Gut bacteria, regulatory T cells and allergic sensitization in early childhood

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Cover illustration: The struggle for tolerance

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To my family…

"If you have knowledge,
let others light their candles at it..."
-Margret Fuller

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ABSTRACT

The hygiene hypothesis postulates that reduced or altered microbial exposure early in life may lead to impaired immune maturation and, as consequence of this, development of allergic disorders. Thus, we examined if the infantile gut microbiota was related to the postnatal T cell development *in vivo* and if certain commensal gut bacteria were able to induce regulatory T cells (Tregs) *in vitro.* We also investigated if the proportion of Tregs was associated with allergic sensitization and allergic disease in the first 3 years of life.

We showed that the gut commensal *Staphylococcus aureus* (*S. aureus*) could convert neonatal $CD4^+$ T cells into $FOXP3^+CD25^+CD127^{low}$ Tregs *in vitro* and that certain culture conditions were required for this conversion. Depletion of pre-existing Tregs before stimulation with *S. aureus* resulted in activated $CD25^+CD127^{low}$ T cells that increased proliferation of $CD4^+$ responder T cells. In contrast, naive $CD4^+$ T cells stimulated in the presence of pre-existing Tregs induced suppressive $FOXP3^+CD25^+CD127^{low}$ Tregs. Finally, blocking programmed cell death ligand-1 (PD-L1) expressed on antigen presenting cells during stimulation with *S. aureus*, reduced or completely inhibited the induction of $FOXP3^+CD25^+CD127^{low}$ T cells.

In the prospective FARMFLORA birth-cohort study, we found that children with an early gut microbiota including bifidobacteria and *Escherichia coli* (*E. coli)* had mononuclear cells with higher capacity to produce proinflammatory and Th2-related cytokines in response to phytohaemagglutinin (PHA) than children not colonized by these bacteria. In contrast, early colonization by *S. aureus* and enterococci was inversely related with the PHA-induced cytokine responses. The early bacterial gut colonization pattern was not associated with the proportion of putative $FOXP3^+CD25^{high}$ Tregs within the circulating $CD4^+$ T cell population during early childhood. However, high proportions of $FOXP3^+CD25^{high}$ cells of the $CD4^+$ T cell population in early infancy were inversely related to the capacity of mononuclear cells to produce cytokines in response to PHA as well as to the proportions of $CD45RO^+$ of $CD4^+$ T cells later in childhood. Moreover, children who were sensitized at 18 and 36 months of age had higher proportions of putative $FOXP3^+CD25^{high}$ Tregs at birth and 3 days of life than children who remained non-sensitized. Allergic disease, on the other hand was not associated with the proportion of putative $FOXP3^+CD25^{high}$ Tregs.

In conclusion, these results indicate that *S. aureus* has an ability to convert naïve neonatal $CD4^+$ T cells into $FOXP3^+CD25^+CD127^{low}$ regulatory T cells *in vitro*, a process which is dependent on the presence of both thymic derived Tregs and of APCs that express PD-L1. However, the early bacterial gut colonization pattern was not related to the proportion of putative $FOXP3^+CD25^{high}$ Tregs within the circulating $CD4^+$ T cell population in children

during the first 3 years of life. Furthermore, as infants who were sensitized had higher proportion of $FOXP3^{+}CD25^{high}$ within the $CD4^{+}$ T cell population early in life compared to healthy children, higher proportions of Tregs early in life do not seem to be protective against atopic disorders. Thus, it is possible that high proportions of putative $FOXP3^{+}CD25^{high}$ Tregs within the $CD4^{+}$ T cell population early in infancy may modulate the effector T cell development in a way that could predispose to allergic sensitization. However, early gut colonization with a gut microbiota including bifidobacteria and *E. coli* might instead enhance the effector T cell development.

Keywords: Regulatory T cells, T cell development, bacterial colonization of the gut, allergy, allergic sensitization, cohort study, children
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ABBREVIATIONS

APC	Antigen-presenting cell
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
DC	Dendritic cell
FOXP3	Forkhead box P3
GALT	Gut associated lymphoid tissue
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked
MLN	Mesenteric lymph nodes
mTOR	mammalian target of rapamycin
OPLS	Orthogonal projection to latent structures by means of partial least squares
PCA	Principal component analysis
PHA	phytohaemagglutinin
PD-1	programmed cell death-1
PD-L1	programmed cell death-ligand 1
PRR	pattern recognition receptor
TLR	Toll like receptor
Tregs	regulatory T cell
iTregs	*in vitro* derived regulatory T cells
pTregs	peripheral derived regulatory T cells
tTregs	thymic derived regulatory T cells
XLAAD	X-linked autoimmune-allergic dysregulation

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunsystem finns till för att förhindra att mikrober såsom bakterier, virus och parasiter infekterar oss. Immunförsvaret består av flera olika sorters vita blodkroppar som har till uppgift att särskilja mikrober från kroppsegna strukturer och ofarliga ämnen som vi kommer i kontakt med via födan och luften. Om de vita blodkropparna angriper kroppsegna strukturer drabbas vi av autoimmuna sjukdomar såsom diabetes, ledgångsreumatism eller multipel skleros. Allergiska reaktioner uppkommer när våra immunceller reagerar mot ofarliga ämnen i vår omgivning som till exempel födoämnen, pollen eller kvalster (så kallade allergen). För att undvika sådana oönskade reaktioner har immunförsvaret utvecklat flera olika regleringsmekanismer. En viktig del av regleringen förmedlas av en speciell typ av immunceller som kallas regulatoriska T-celler. De hämmande regulatoriska T-cellerna har förmågan att stänga av eller dämpa andra immunceller till exempel T-celler som kan orsaka stor skada om de reagerar mot kroppsegna eller ofarliga främmande ämnen.

Under de senaste decennierna har förekomsten av både autoimmuna och allergiska sjukdomar ökat i västvärlden. En tänkbar förklaring är den så kallade hygienhypotesen som föreslår att ökningen av de ovanstående sjukdomarna beror på att vi är mindre utsatta för mikroorganismer och drabbas av färre infektioner. Därmed skulle immuncellerna inte få tillräckligt med aktivering för att kunna utbildas och mogna på ett korrekt sätt. Flera studier stödjer hygienhypotesen, bland annat är starkaste skyddet mot allergi att växa upp på bondgård med djur. Det har även visats att barn som har en bakterieflora i tarmen som är komplex med många olika bakteriearter har lägre förekomst av allergier jämfört med barn med en mer artfattig tarmflora. Detta skulle kunna tyda på att den tidiga bakteriekoloniseringen av tarmen kan aktivera immunförsvaret och därför är viktig för immunförsvarets utmognad.

I Sverige har det blivit vanligt att svenska barn koloniseras av hudbakterien *Staphylococcus aureus* (*S. aureus*) i tarmen under de första månaderna i livet. Det faktum att *S. aureus* kan kolonisera tarmen hos små barn idag tros bero på en minskad konkurrens av klassiska tarmbakterier så som *Escherichia coli* (*E. coli*) i omviningen. Det har visats att barn som koloniserats med *S. aureus* i tarmen har en lägre förekomst av födoämnesallergi jämfört med de barn som saknade *S. aureus*. Barn som fått orala droppar av laktobakterien (*Lactobacillus reuteri*) har också har en lägre förekomst av eksem vid 2 års ålder än de barn som inte fått laktobakterier. I delarbete I ville vi därför genom experimentella försök utröna om *S. aureus* och *Lactobacillus paracasei* (*L. paracsei*) kunde stimulera T-celler att utvecklas till regulatoriska T-celler. Vi fann att *S. aureus* kunde stimulera bildningen av regulatoriska T-celler och att detta troligtvis var beroende av förekomsten av redan existerande regulatoriska T-celler i cellodlingen. Vi fann även att *L. paracasei* inte hade samma förmåga att stimulera till nybildningen av regulatoriska T-celler eftersom de stimulerade till lägre andelar av dessa celler jämfört med vad *S. aureus* gjorde. Nybildningen av regulatoriska T-celler var också beroende av om T-cellerna kunde binda till speciella immunceller som

kallas antigenpresenterande celler. Antigenpresenterade celler är viktiga för att aktivera T-celler så att de kan utvecklas till antingen regulatoriska T-celler eller effektor T-celler som kan hjälpa till att döda mikrober. Vi fann även att det var viktigt att antigenpresenterade celler som uttryckte proteinet PD-L1 band till proteinet PD-1 som fanns på T-cellerna. När PD-1 och PD-L1 förhindrades från att samspela med varandra medförde stimulering med *S. aureus* att färre eller inga regulatoriska T-celler bildades.

I arbete II ville vi ta reda hur den tidiga bakteriella koloniseringen av tarmen är kopplad till andelar regulatoriska T-celler i blodet och förmågan hos immunceller att producera immunologiska signalmolekyler (cytokiner) hos barn under de tre första levnadsåren. Vi fann att barn som var koloniserade med bifidobakterier eller *E. coli* under de två första veckorna i livet hade immunceller med högre förmåga att producera cytokiner senare i barndomen än barn som inte hade dessa bakterier. Barn som däremot var koloniserade med *S. aureus,* enterokocker eller clostridier hade immunceller med lägre förmåga att producera cytokiner. En tarmflora som inkluderar bakterierna bifidobakterier och *E. coli* tidigt i livet verkar därför ha en bättre förmåga att aktivera immunförsvaret hos små barn. Däremot fann vi inte att andelen regulatoriska T-celler i blodet var förknippat med någon bakterie som koloniserade tarmen under spädbarnstiden. Vi fann även att barn som hade höga andelar regulatoriska T-celler i blodet tidigt i livet hade lägre andel aktiverade T-celler senare i barndomen, vilket skulle kunna tyda på att de regulatoriska T-cellerna har en förmåga att motverka aktivering och därmed mognadsprocessen av T-celler under tidig barndom.

Allergier uppkommer oftast då speciella vita blodkroppar (B-celler) bildar IgE antikroppar mot ett allergen (t.ex. födoämnen, pollen, djurepitel eller kvalster) vilket kallas för sensibilisering. Även om inte all allergi orsakas av sensibilisering, har det visats att barn som är sensibiliserade har en högre risk att utveckla födoämnesallergi, hösnuva eller astma senare i barndomen. Enligt hygienhypotesen leder låg stimulering av immunsystemet till en försämrad utmognad av immunförsvaret, vilket resulterar i nedsatt tolerans mot ofarliga ämnen. I det sista delarbetet ville vi därför undersöka om barn som utvecklade allergier eller var sensibiliserade skiljde sig åt vad det gällde utvecklingen av regulatoriska T-celler och aktiverade T-celler jämfört med friska barn. Vi fann att barn som var sensibiliserade vid 18 och 36 månaders ålder hade en högre andel regulatoriska T-celler vid födelsen och 3 dagars ålder än friska barn. Däremot fanns det ingen skillnad på andelar regulatoriska T-celler mellan allergiska och friska barn. Det verkar därför som att höga andelar regulatoriska T-celler tidigt i livet inte har en skyddande effekt mot sensibilisering.

Sammanfattningsvis tyder våra resultat på att höga andelar regulatoriska T-celler tidigt i livet kan motverka aktivering av T-celler och på så sätt kanske också öka benägenheten att bli sensibiliserad. Kolonisering med en tidig tarmflora som innehåller bifidobakterier eller *E. coli*, motverkar däremot den hämmande effekten av regulatoriska T-celler genom att istället stimulera aktivering och utmognad av immunsystemet.

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1. THE IMMUNE SYSTEM

1.1 Introduction

The immune system has several important roles in our body; to eliminate harmful microbes, to distinguish these from self-antigens and environmental antigens and to regulate the immune responses towards microbes that live in symbiosis with us. For these purposes, the immune system contains a first and second line of defense known as innate and adaptive immunity, respectively.

