Th17 cells in early life immune development: a follow-up II

- **Poster presentation: World Immune Regulation Meeting IV**, 2010, Davos, Switzerland
  FOXP3 demethylation: a reliable quantitative marker for nTreg cells in early immune regulation

- **Oral presentation: Annual GALTRAIN meeting**, 2009, Borstel, Germany
  Th17 cells in early life immune development: a follow-up

- **Poster presentation: World Immune Regulation Meeting III**, 2009, Davos, Switzerland
  Polymorphisms in the Th17 pathway were associated with downregulation of Treg markers in cord blood

- **Poster presentation: European Respiratory Society Annual Congress**, 2008, Berlin, Germany
  Role of Th17 cells in neonates related to maternal atopy

- **Oral presentation: Annual GALTRAIN meeting**, 2008, Munich, Germany
  Th17 cells in early life immune regulation

- **Poster presentation: World Immune Regulation Meeting II**, 2008, Davos, Switzerland
  Th17 cells in relation to maternal atopy in neonatal cord blood

References

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