1.2 The innate immune system

The innate immune system consists of many different cells, e.g. neutrophils, eosinophils, basophils, natural killer (NK) cells, mast cells, monocytes, macrophages and dendritic cells (DCs). These cells are able to recognize intruding microorganisms, i.e. bacteria, viruses, fungi and parasites, by the use of diverse pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLRs) [1]. PRRs bind to certain microbial products or molecules expressed exclusively by microorganisms, e.g. lipopolysaccharide, peptidoglycan, lipoteichioc acids, bacterial DNA and double-stranded RNA [2]. The innate immune cells do not require previous exposure to specific microbes to identify and kill them.

For an invasive infection to occur a microbe must either colonize the skin or the mucosal surfaces and penetrate them to infect the tissue underneath. The first innate cells to encounter the pathogens are tissue resident macrophages and dendritic cells. These cells recognize the intruding microbes via PPRs, become activated and phagocytose the pathogen. The activated macrophages and DCs also secrete signal mediators, e.g. cytokines, which initiate the inflammatory response. Nearby blood capillaries dilate in response to proinflammatory cytokines. Moreover, endothelial cells lining the capillary walls are activated and increase their expression of adhesion molecules. These alterations enable the adherence of circulating neutrophils to the endothelium. Moreover, chemokines secreted by activated endothelial cells and macrophages facilitate the migration of the neutrophils into the inflamed tissue where they kill the pathogens via phagocytosis and degranulation (emptying their granule content into the extracellular milieu).

At the site of inflammation, activated DCs take up entire microorganisms via phagocytosis, or parts of the microbes via macropinocytosis, a process in which large amount of extracellular fluid and its contents are ingested and processed. During this process the DCs upregulate CD80 and MHC on the cell surface [3], which is necessary for the activation of naïve T cells. Furthermore, the homing receptor CCR7 is also upregulated on the DCs that enables their migration towards the peripheral lymphoid tissue [4, 5]. Thus, activated $CCR7^+$ DCs migrate via the draining lymphatic system to nearby peripheral lymph nodes and activate naïve T cells by antigen presentation. This process induces the adaptive immune response.

1.3 The adaptive immune system

In addition to eliminating and neutralizing pathogens and their toxins the adaptive immune responses (also known as acquired) have the specific ability to create an immunological memory towards specific microbes. This is performed by lymphocytes, which consist of B and T cells.

B and T cells can recognize and react to millions of different structures termed antigens. The lymphocytes recognize specific antigens via antigen receptors, i.e. the B cell receptor (BCR) and the T cell receptor (TCR), respectively. Antigens are often of microbial origin, but adaptive immune responses can also be induced to certain self-antigens and to proteins and/or carbohydrates in food and inhaled air. Unlike the PRRs on innate immune cells, antigen receptors expressed by each lymphocyte are unique. Thus, the antigen receptor repertoire of lymphocytes are diverse and are able to recognize any substance or pathogen. After encounter with their specific antigen the naïve lymphocytes are activated and start to proliferate and differentiate to clones of effector or memory cells that have identical antigen receptors.

The activation of naïve lymphocytes occurs in the secondary lymphoid tissues, such as the spleen, the lymph nodes and the Peyer's patches (PP). Here naïve T cells are activated by specialized antigen-presenting cells (APCs), i.e. monocytes (*in vitro*), DCs and macrophages, but also activated B cells. The activation of B cells occurs via T helper cells. Upon activation, expansion and differentiation, the effector lymphocytes either migrate to the site of inflammation and contribute to the elimination of the pathogen or remain in the lymphoid tissue and continue activating the adaptive immune system.

1.3.1 T cells

The TCR recognizes peptides that are presented by two different types of MHC molecules; MHC class I that interact with cytotoxic $CD8^+$ T cells and MHC class II that binds to $CD4^+$ helper T cells. Thus, T cells can be divided into two subtypes; the cytotoxic $CD8^+$ T cells that upon activation are specialized to kill infected cells or tumor cells in the host; the $CD4^+$ T helper cells that are able to activate B cells and macrophages. Depending on the type of infection and cytokines released by the APCs, T helper cells (Th cells) can differentiate in to several subtypes, e.g. Th1, Th2, Th17 and regulatory T cells (Tregs), with different effector functions.

1.3.2 B cells

The BCR is an immunoglobulin molecule that recognizes both conformational and linear epitopes on macromolecules (proteins, lipids, polysaccharides, nucleotides or certain haptens). Following activation the naïve B cell will differentiate into plasma cells that secrete antibodies or into memory B cells that will respond rapidly when they re-encounter

the antigen. Activated B cells may also act as APCs and present antigen to naïve T cells [6]. The constant part of the antibody determines the isotype of the immunoglobulin (IgM, IgD, IgG, IgE or IgA). IgM and IgD are expressed by naïve B cells [7], but after activation B cells may undergo isotype switch that results in B cells that express or secrete IgG, IgE or IgA antibodies. IgG is the most common immunoglobulin found in serum, whereas IgA is mainly transported across the mucosal surfaces, e.g. the respiratory tract and the gut, to prevent microorganisms to adhere to the epithelial cells and infect the tissue underneath. IgE, on the other hand, is important for the defense against parasites. However, allergen-specific IgE antibodies are essential in immune responses in many allergic individuals [8]. The effector functions of antibodies are to opsonize the pathogens and thereby facilitate phagocytosis, to activate the complement system that will eliminate the pathogen and to neutralize pathogens and toxins.

1.4 Cytokines

As mentioned above, cytokines are proteins that function as mediators between different cells and are essential in the activation of immune cells. Cytokines also have a role in stopping the inflammation, as certain cytokines are able to inhibit activation of APCs, lymphocyte proliferation and cytokine release. The specific functions of some cytokines involved in the innate and adaptive immune responses are described in Table I.

1.5 Conclusion

Innate immune cells are essential for the activation of lymphocytes and to restrain the pathogen until effector T or B cells are functional. The adaptive immune cells on the other hand are pivotal for specific elimination of the pathogen and to establish an immunological memory. The $CD4^+$ helper T cells are able to direct the immunological response by being involved in the activation of B cells and by secretion of cytokines that influence B and T cell differentiation.

Table I. Cytokines involved in the innate and adaptive immune response

Cytokine	Cell source	Effect
IL-1β	Monocytes Macrophages DCs	Enables infiltration of immune cells via the blood to the site of inflammation Induces production of IL-6
TNF	Monocytes Macrophages DCs Th1 cells	Enables infiltration of immune cells via the blood to the site of inflammation Activates DCs
IL-6	Macrophages DCs	Activates lymphocytes Increases antibody production Induces acute phase protein production
IL-12	Monocytes Macrophages DCs	Activates NK cells Stimulates induction of IFN-γ Induces differentiation of Th1 cells
IL-2	Activated T cells	Induces T cell proliferation
IL-17	Th17 cells	Stimulates neutrophil recruitment
IL-4	Th2 cells	Stimulates B cell proliferation and differentiation Stimulates class-switch to IgE Induces differentiation of Th2 cells
IL-5	Th2 cells Mast cells	Stimulates proliferation and survival of eosinophils
IL-13	Th2 cells eosinophils	Stimulates class-switch to IgE Stimulates B cell proliferation and differentiation
IL-9	Th2 cells	Enhances IL-4 mediated IgE and IgG production from B cells Promotes mast cell growth and function Promotes airway hyperresponsivness and overproduction of mucus
IFN-α IFN-β	Plasmacytoid DCs	Increases killing capacity of NK cells Increases MHC class I expression
IFN-γ	NK cells Th1 cells	Activates macrophages to kill engulfed bacteria
TGF-β	DCs T helper cells	Inhibits activation and proliferation of T cells Directs differentiation of T cells to Tregs
IL-10	DCs Tregs	Inhibits activation and proliferation of T cells Inhibits cytokine release from macrophages

2. T CELLS

2.1 Introduction

T cells are specialized to recognize antigens that have been intracellularly processed by APCs and subsequently presented as a small peptide on their MHC molecules. These peptides might be part from proteins produced in virus infected cells, tumor cells or extracellular microbes that have been digested by the APCs. Peptides from virus-infected cells or tumor cells primarily activate the cytotoxic $CD8^+$ T cells, while peptides from extracellular bacteria or parasites mainly activate $CD4^+$ helper T cells that direct B cells to produce antibodies with the proper effector functions. In this thesis I have studied the $CD4^+$ T cell development including $CD4^+$ regulatory T cells in children and I will therefore focus on these cells.

2.2 T cell maturation

T cells originate from precursor cells in the bone marrow. The precursor cells migrate at an early stage to the thymus to mature into T cells. Here cells that are able to bind to MHC class molecule I or II during positive selection develop into either $CD8^+$ cytotoxic T cells or $CD4^+$ helper T cells, respectively [9]. During negative selection thymocytes that bind strongly to self-peptides and MHC molecules will be clonally deleted [9], a process termed central tolerance. However, certain T cells that bind strongly to self-antigens during negative selection will mature into $FOXP3^+CD25^+$ regulatory T cells that are involved in the peripheral tolerance [10]. There will also be some leakage of self-reactive T cells into the periphery. Mature T cells leaving the thymus are naïve and require activation to become fully functional.

2.3 Activation of naïve T cells

The naïve T cells express CD45RA [11] and are activated via antigen presentation in the peripheral lymphoid tissue. In order to activate naive T cells, the APCs have to present an antigen specific for the TCR expressed by the T cells. Moreover, the APCs also have to provide costimulation via expression of CD80 or CD86 that interact with CD28 expressed by the T cells [3]. In the absence of costimulation, the activation will be incomplete and the naïve T cell become anergic, i.e. unresponsive to antigen [3]. The combination of specific antigen encounter and costimulatory signals lead to activation of the naïve T cells, which start to secrete IL-2 [3]. IL-2 will drive the T cell proliferation and differentiation. Depending on the cytokines secreted by the APCs during antigen presentation, the naïve $CD4^+$ T cell differentiate into different T helper subsets, e.g. Th1, Th2, Th17 or peripherally induced Tregs (pTregs).

2.4 T cell differentiation

The cytokine milieu provided by the APCs during antigen presentation directs the differentiation of $CD4^+$ T cells. Cytokine signaling initiates the activation of STATs (signal-transducing activators of transcriptors) and up-regulates specific transcription factors that control expression of a panel of genes that are specific for each T cell phenotype. For example, the presence of IL-12 and IFN-γ during antigen presentation stimulates differentiation into Th1 cells via the activation of STAT4 and the upregulation of the transcription factor T-bet (Figure 1) [12]. T cells differentiated to Th1 primarily secrete the cytokines IL-2 and IFN-γ [13]. IFN-γ is involved in the eradication of intracellular bacteria or viruses as it activates macrophages via IFN-γ receptors that stimulates a more effective breakdown of phagocytosed microbes [12].

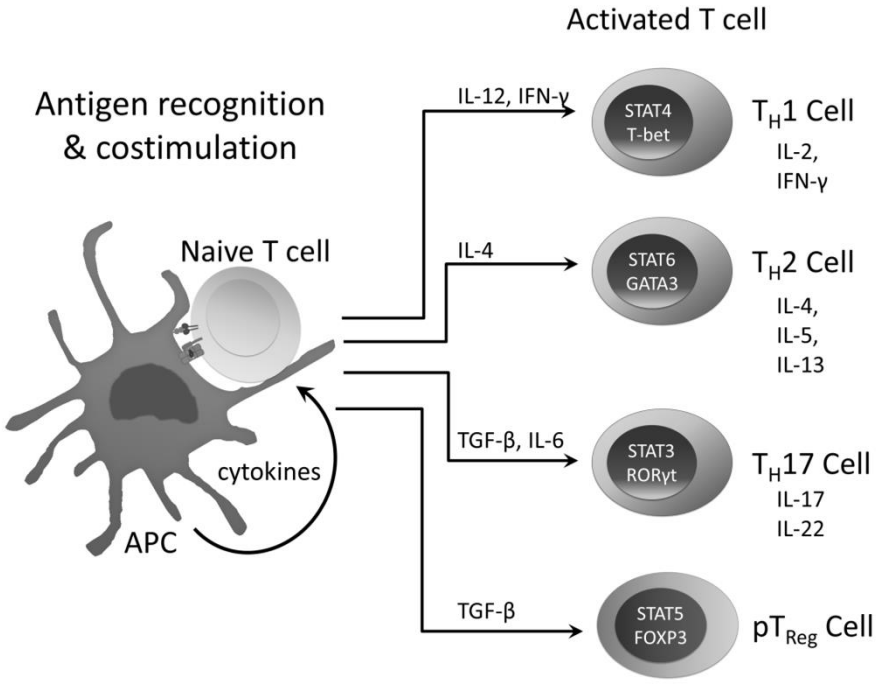

Figure 1. T cell differentiation.
Cytokines, STATs and transcriptor factors that are involved in the conversion of naïve $CD4^+$ T cells into Th1, Th2, Th17 and pTregs.

Th2 differentiation, on the other hand, are primarily driven by IL-4 that activates STAT6 and upregulates the transcription factor GATA3 (Figure 1) [13]. Th2 cells are involved in the defense against helminths and other extracellular parasites, but these cells are also involved in allergic inflammation [14]. Th2 cells secrete mainly the cytokines IL-4, IL-5, IL-13 and IL-9 [13, 14].

The combination of TGF-β and IL-6 leads to the activation of STAT3 and the transcriptor factor RORγT, whereas TGF-β alone is thought to activate STAT5 and Foxp3 that results in differentiation into Th17 and Tregs, respectively (Figure 1) [13]. Th17 cells produce mainly IL-17 and IL-22 and are important in the protection against extracellular pathogens such as bacteria, yeastsand fungi [15]. However, this subset is also involved in the inflammation of autoimmune and inflammatory disorders such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, contact dermatitis, and allergic asthma [16, 17]. The phenotype and function of pTregs will be further discussed in chapter 3.

2.5 T cell migration

T cells circulate in the bloodstream to secondary lymphoid tissues and non-lymphoid tissue to ensure contact of naïve and memory T cells with their antigens and to distribute effector cells to their target tissues. This is dependent on the binding between certain surface molecules on the T cells and molecules expressed by the vascular endothelia, a process termed homing [18].

Migration involves four adhesion steps: rolling, activation, firm adhesion and transmigration [18]. During rolling, the circulating cells that express selectins must first bind loosely to their ligand. CD62L (L-selectin) expressed by the T cells bind to peripheral lymph node addressin (PNAd), glycosalation-dependent cell adhesion molecule 1 (Gly-CAM-1) or mucosal addressin CAM-1 (MadCAM-1) [19, 20]. This tethering will allow the cells to roll on the endothelial cells, which facilitate T cells to encounter chemokines in the local environment. For example, the chemokines CCL19 and CCL21, which are found in high endothelial venules near secondary lymphoid tissue interact with the chemokine receptor CCR7 expressed by naïve T cells. The interaction between chemokines and chemokine receptors leads to the second step of migration, i.e. activation and conformation of integrins expressed on T cell surface. The integrins αLβ2 (VLA-4), α4β1 (LFA-1) and α4β7, will obtain higher affinity to their ligand ICAM, VCAM and MAdCAM-1, respectively [21]. Thus, the T cell is able to adhere strongly to the endothelial surface via the integrins and their ligands, known as the third and fourth steps in migration. The T cell will subsequently crawl through the interendothelial junctions. Thereafter, the cell can respond to the gradient of chemokines and migrate into the secondary lymphoid tissue.

Naïve $CD4^+$ T cells express the homing receptors CCR7 and CD62L that enable migration into lymphoid tissue [22, 23]. In children naïve T cells also express the integrin α4β7 that binds to MAdCAM-1 [24]. During fetal development and in early childhood MadCAM-1 is expressed in peripheral lymph nodes [25], as well as on high endothelial venules in gut-associated lymphoid tissues (GALT), and on postcapillary venules in the gut and in the mammary gland [26, 27]. However, in adults MadCAM-1 is only expressed in the GALT.

Upon activation the T cells down-regulate the expression of CCR7 and CD62L and acquire a new tissue-specific homing phenotype. The site of activation will dictate the homing

phenotype obtained, e.g. T cells activated in cutaneous lymph nodes express cutaneous CCR4 and CCR10 that will direct the T cells to normal and inflamed skin [28, 29]. Moreover, T cells activated in the MLN or PP of the intestine express the chemokine receptor CCR9 or CCR10 directing them back to the small intestine or colon, respectively [30, 31].

2.6 Memory T cells

As mentioned in chapter 1, naïve T cell that are activated in the peripheral lymph nodes proliferate and differentiate to either effector T cells or memory T cells. Memory $CD4^+$ T cells are long-lived cells that are able to respond rapidly upon a reinfection. These cells express CD45RO as the longer CD45RA molecule is spliced during activation of the naïve T cell [11]. There are two types of memory T cells; central memory T cells and effector memory T cells [32, 33]. As the central memory T cells mainly infiltrate the secondary lymphoid tissue, similarly to naïve T cells, they also express the homing receptors CCR7 and CD62L [32, 33]. In contrast, effector memory T cells do not express CCR7 or CD62L and instead migrate towards the peripheral tissue where they convey a range of effector functions [32, 33].

2.7 Conclusion

Central tolerance is one mechanism by which self-reactive T cells can be stopped from reaching the periphery. However, there is leakage of T cells that react to self-antigens and to antigens of non-microbial origin, such as food proteins. It is the task of regulatory T cells to confer tolerogenic and non-aggressive reactions to innocuous antigens. Thus, it is important to study the factors that influence development of Tregs in the periphery to better understand the immunoregulatory mechanisms that may hinder the development of autoimmune and allergic disorders.

3. REGULATORY T CELLS

3.1 Introduction

Mouse models of neonatal thymectomy demonstrated the existence of T cells that were capable of suppressing other cells and maintaining peripheral tolerance. Mice that were thymectomiced on day 3 of life (d3Tx) developed T cells that mediated autoimmune disease [34]. Interestingly, thymectomy before 3 days or after 7 days of life did not result in disease. D3Tx mice could be rescued from disease if they were reconstituted with thymocytes from adult mice that had not been thymectomized at day 3 [35]. Thus, it seemed as if there was an essential difference in T cells leaving the thymus before day 3 of life and those leaving later. In 1995, Sakagushi et al reported that depletion of $CD25^+CD4^+$ T cells in mice followed by transfer of $CD4^+CD25^{neg}$ T cells from another mouse resulted in similar autoimmune diseases as those observed in d3Tx mice. However, disease could be prevented by co-transfer of $CD4^+CD25^+$ T cells in both the cell-transfer model and the d3Tx model [36]. Today, $CD4^+CD25^+$ regulatory T cells (Tregs) are recognized as a central T cell population for preserving peripheral tolerance.

3.2 Regulatory T cells

Approximately 5-10% of the circulating $CD4^+$ T cells are Tregs in children and adults [37, 38]. Human Tregs suppress T cell proliferation and cytokine production in response to self-, tumor, microbial or environmental antigens [37, 39]. Tregs are traditionally characterized by high expression of CD25 and the expression of transcription factor *Foxp3*. *Foxp3* is thought to have a critical role in the development of Tregs. Infants born with a mutation in the *Foxp3* gene develop IPEX/XLAAD syndrome and succumb to several organ-specific autoimmune diseases, food allergy, severe dermatitis, high levels of IgE and sometimes eosinophilia [40]. Moreover, studies have shown that forced *Foxp3* gene expression is able to convey Treg-like suppressive function on conventional T cells [41, 42]. *Foxp3* is therefore considered as a lineage-specific transcription factor for Tregs.

In human adults, however, FOXP3 protein has been shown to be momentarily up-regulated in conventional $CD25^{neg}$ T cells upon TCR stimulation *in vitro* [43, 44]. Indeed the circulating conventional $CD4^+$ T cell pool contains a small population of cells that express FOXP3. These T cells express intracellular proinflammatory cytokines and are not suppressive [45]. Thus, FOXP3 expression *per se* is not sufficient to define functional Tregs, at least not in human adults.

In 2006 it was reported that human Tregs express little or no CD127, the α-chain of the IL-7 receptor, on the cell surface [38, 46]. Indeed, the expression of CD127 on $CD4^+CD25^+$ T cells were found to be inversely correlated with the expression of FOXP3 and the inhibitory function [38, 46]. Two advantages with the discovery of this marker was that it made it possible to differentiate between $CD25^+$ cells that were activated $CD4^+$ T cells and $CD25^+CD4^+$ Tregs and it also enabled isolation of viable Tregs for functional studies.

3.3 Thymus-derived and peripherally induced Tregs

As mentioned in chapter 2, certain thymocytes mature into $FOXP3^+CD25^+$ Tregs in the thymus. The exact mechanism behind this selection is still unknown, but it has been suggested that only thymocytes with TCRs with high affinity towards self-antigen during negative selection induce FOXP3, which leads to survival and subsequently maturation into $FOXP3^+$ Tregs [47, 48]. Thus, these so called thymus-derived Tregs (tTregs) have TCRs that are self-reactive [10]. It has been shown that circulating human tTregs can be divided into resting $CD45RA^+FOXP3^+$Tregs and activated $CD45RA^{neg}FOXP3^+$ Tregs, both of which are suppressive [45]. Miyara et al. also showed that stimulated resting $CD45RA^+$ Tregs upregulate FOXP3, become activated Tregs and proliferate [45]. Although the majority of the activated Tregs are thought to originate from resting Tregs, activated Tregs can also be generated in the periphery from conventional $CD4^+$ T cells.

Peripherally induced Tregs (pTregs) originate from non-regulatory $CD4^+CD25^{neg}$ T cells [49]. It is difficult to discriminate between tTregs and pTregs as both subtypes share the similar molecular signature, including high surface expression of CD25, intracellular expression of FOXP3 and CTLA-4, but low or no expression of surface CD127. The transcription factor Helios, a member of the Ikaros family, was suggested to be exclusively expressed in tTregs compared to pTregs [50]. However, Helios expression has been shown to be induced upon activation in conventional $CD4^+$ T cells, $CD8^+$ T cells as well as in Tregs [51]. Moreover, humans have been found to have a population of $Helios^{neg}$ naïve Tregs that express the recent thymic emigrant marker CD31, indicating that not all thymus derived-Tregs express Helios [52].

3.4 Induction of pTregs

The mechanisms involved in the conversion of conventional $CD4^+$ T cell into $FOXP3^+$ Tregs are still unclear. However, antigen stimulation of $CD4^+CD25^{neg}$ T cells in the presence of TGF-β induces $FOXP3^+CD25^+CD4^+$ T cells [53, 54]. These induced $FOXP3^+$ Tregs are able to suppress other T cells [53, 54]. Moreover, TGF-β in combination with retinoic acid, a vitamin A metabolite produced by specialized DCs in the gut, has also been shown to direct naïve T cell differentiation into $FOXP3^+$ Tregs [55].

In mouse models, low antigen dose and costimulation by APCs results in induction of functional $FOXP3^+$ Tregs [56]. Accordingly, *in vitro* studies have shown that robust TCR signaling activates the intracellular Akt-PI3K-mTor pathway, which leads to T cell differentiation but inhibits induction of *Foxp3* [57, 58]. Accordingly, blocking the mTor pathway induces functional Tregs [59]. Thus, Treg induction seems to occur during conditions that are suboptimal for general T cell activation.

The programmed cell death ligand-1 (PD-L1) expressed by APCs during certain conditions is important for the induction of pTregs. PD-L1 interacts with programmed cell death 1

(PD-1) expressed on newly activated T cells and impede the TCR signaling pathway by the recruitment of phosphatases such as SHP-2 (Src homology region 2 domain-containing phosphates) and PTEN (Phosphatase and tensin homolog) (Figure 2) [60-62]. Amarnath et al showed that interaction between PD-L1 and PD-1 during activation of TBET^{+}Th1 cells converted these cells into FOXP3^{+} Tregs [63, 64]. However, blocking phosphorylation of SHP-2 during PD-1/PD-L1 interaction hindered the conversion into Tregs [63, 64].

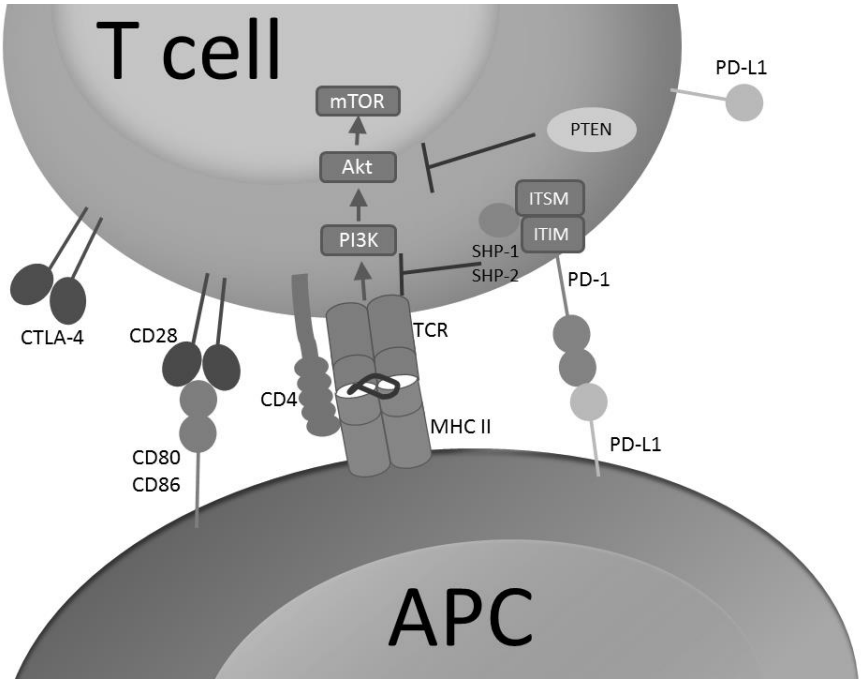

Figure 2. The interaction between PD-1 and PD-L1.
Interaction between PD-1 and PD-L1 during antigen presentation results in recruitment of the phosphatases SHP-1 and 2 as well as PTEN. These molecules will hinder the TCR signaling pathway and mTOR, which results in differentiation of regulatory T cells.

3.5 Treg mechanisms of suppression

Tregs have several mechanisms by which they regulate other cells.

Targeting APCs: The regulatory molecule cytotoxic T lymphocyte associated antigen-4 (CTLA-4) seems to be essential for Treg function [65-68]. Mice that lack CTLA-4 develop autoimmune disorders that are characterized by infiltration of CD4^{+} T cells into non-lymphoid tissues [69, 70]. However, transfer of CTLA-4^{+} Treg or CTLA-4^{+} conventional CD4^{+} T cells prevented the migration and accumulation of autoantigen-specific T cells into the target tissues [71, 72]. CTLA-4 is a homolog to the costimulatory molecule CD28 and is therefore also able to bind to CD80 and CD86 expressed on APCs. However, CTLA-4 have higher affinity to CD80 and CD86 compared to CD28 [73]. In contrast to CD28, interaction between CTLA-4 and CD80 or CD86 inhibit the production of IL-2 and proliferation of T cells [74]. Furthermore, CTLA-4 down-regulate CD80 and CD86 on DCs by binding and removing these molecules via trans-endocytosis, independently on which cell they are expressed on [75]. The snatching of CD80 and CD86 from the DCs will inhibit activation

and differentiation of other T cells. Consequently, blocking the interaction between CTLA-4 and CD80 and CD86 expands the activated T cell population [76].

Competition: Also, FOXP3$^+$ Tregs have been shown to out-compete naïve T cells by forming aggregates around DCs *in vitro* [77] and thereby hindering T cell activation. These formations are dependent on high expression of the adhesion molecule LFA-1 (lymphocyte function-associated antigen-1) expressed on Tregs [77].

Metabolic disruption: The cell surface molecules CD39 and CD73 have been suggested to be involved in the suppressive ability of Tregs [78-80]. CD39 is expressed by Tregs in mice, but to a lesser extent in humans [78]. Both molecules are ectoenzymes and are involved in the generation of adenosine [78]. Adonesine triphosphates (ATP) is an indicator of tissue destruction and CD39 has the ability to degrade ATP to AMP [78]. Next, CD73 in combination with CD39 further converts AMP to adenosine [79]. Adenosine has immunosuppressive properties as it inhibits proliferation of effector T cells [79, 80]. Furthermore, Tregs express CD25 to a high extent as they require IL-2 for their cell survival. However, the high levels of CD25 on the cell surface may deprive effector T cells of IL-2 and thus inhibit their proliferation [80].

3.6 Conclusion

The regulatory T cells may hinder development and/or impede the function of autoreactive T cells as well as other activated T cells. However, a balance is needed between the regulation of harmful self-reactive effector T cells, yet allowing effector T cells to function and be involved in the elimination of harmful microbes. Factors that enhance the Treg function and numbers *in vivo* during early childhood still need to be elucidated, but several mouse models have suggested the gut microbiota to be an important stimulus for the induction of Tregs.

4. THE GUT MICROBIOTA

4.1 Introduction

The gut microbiota has several critical physiological roles, such as digestion of carbohydrates that otherwise would pass the gut undigested, suppression of growth of invasive and resident pathogens, as well as activation of the immune system of the host. The stomach and the small intestine contain only few and low numbers species of bacteria. The low numbers of bacteria in this area is due to environmental factors such as acid, bile and pancreatic secretions that kill most ingested microorganisms, as well as the phasic propulsive motor activity towards the ileal end that impedes stable bacterial colonization [81]. In contrast, the large intestine contains a complex and dynamic microbiota. The large intestine of an adult harbors approximately 10^{11} bacteria/g faeces, which is ~60% of the fecal mass [82]. The numbers of microbial cells in the gut lumen is 10 times larger than the number of eukaryotic cells in the human body [81].

4.2 The "classical" gut bacterial colonization pattern

The establishment of the gut flora may start during or directly after birth when the neonate is first exposed to bacteria. The neonatal gut is rich in oxygen and favors the colonization of facultative bacteria that can perform either aerobic or anaerobic metabolism. The "classical colonization" pattern, as described in culture-based studies from the 1970s and 1980s, implies that *Escherichia coli* (*E. coli*) and enterococci are the first bacteria that colonize the large bowel [83, 84], but enterobacteria other than *E. coli* e.g. Klebsiella and *Enterobacteria* species are also common. After the facultative bacteria have consumed the oxygen, the anaerobic bacteria *Bacteroides*, bifidobacteria and clostridia have the opportunity to colonize the gut and a majority of infants acquire these bacteria the first weeks of life [82]. These anaerobes are followed by other more anaerobic bacteria until a more complex microbiota is established in the gut [83, 84]. This infantile bacterial colonization pattern was common in both the developing countries and the Western world 40 years ago [82], although colonization seemed to occur more rapidly and with a higher strain-turnover rate in infants in developing countries [85, 86].

Not much is known regarding the sources of the bacteria that colonize gut of the neonates have been little studies. However, approximately half of the of *E. coli* strains in neonates originate from the maternal fecal flora [82]. Other strains may come from infants at the same ward and are passed on by the staff [87]. Bifidobacteria have also been found to originate from maternal feces, but may also be spread between infants [82].

4.3 An altered gut bacterial colonization pattern

Today colonization with *E. coli* is delayed in Swedish infants and the turnover rate of individual bacterial strains is slower than in studies performed in the 1980s [85, 88]. Colonization by *Bacteroides* also seems to be delayed in today's Swedish infants. As *E. coli*

and *Bacteroides* are found only in the gut of humans and other mammals the delayed colonization by these bacteria indicate reduced exposure to fecal bacteria in Sweden today, which reflects high hygienic standards. The colonization rate of enterococci and enterobacteria other than *E. coli*, such as *Klebsiella* does not seem to have changed, which could be due to the fact that these bacteria are common in various environmental niches also in very hygienic society [82].

During the last decades, *Staphylococcus aureus* (*S. aureus)* has emerged as one of the first gut colonizers in Swedish infants [89]. About 65% of the children born in the late 1990s were colonized by this bacterium at 2 weeks of life [90]. *S. aureus* is normally a commensal skin bacterium and the majority of the infants that harbor *S. aureus* in the intestine acquire the bacteria from the skin of their parents [91]. The increased frequency of intestinal colonization with *S. aureus* is probably due to decreased competition by classical fecal bacteria as a result of improved sanitary conditions. Thus, colonization by *S. aureus* may indicate that Swedish infants have an undeveloped gut flora of low complexity, which allows bacteria that normally colonize the skin to be established in the gut.

4.4 Mucosal immune system

The intestinal microbiota is a strong stimulant for the neonatal immune system. The intestinal epithelium provides a barrier against invasion of microbes. However, there are several mechanisms by which the gut bacteria and the bacterial products may cross the epithelium. In infants, bacteria that reach high counts in the intestine may cross the immature gut barrier and stimulate immune cells via a process termed translocation (Figure 3) [92]. Moreover, the small intestine contains specialized M cells located between the epithelial cells. The M cells transport macromolecules, particles and microorganisms across the epithelium to underlying structures called Peyer's patches [93], which are included in the gut associated lymphoid tissue (GALT). GALT also includes small lymphoid aggregates in the small intestine and the lymphoid follicles in the large bowel [94]. These structures resemble lymph nodes as they contain B cell follicles and T cell areas and are considered to be the induction sites for the gut immune responses. Bacteria or bacterial products that have crossed the epithelium are engulfed and processed by APCs that present antigens to T cells in the Peyer's patches or mesenteric lymph nodes (MLNs) that drain the gut mucosa (Figure 3). The activated T cells activate B cells that may undergo class-switch from IgM to IgA due to retinoic acid released by the gut DCs [95]. Activated lymphocytes leave the MLNs via the efferent lymphatic vessels that gather in the thoracic duct and are thereafter transported via the blood to the mucosal effector sites. By the expression of homing receptors such as α4β7, CCR9 and CCR10, the circulating lymphocytes will be able to migrate into the mucosal effector sites (as described in chapter 2) and reach the lamina propria [26, 27, 30].

In the lamina propria, B cells will further differentiate into IgA-secreting plasma cells. The secreted IgA is a molecule that is dimeric and is transported through the epithelial cells into the gut lumen (Figure 3). Secretory IgA in the lumen bind to bacteria and bacterial components and thereby impede translocation (Figure 3). Thus, the immune system is stimulated every time a new bacterial strain succeeds to reach the mucosal lymphoid tissue. However, once a specific IgA response is developed the translocation of the strain is prevented. Therefore, bacteria that colonize the gut for an extended period of time only stimulate the immune system upon the initial colonization.

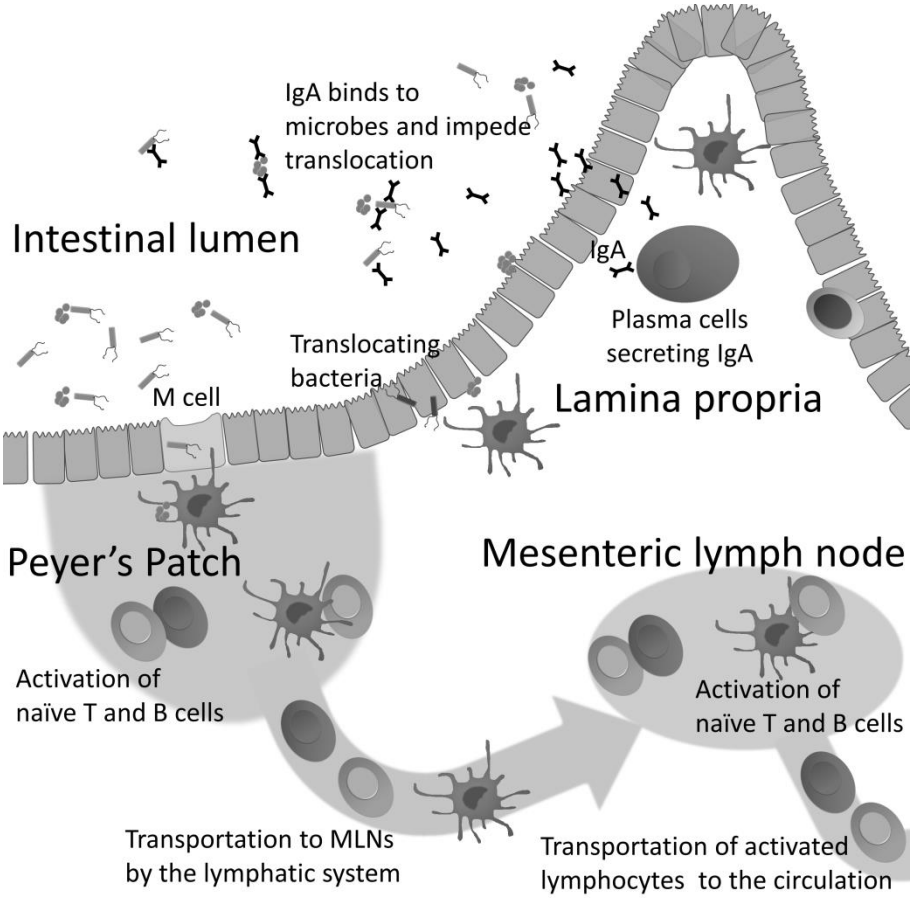

Figure 3. Mucosal immunity

M cells found between epithelial cells of the intestine are able to transport microorganisms or compounds to the Peyer's patches situated underneath M cells. These compounds are digested by DCs that in turn activate naïve T cells. Here B cells also encounter their antigen and are activated. Thereafter, activated lymphocytes and antigen-loaded DCs travel to the mesenteric lymph nodes (MLNs). In the MLNs DCs continue to activate T cells. Activated lymphocytes travel via the efferent lymphatic to the circulation. Circulating effector T and B cells then return to the gut. Plasma cells situated in the lamina propria secrete IgA. The IgA is transported through the epithelium to the intestinal lumen and hinder microorganisms to translocate.

Germ-free mouse models have demonstrated the importance of bacterial gut colonization for the maturation of the immune system as these mice have smaller Peyer's patches and mesenteric lymph nodes and reduced number of T cells and IgA-producing plasma cells in

the intestine than conventionally raised mice [96, 97]. Accordingly, after the introduction of bacteria to the intestine, germinal centers start to appear followed by increased numbers of IgA-expressing B cells [97]. Furthermore, intestinal bacterial colonization has also been shown to be important for the induction and function of $FOXP3^+$ Tregs [98-100]. Germ-free mice have lower proportions of $FOXP3^+$ Tregs and reduced suppressive capacity than wild type mice [98-100]. Colonization with either a mixture of bacteria or monocolonization with certain bacterial species resulted in *de novo* generation of $FOXP3^+$ Tregs in the intestine [98-100].

In humans, the influence of the infantile gut bacterial colonization on the developing immune system is still largely unknown. It has been shown that early colonization by *E. coli* and bifidobacteria is positively associated with higher numbers of circulating $CD27^+$ memory B cells at 4 and 18 months of life [101]. In contrast, colonization with *S. aureus* was inversely related to memory B cell counts [101], indicating that the colonization pattern early in infancy might influence the maturation of the adaptive immune system later in childhood.

4.5 Conclusion

The intestinal tract contains an enormous variety of foreign antigens that are a strong stimuli for the developing immune system. During the last 40 years the lifestyle has changed in the Western world and the acquisition of typical gut bacteria such as *E. coli* and *Bacteroides* is delayed. It is important to study how this affects the maturation of the human infantile immune system and if alterations in the immune development early in life may lead to immune disorders such as allergies.

5. ALLERGY

5.1 Introduction

In allergic individuals the immune system reacts against harmless antigens in food or in the inhaled air, such as pollen or animal dander. Healthy persons do not react towards these substances as they are tolerant to allergens. Interestingly, allergic disorders are most common in societies with high hygienic standards, small families and few infections, suggesting that environmental factors such as microorganism may influence the development of tolerance to innocuous antigens.

5.2 Allergic sensitization and inflammation

Allergic sensitization is caused by the formation of IgE antibodies to harmless environmental antigens, termed allergens [102]. For some individuals, a second exposure to the allergen leads to immune-mediated hypersensitivity, i.e. allergy. However, not all individuals with allergic symptoms are sensitized, and not all sensitized individuals develop allergies. The most common target organs for allergic symptoms are the respiratory tract (rhinitis and asthma), the eyes (conjunctivitis), the skin (atopic eczema) and the gastrointestinal tract (food allergies).

The initiation of allergy starts with sensitization towards a specific allergen. The allergen is taken up by APCs in the eyelids, nose, lungs, skin, and intestine. The APCs then migrate to the nearest lymphoid tissue were they present the allergens to naïve T cells. In this case, antigen presentation leads to differentiation into Th2 cells, which secrete the cytokines IL-4, IL-5, IL-13 and IL-9. The cytokines IL-4 and IL-13 are associated with isotype-switch of B cells, which results in allergen-specific IgE antibodies [103]. IgE is able to bind to mast cells via Fcε-receptors, which are then ready to be activated upon encountering the allergen. Upon second exposure allergen binds to the IgE antibody on the mast cells that directly respond to the allergens and release inflammatory mediators such as histamine and proteases. Histamine stimulates the smooth muscles in arteries, respiratory tract and intestinal tract to contract, whereas arterioles are dilated and permeability in post-capillary vessels are decreased. The proteases degrade neuropeptides and induce secretion of mucus. This reaction occurs within 10 minutes after exposure to allergen and is followed by a late phase reaction.

The late phase reaction causes symptoms several hours after exposure to the allergen. The symptoms are maintained by cytokines produced from mast cells and Th2 cells. TNF induce an upregulation of adhesion molecules on the endothelial wall, which results in increased attachment as well as increased migration of eosinophils, mast cells, monocytes and neutrophils to the mucosa. Activated Th2 cells infiltrate the tissue and secrete cytokines that contribute to the late phase reaction. IL-13 stimulates differentiation and survival of eosinophils and mast cells. Th2 cells, mast cells and eosinophils secrete IL-5 that promotes development, recruitment and survival of eosinophils. Furthermore, IL-9 is also secreted by

Th2 cells and has been shown to be involved in the growth of mast cells, promote airway hyperresponsivness and overproduction of mucus.

5.3 Allergy in children

The most common allergic disease in early childhood is atopic eczema that is an inflammatory skin disease that can appear as early as at 1-2 months of age [104]. Generally, atopic eczema is diagnosed in children that have had itchy skin conditions over the last 12 months and visible flexural dermatitis, generally dry skin over the past year and a onset before 2 years of age [105]. In a Danish cohort, approximately 17% of the children had developed eczema by 1.5 years of age, but at 5 years of age the symptoms remained only in 2% of these children [106]. The observed decrease of eczema at 5 years of age is common as this disorder is often outgrown later in childhood. Thus, for diagnosis of atopic eczema after 4 years of age, the child must also have a history of asthma or rhinitis [105]. Approximately 40% of skin rashes observed in children with moderate or severe atopic eczema are caused by food allergens [107]. Indeed, 35% of children with eczema have IgE-mediated food allergy [108]. Notably, T cell clones against specific food allergens have been isolated in skin lesions of eczematous patients, suggesting a connection between food allergy and skin inflammation [109].

Food allergies are normally acquired during the 2 first years of life, but peak at 1 year of age [106, 110]. Relatively few food stuffs account for most food allergies, i.e. hen's egg, cow's milk, peanut and tree nuts. Although most of the food allergies in early childhood are outgrown, e.g. egg and milk, allergic reactions to peanut and tree nuts can be life long [110]. The symptoms of food allergy may include nausea, abdominal pain, abdominal cramping, vomiting or diarrhea. As there is a connection between IgE-mediated food allergy and atopic eczema, the ingestion of food allergens can also lead to immediate cutaneous symptoms and/or worsen chronic symptoms. Asthma and allergic rhinoconjunctivitis are more common allergic disorders later in childhood [104]. Recurrent wheezing and/or problems of breathing in between colds are typical symptoms of asthma. Allergic rhinoconjunctivitis is characterized by nasal congestion, runny rose, sneezing, red eyes and itching of the nose or eyes after exposure to pollen or animal dander.

The progression of allergic disorders during childhood starting with eczema and food allergy in early childhood, followed by asthma and allergic rhinoconjunctivitis is referred to as the atopic march [104, 111]. Accordingly, atopic eczema is associated with an increased risk of developing food allergy, asthma and allergic rhinoconjunctivitis [111]. It has been estimated that 30% of children with eczema develop asthma, whereas 60% develop allergic rhinoconjunctivitis [112, 113]. Moreover, children who are sensitized to any allergen at 18 months of age but without allergic symptoms, are at a higher risk to develop symptoms of wheezing, asthma and rhinitis at 5 years of age compared to non-sensitized children [114].

5.4 The hygiene hypothesis

The prevalence of allergy has increased along with an increased hygienic lifestyle [115]. Epidemiological studies have shown that the risk of developing IgE-mediated allergies is related to social status, good housing standard, numbers of siblings and sanitary conditions [116]. These observations lead to the postulation of the hygiene hypothesis, formulated by David Strachan 1989 [117]. Strachan proposed that exposure to microorganisms educates the immune system and that reduced microbial exposure may lead to allergy development. Accordingly, infants growing up in a farming environment with close contact to animals during their first year of life have lower prevalence of allergic disease and sensitization in school-age compared to non-farmers' children [118]. Moreover, high enterotoxin level in the dust from mattresses, which was considered to represent a high bacterial content in the environment, is inversely related with hay fever, asthma and atopic sensitization [119]. It was also recently shown that infants whose parents cleaned their pacifiers by sucking at it were partially protected against early eczema development and asthma symptoms compared to children whose parents cleaned the pacifiers in boiling water [120]. The allergy protective effect was suggested to result from transfer of parental oral bacteria to the infants [120]. Taken together, these studies indicate that, early exposure to microbes is important for the protection against allergies.

The gastrointestinal microbiota has also been suggested to be important for the prevention of allergic disorders. Accordingly, a high diversity of the early gut flora is negatively associated with later allergy development [121, 122]. Others have shown that oral administration of *Lactobacillus reuteri* early in life was negatively associated with IgE-mediated eczema at 2 years of age [123]. However, at school age these children do not have lower prevalence of respiratory allergies, suggesting that the effect of *L. reuteri* is transient [124]. Moreover, children colonized with *S. aureus* in the gut early in infancy have lower prevalence of food allergy compared to non-colonized children [125]. However, it is unclear whether or not the protective effects by gut microbiota against allergic disorders are due to maturation of the immune system.

5.5 Regulatory T cells and allergy

FOXP3$^+$ Tregs are thought to play an important role in the development of allergic disorders. Children with dysfunctional *Foxp3* gene not only develop autoimmune diseases, but they also develop severe dermatitis, high levels of IgE and sometimes eosinophilia [40]. Furthermore, children who had outgrown their cow's milk allergy developed functional Tregs one week after challenge with milk while infants that were still allergic did not [126]. Others have found that children who developed tolerance towards heated milk had a higher proportion of proliferating and functional Tregs after stimulation with allergen compared to children who were allergic to heated milk [127]. Thus, Tregs seem to be important in the down-regulation of allergic inflammation.

Moreover, Tregs from birch allergic adults are not able to suppress birch-allergen induced IL-5 and IL-13 responses during birch-pollen season compared to outside the season [128]. Accordingly, patients with allergic rhinitis have higher IL-4, IL-5 and TNF levels in nasal lavage, but lower proportions of circulating putative $CD25^{+}FOXP3^{+}$ Tregs than healthy controls [129]. Tregs may therefore also have an impaired capacity to inhibit Th2 responses during allergic inflammation.

It has also been suggested that infants have an impaired function of Tregs before the onset of allergy. Smith et al found that infants with hen's egg allergy had neonatal $CD25^{+}CD127^{low}$ T cells with lower capacity to suppress IL-13 responses from $CD4^{+}$ responder cells compared to children who remained non-allergic [130]. Moreover, neonates with higher risk to develop allergy, i.e. having parents with allergic disorders, were shown to have lower numbers of Tregs at birth than children with lower allergic risk [131]. However, allergic children have also been found to have equally suppressive Tregs as non-allergic children before allergy onset [132]. Thus, it is still unclear if the proportion of Tregs and their regulatory capacity have an influence on later onset of allergy or sensitization.

5.6 Conclusion

The prevalence of allergic disorders has increased more than threefold during the last 40 years. The hygiene hypothesis postulates that the immune system needs microbial stimulation to develop properly and respond with tolerance towards harmless antigens. Thus, several studies have examined the association between allergy and microbial exposure. However, to fully understand the underlying mechanisms in allergy development it is also important to study the association between the immunological maturation early in life and allergy or sensitization later in childhood.

6. AIM OF THE PRESENT STUDY

6.1 The aim of the thesis

The overall aim of the study was:

- To investigate if the infantile gut microbiota was related to Treg induction and the postnatal T cell development *in vitro* and *in vivo,* respectively.
- To study if the proportion of Tregs within the $CD4^+$ T cell population is associated with allergic sensitization and allergic disease in the first three years of life.

6.2 Questions for each paper

Paper I:
Are the gut commensals *Staphylococcus aureus* or *Lactobacilli paracasei* able to convert neonatal $CD4^+$ T cells into $FOXP3^+CD25^+CD127^{low}$ T cells *in vitro*?

Paper II:
Is the early bacterial gut colonization pattern associated with the capacity of T cells to produce cytokines and T cell memory conversion later in childhood?

Paper III:
Is the proportion of $FOXP3^+$ Tregs early in life associated with the proportion of activated or memory T cells later in childhood?

Paper IV:
Is the proportion of $FOXP3^+$ Tregs early in life associated with allergic sensitization or allergy later in childhood?

7. MATERIAL AND METHODS

The purpose of this section is to give an overview on material and methods used in the experimental study (paper I) and in the prospective FARMFLORA birth-cohort study (paper II-IV).

7.1 The experimental study (Paper I)

7.1.1 Study subjects

Cord blood samples were obtained from unselected healthy newborn infants born at term (≥38 gestational weeks) at the Sahlgrenska University Hospital. Peripheral blood was collected from healthy adult volunteers with no relation to the newborn children.

7.1.2 Cell cultures

In initial experiments, mononuclear cells from cord (CBMC) or adult peripheral blood (PBMC) were isolated by density gradient centrifugation. CBMC or PBMC were cultured with UV-killed *Staphylococcus aureus* (a strain able to produce staphylococcal enterotoxin C before being killed) or *Lactobacillus paracasei* for 3 days. The bacterial strains were originally isolated from stool samples from healthy Swedish infants.

Several groups have studied the induction of Tregs *in vitro* by either stimulating mononuclear cell cultures or depleting the pre-existing Tregs. Here we have set up a novel experimental model in which conventional $CD4^+$ T cells or pre-existing Tregs could be tracked and distinguished from induced Tregs. Thus, to be able to trace the conventional $CD4^+$ T cells, $CD4^+CD25^{+/neg}CD127^+$ T cells (non-Tregs), $CD4^+CD25^+CD127^{low}$ (Tregs) and remaining mononuclear cells were sorted using an iCyt Synergy™ cell sorter. The non-Tregs were stained with CellTrace Violet and co-cultured with autologous $CD4^+CD25^+CD127^{low}$ Tregs and remaining mononuclear cells. The cells were stimulated with $5x10^7$/ml of *S. aureus* or *L. paracasei* (Figure 4).

In cord blood the majority of $CD4^+$ T cells are of a naïve $CD45RA^+$ phenotype, while ~40% of $CD4^+$ T cells are $CD45RA^+$ in adults. Thus, to examine if *S. aureus* could convert naïve $CD4^+$ T cells from adults into $FOXP3^+CD25^+CD127^{low}$ T cells, naïve $CD4^+CD45RA^+CD25^{neg}CD127^+$ T cells were sorted and stained with CellTrace violet before stimulation with *S. aureus* in co-culture with autologous $CD4^+CD25^+CD127^{low}$ Tregs and remaining mononuclear cells.

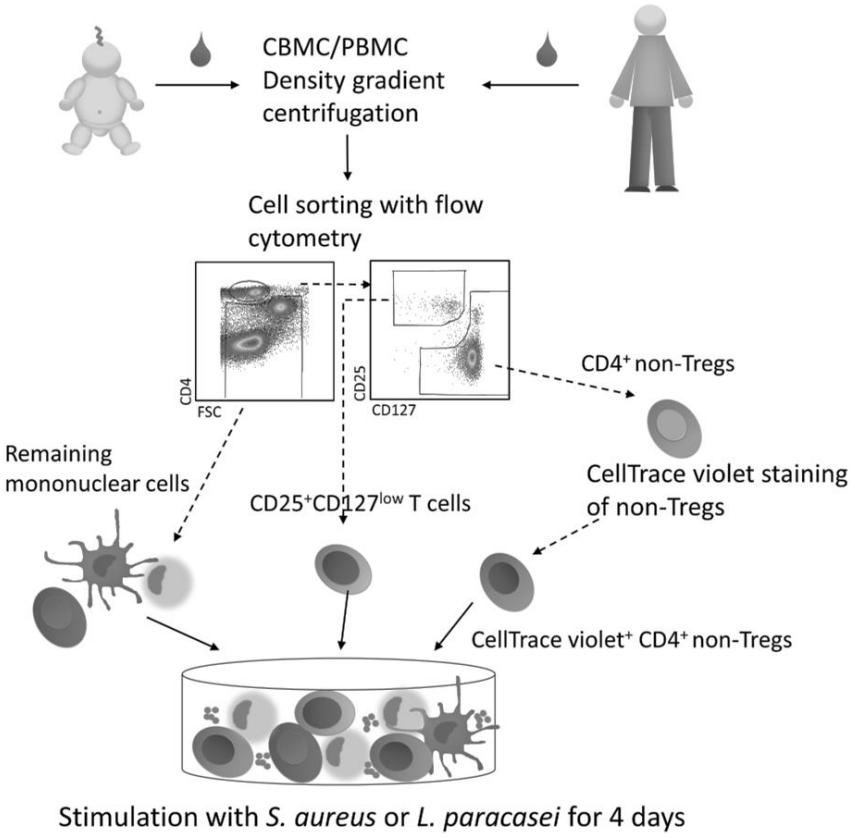

Figure 4. Tracing conventional CD4⁺ T cells during stimulation with *S. aureus*.
Mononuclear cells were isolated from cord or peripheral blood from adults. With the use of flow cytometry, $CD4^+CD25^{neg/+}CD127^+$ (non-Tregs), $CD4^+CD25^+CD127^{low}$ T cells (Tregs) and remaining mononuclear cells were sorted. Next, non-Tregs were stained with CellTrace violet before they were co-cultured with Tregs and the remaining mononuclear cells in the presence of *S. aureus* or *L. paracasei* for 4 days.

In PD-L1 blocking experiments, the remaining mononuclear cells were first stimulated with *S. aureus* in the presence or absence of PD-L1-antibodies or isotype control IgG_1 for 45 minutes before adding the autologous Tregs and CellTrace violet stained non-Tregs.

All cultures were performed in U-bottomed 96-well culture plates in RPMI 1640 media supplemented with 5% heat-inactivated autologous plasma, 1mM L-glutamine and 50µg/ml gentamycin for 3-4 days, kept in 5% CO_2 at 37°C.

7.1.3 Suppression assays

In all suppression assays autologous $CD4^+$ responder T cells ($CD4^+CD25^{neg}$) were isolated using Miltenyi's $CD4^+$ T cell Isolation Kit II and thereafter gradually cooled and stored at -70°C. At the time for use, the responder T cells were gradually thawn and stained with CellTrace violet.

To analyse whether *S. aureus*-induced $CD25^{+}CD127^{low}$ T cells were suppressive, $CD4^{+}CD25^{+/neg}CD127^{+}$ T cells (non-Tregs), $CD4^{+}CD25^{+}CD127^{low}$ (Tregs) and remaining mononuclear cells from cord blood were sorted. The pre-existing $CD4^{+}CD25^{+}CD127^{low}$ Tregs were discarded. Next, $CD4^{+}CD25^{+/neg}CD127^{+}$ non-Tregs were co-cultured with autologous remaining mononuclear cells, and stimulated with or without *S. aureus* in 24-well culture plates (Figure 5A). On day 3, $CD25^{+}CD127^{low}$ T cells were sorted from *S. aureus*-stimulated cultures. CellTrace violet stained responder T cells were co-cultured in a ratio of 1:2, 1:4 or 1:5 with the sorted $CD25^{+}CD127^{low}$ T cells, depending on how many $CD25^{+}CD127^{low}$ T cells that were collected during sorting.

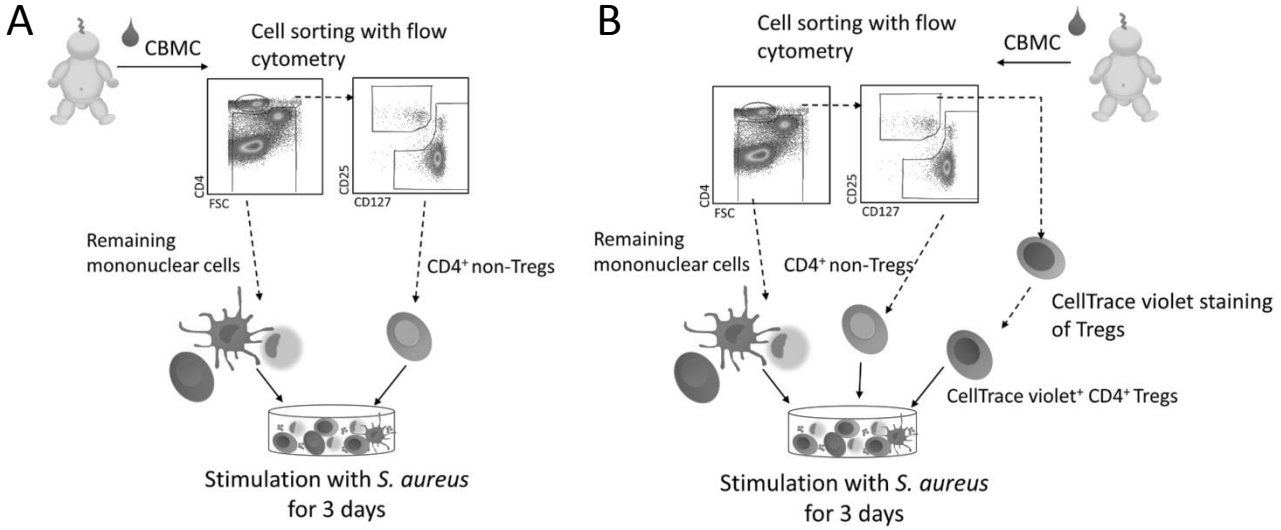

Figure 5. The bacterial stimulation phase for the described suppression assays.
(A) Sorted non-Tregs and remaining mononuclear cells were stimulated for 3 days with *S. aureus*. Pre-existing Tregs were discarded. (B) Sorted pre-existing Tregs were stained with CellTrace violet and co-cultured with autologous non-Tregs and remaining mononuclear cells and stimulation with *S. aureus* for 3 days.

We managed to perform one experiment in which pre-existing Tregs were included during the stimulation phase with *S. aureus*. In this experimental set up, non-Tregs, pre-existing Tregs and remaining mononuclear cells from cord blood were sorted. Next, pre-existing Tregs were stained with CellTrace violet before stimulation with *S. aureus* in co-culture with non-Tregs and remaining mononuclear cells (Figure 5B). On day 3, CellTrace $violet^{neg}$ $CD25^{+}CD127^{low}$ T cells (induced cells) were sorted and co-cultured with CellTrace violet stained $CD4^{+}$ responder T cells in a ratio of 1:20 for 5 days.

In both variants of suppression assays, the cells were cultured in the presence of plate-bound α-CD3 and soluble α-CD28 and cultured for 4-5 days in 5% CO_2 at 37°C in X-vivo 15 medium.

7.1.4 Flow cytometry

Flow cytometric analysis was performed on mononuclear cells that were prepared for sorting or on cells that had been cultured for 3-4 days in the presence or absence of commensal bacteria. The samples were analyzed on a FACSCanto II, equipped with FACSDiva software. The antibodies and fluorochromes used are shown in Table II. All flow cytometry data were analyzed by the use of Flow Jo software.

Table II. Monoclonal antibodies and fluorochromes used in paper I

Experiments	FITC	PE	PerCP	APC	Alexa fluor 647	APC-H7	Brilliant violet 421 /CellTrace violet
Paper I							
Cell sorting	anti-CD4				anti-CD127		anti-CD25
Cell sorting of naïve T cells	anti-CD45RA				anti-CD127	anti-CD4	anti-CD25
Apoptosis	Annexin V	anti-CD25			anti-CD127	anti-CD4	CellTrace violet
Induction experiments		anti-FOXP3		anti-CD25	anti-CD127	anti-CD4	CellTrace violet
Induction experiments PD-1		anti-PD-1		anti-CD25	anti-CD127	anti-CD4	CellTrace violet
PD-L1 on B cells				anti-PD-1		anti-CD20	anti-PD-L1
Suppression assays	anti-CD4			anti-CD25			

7.1.5 Univariate analysis

All univariate statistical analyses were performed with the use of GraphPad Prism program. As the data was generally not normally distributed non-parametric test were used. Thus, Mann-Whitney U test was used to compare two groups, whereas Kruskal-Wallis test followed by Dunn's multiple comparison test was used to compare three or more groups. Wilcoxon signed rank test was used to compare two paired groups. $P \leq 0.05$ was considered as significant (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$).

7.2 The FARMFLORA study (Papers II-IV)

7.2.1 Study subjects

The prospective FARMFLORA birth-cohort study included 65 children born in rural areas in South-West of Sweden. This cohort study was designed to examine the relation between the early bacterial colonization of the intestine, the maturation of the immune system and allergy development during early childhood. Twenty-eight children were raised on dairy farms, whereas 37 children lived on the countryside but not on farms. Blood samples were obtained from the umbilical cord at birth (≥38 gestational weeks) and from the peripheral blood at 3-5 days, 1, 4, 18 and 36 months of age (Figure 6).

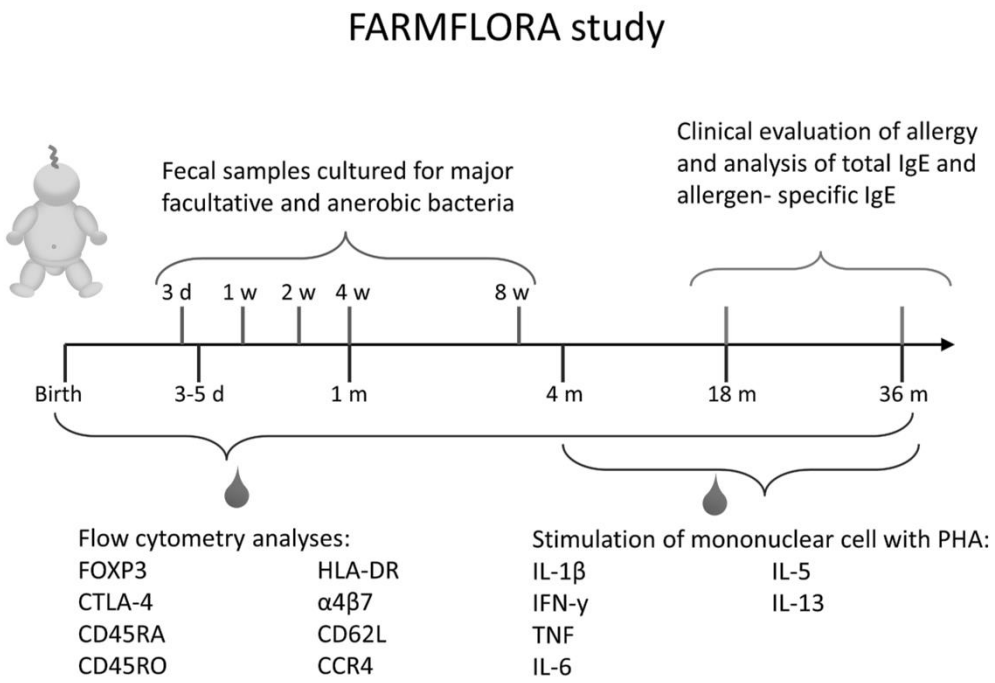

Figure 6. Overview of the sampling points and clinical evaluation of allergy in the prospective FARMFLORA study. Fecal samples were collected at 3 days, 1, 2, 4 and 8 weeks of life for analysis of the gut microbiota. Blood samples were obtained at birth, 3-5 days, 1, 4, 18 and 36 months of age for analysis of different immune parameters. At 18 and 36 months of age the children were examined for allergic disorders.

7.2.2 Flow cytometry

Flow cytometric analyses were performed on whole blood. The staff at the Clinical Immunology Laboratory of the Sahlgrenska University Hospital performed all stainings of the blood samples and analyzed the cells in a FACSCalibur equipped with CellQuestPro software. The flow cytometry panel in the FARMFLORA study is depicted in Table III. All flow cytometry data were analyzed by the use of Flow Jo software.

To set the gate for FOXP3$^+$ within the CD25^{high}CD4$^+$ T cell population, the lymphocyte population was first recognized using forward and side scatter. Next, CD4$^+$ lymphocytes were gated and thereafter gates for CD25^{neg}, CD25$^+$ and CD25^{high} (the top 2% of CD4$^+$ T cells that expressed CD25) were set within the CD4$^+$ T cell population. The FOXP3$^+$ T cell population was identified within the CD25^{high} T cells by comparing the expression of this marker within the CD4$^+$, CD25^{neg}, CD25$^+$, CD25^{high} T cell populations and the lack of expression in the isotype control, as shown in [24]. Finally, the proportion of FOXP3$^+$CD25^{high} cells within the CD4$^+$ T cell population was calculated by multiplying the proportion of CD25^{high} cells within the CD4$^+$ T cell population (~2%) with the percentage of FOXP3$^+$ in the CD25^{high}CD4$^+$ T cell population x 100. For example, 87% of FOXP3$^+$ cells within the CD25^{high}CD4$^+$ T cell population corresponds to 1.74% of FOXP3$^+$CD25^{high} cells within the CD4$^+$ T cell population calculated via the formula 0.02*0.87*100=1.74%.

Table III. Antibodies and fluorochromes used in papers II-IV

Analyses	FITC	PE	PerCP	APC
Paper II-IV				
CD45RA/CD45RO	anti-CD45RA	anti-CD45RO	anti-CD4	anti-CD25
α4β7	anti-CD49d	anti-β7	anti-CD4	anti-CD25
CD62L/CCR4	anti-CD62L	anti-CCR4	anti-CD4	anti-CD25
HLA-DR	anti-CD69	anti-CD62L	anti-CD4	anti-HLA-DR
FOXP3	anti-CD27	anti-FOXP3	anti-CD4	anti-CD25
CTLA-4	anti-CD27	anti-CTLA-4*	anti-CD4	anti-CD25

*anti-CTLA-4 was conjugated with biotin, followed by staining with PE-conjugated streptavidin.

7.2.3 Cytokine analysis

Flow cytomix was used to determine the concentrations of IL-1β, IL-6, TNF, IFN-γ, IL-5 and IL-13 in the supernatants after stimulation of mononuclear cells with phytohaemagglutinin (PHA), birch allergen extract or ovalbumin (OVA) at 4, 18 and 36 months of age. Flow Cytomix allows analyses of a range of cytokines in small sample volumes. To confirm the cytokine concentrations, the levels of IFN-γ and IL-13 were also determined by ELISA as described in detail previously [133].

7.2.4 Sampling and culture of the gut bacteria

Rectal swabs samples obtained at 3 days were cultured for aerobic bacteria and fecal samples obtained at 1, 2, 4 and 8 weeks of age were cultured quantitatively for major groups of aerobic and anaerobic bacteria. Table IV summarizes how the various bacteria and bacterial groups were identified.

Table IV. Methods used to identify the different bacterial species or groups of bacteria

Bacteria	Culture conditions	Culturing media	Microscopical appearance	Phenotypic identification	Genotypic identification
Staphylococcus aureus	aerobic 2 days	*Staphylococcus* agar	Gram-positive cocci	positive in coagulase test	
Escherichia coli	aerobic 2 days	Drigalski agar	Gram-negative rods	API20E	
enterobacteria other than *E. coli*	aerobic 2 days	Drigalski agar	Gram-negative rods	API20E	
enterococci	aerobic 2 days	Enterococcosel agar	Gram-positive cocci	Hydrolyze esculin on enterococcosel agar	
Bacteroides	anaerobic 3 days	*Bacteroides* Bile Esculin agar	Gram-negative rods	RAPID ID32A	
bifidobacteria	anaerobic 3 days	Beerens agar	Gram-positive rods		*Bifidobacterium*-specific PCR
lactobacilli	anaerobic 3 days	Rogosa agar	Gram-positive rods		Negative in *Bifidobacterium*-specific PCR. Positive in a *Lactobacillus*-specific PCR
clostridia	anaerobic 3 days	alcohol-treated and serially diluted feces cultured anaerobically on Brucella blood agar	Gram-positive or Gram-variables rods	RAPID ID 32A	
yeast	aerobic 2 days	Saboraud agar (supplemented with antibiotics)	Yeasts		

7.2.5 Clinical examination and laboratory tests for allergy diagnosis

The small size of the cohort made it possible for paediatricians to examine all children carefully at 18 and 36 months of age, as well as between follow-ups if the children developed symptoms suggestive of allergic disease. The criteria for clinical assessment of eczema, food allergy, asthma and allergic rhinoconjunctivitis are shown in Table V.

Table V. Criteria for clinical diagnosis of different allergic disorders and their prevalence at 18 and 36 months of age

Diagnosis	Criteria	18 months		36 months	
		n=64	(%)	*n*=63	(%)
Eczema	*Eczema*, diagnosed according to Williams' criteria [105]. Eczema at 18 months denoted diagnosis at any time before or at 18 months, while eczema at 36 months required symptoms to be present after 24 months of age	13	(20)	7	(11)
Food allergy	An immediate or late-onset reaction after ingestion of the specific food, followed by a clear and prompt clinical improvement once the food allergen was eliminated. Diagnosis was supported by an open food challenge tests, and/or a positive specific allergy test (specific IgE ≥ 0.35 KU/L or skin prick test wheal ≥3 mm), and/or eosinophilic inflammation in mucosal gastro-intestinal biopsies, and/or multi-organ reactions.	2	(3)	2	(3)
Asthma	Persistent wheezing for ≥4 weeks or ≥3 episodes of wheezing in the first 18 months of life in combination with other manifestations of allergy (eczema, rhinoconjunctivitis or food allergy) or with wheeze/breathing problems in between colds. For asthma at 36 months, ≥1 wheezing episode should have occurred after 24 months of age, and response to inhaled glucocorticoids or leukotriene antagonists was included among the minor criteria	5	(8)	4	(6)
Allergic rhino-conjunctivitis	Symptoms in the eyes and/or nose upon exposure to pollens or animal dander, together with a positive allergen-specific IgE test directed against a corresponding allergen.	1	(2)	1	(2)

Children who were allergic at 18 months of age were compared to those who were not allergic at 18 months of age, whereas children who were allergic at 36 months of age were compared with those who were not allergic at either 18 or 36 months of age. Eight children who were allergic at 18 but not at 36 months of age were excluded, as they could neither be included in the allergic nor in the non-allergic group. One child who did not undergo clinical examination at 18 months of age was also excluded from both the 18 and 36 month analyses.

Regarding sensitization, venous blood was obtained at 18 and 36 months of age for the analysis of total and allergen-specific IgE (Table VI). In children who were examined by the study paediatrician because of allergy symptoms before 18 months of age, allergen-specific IgE-tests or skin prick tests were performed guided by symptoms. Sensitized and non-sensitized children at the 18 and 36 months of age were compared in the same fashion as children diagnosed with allergy or not.

Table VI. Criteria for sensitization and prevalence at 18 and 36 months of age

	Criteria	Allergen-specific IgE at 18 months		Allergen-specific IgE at 36 months	
		n=62	(%)	*n*=56	(%)
Sensitization	Screening for allergen-specific IgE, i.e. 6-mix food test and Phadiatop, followed by analysis for specific IgE against cow's milk, hen's egg, fish, wheat, soy, peanut, birch, timothy, mugwort, dog, cat, horse and house dust mite. An allergen-specific IgE level of ≥0.35 kU/L was considered positive.	9	(15)	14	(25)

7.2.6 Multivariate factor analysis

Multivariate factor analysis (SIMCA-P+ software version 12 or 13) was used to study the relations between the various variables collected in the FARMFLORA study. Multivariate factor analysis reveals patterns that may help to find relations between the variables. If all data collected in the cohort were studied by univariate analyses mass significance would be created and relations between all the variables would be impossible to interpret.

The first analysis used is normally principal component analysis (PCA), which helps to get an overview of groupings and trends of the different variables. In **paper II**, PCA was used to examine the relationship between the early gut bacterial colonization pattern and the proportions of $CD45RO^+$, $FOXP3^+CD25^{high}$ or $CTLA\text{-}4^+CD25^+$ cells within the $CD4^+$ T cell population and PHA-induced cytokine response from mononuclear cells. In **paper III,** associations between $FOXP3^+$ or $CTLA\text{-}4^+$ cells within the $CD25^+CD4^+$ T cell populations and the $CD4^+$ T cells that expressed CD45RA, $\alpha_4\beta_7$, CD62L, CD45RO, HLA-DR and CCR4 was first analyzed with PCA. As shown in Figure 7, there is a negative association between X-variables that are projected on the opposite side of the Y-axis. A positive association is observed between X-variables that are projected on the same side of the Y-axis (Figure 7), which is stronger between X-variables that are projected in the same quadrant in the PCA loading plot.

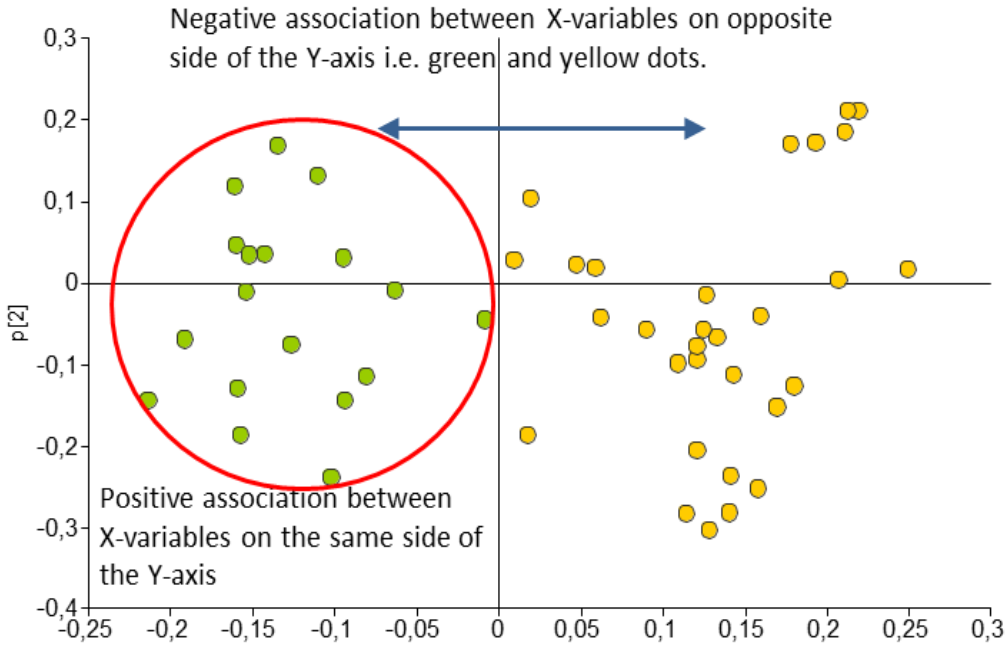

Figure 7. Principal component analysis
A pedagogical example of principal component analysis (PCA) loading plot that depicts the relations between the X-variables. X-variables that are projected on the same side of the Y-axis are positive associated to each other. A negative association is observed between X-variables that are projected on the opposite side of the Y-axis.

Orthogonal projection to latent structures by means of partial least squares (OPLS) was used to correlate the X and Y data matrices in **papers II and III**. With the use of OPLS, we were able to study how the different X-variables were related to a specific chosen Y-variable. For example, in paper II we studied the relationship between cytokine responses (Y-variables) and the bacteria colonizing the gut (X-variables). X-variables represented by bars pointing in the same direction as the Y-variable are positively associated, whereas X-variables pointing in the opposite direction are negatively associated with Y (Figure 8). The final OPLS loadings column plots in the papers are models based on X variables with variable influence of projection (VIP) values > 0.8. VIP-values were used to discriminate between important and unimportant predictors for the overall model, i.e. X-variables that contributed most to the OPLS model (Figure 8).

In **paper IV**, OPLS-discriminant analyses (OPLS-DA) were used to examine if classes of observations, i.e. sensitized compared to non-sensitized children, allergic compared to non-allergic children, and farmers' compared to non-farmers' children could be distinguished based on the associations between the X-variables, i.e. the different immune parameters.

The quality of the PCA, OPLS or OPLS-DA was assessed based on the parameters R2 and Q2, i.e. the percentages of the variation of the data set explained (R2) and predicted (Q2) by the model, respectively.

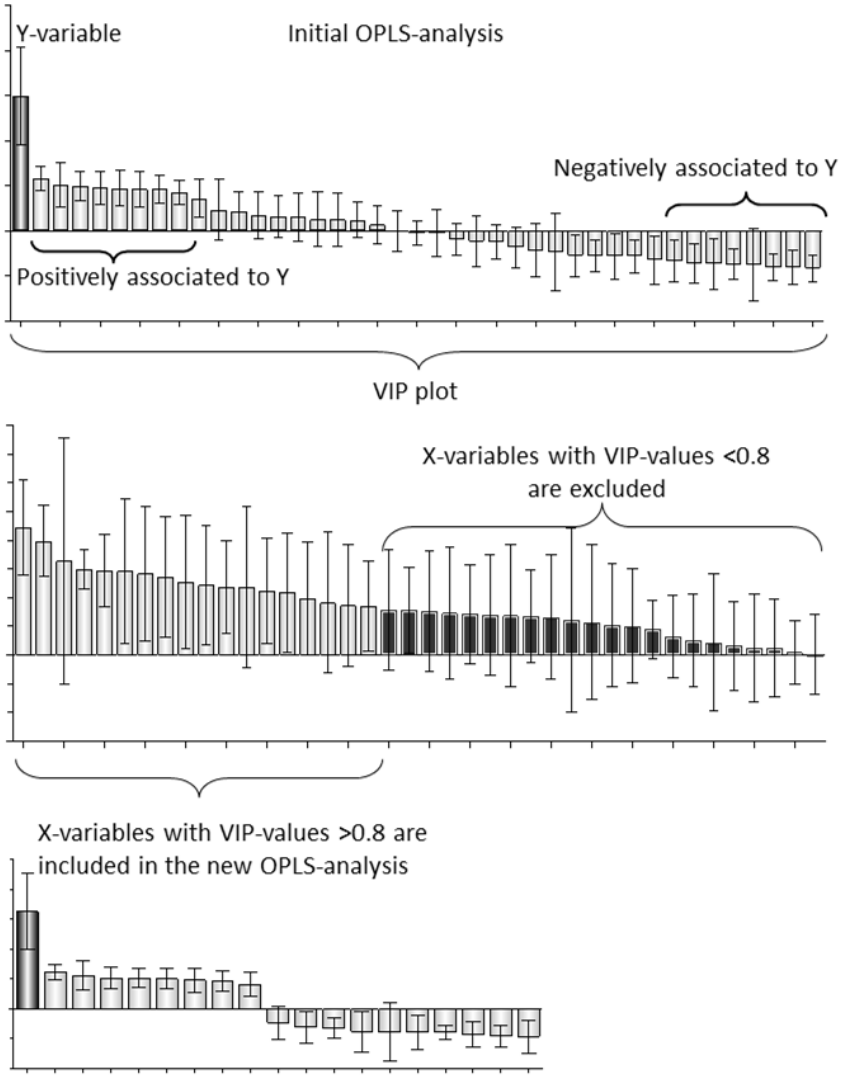

Figure 8. A pedagogical example for the use of variable influence of projection (VIP)-values.
In the initial OPLS-analysis not all of the X-variables included in the analysis will have an association to the chosen Y variable and thus they will not contribute to the overall model. These X-variables can be excluded with the use of variable influence of projection (VIP)-values. X-variables in the OPLS-analysis with a VIP-value below, e.g. 0.8 will be excluded from the model and a new OPLS-analysis will be created. The new analysis will have better R2 and Q2, as it lacks X-variables that do not contribute to the model, but rather introduced noise and disturbed the model.

7.2.7 Univariate analysis

Multivariate findings were corroborated by univariate analyses. To avoid mass significance, univariate analyses were performed exclusively on the X-variables that contributed most to the respective models. Spearman's rank correlation test was used in paper III, whereas Mann-Whitney U test was used in papers II-IV. $P \leq 0.05$ was considered as significant (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$).

8. RESULTS AND COMMENTS

8.1 Induction of regulatory T cells after stimulation of mononuclear cells with *Staphylococcus aureus* or *Lactobacillus paracasei* (Paper I)

In Swedish infants colonization of the intestine by *S. aureus* has become almost as common as colonization with *E. coli* in the first months of life. Infants harboring *S. aureus* during the first week(s) of life have been shown to have a lower risk of developing food allergy compared to non-colonized children [125]. Also, infants receiving oral supplementation with lactobacilli (*L. reuteri*) had a lower prevalence of IgE-mediated eczema at 2 years of age compared to the placebo-treated children [123]. As Tregs are thought to be involved in the prevention of allergic diseases, we hypothesized that stimulation with these two commensals would be able to induce Tregs in newborn infants. However, since it is not possible to monocolonize the gut of human infants *in vivo* with specific bacterial strains, we studied the effect of bacterial stimulation on $CD4^+$ T cells *in vitro*.

In initial experiments, the proportions of $CD25^+CD127^{low}$ T cells were examined after stimulating mononuclear cells from cord blood and peripheral blood from adults with *S. aureus* or *L. paracasei*. We found that stimulation with *S. aureus*, but not *L. paracasei*, increased the proportions of these cells in cultures from both newborns and adults (Figure 9A). However, it has been shown that conventional $CD4^+$ T cells from cord blood are more prone to undergo apoptosis compared to Tregs after primary stimulation *in vitro* [134], which could result in a false increase of Tregs. Therefore, we set up an experimental model in which we could track either conventional $CD4^+$ T cells or pre-existing $CD25^+CD127^{low}$ Tregs with the use of CellTrace violet during bacterial stimulation. In agreement with previous studies, we found that the proportions of apoptotic Annexin V^+CD4^+ T cells were higher in the non-Treg population compared to the CellTrace violet stained pre-existing Tregs in both *S. aureus*- and *L. paracasei*-stimulated cultures from newborns (Figure 9B). These results suggest that the increased proportion of $CD25^+CD127^{low}$ T cells after bacterial stimulation of mononuclear cells observed in Figure 9A could be due to apoptosis within the conventional $CD4^+$ T cell population.

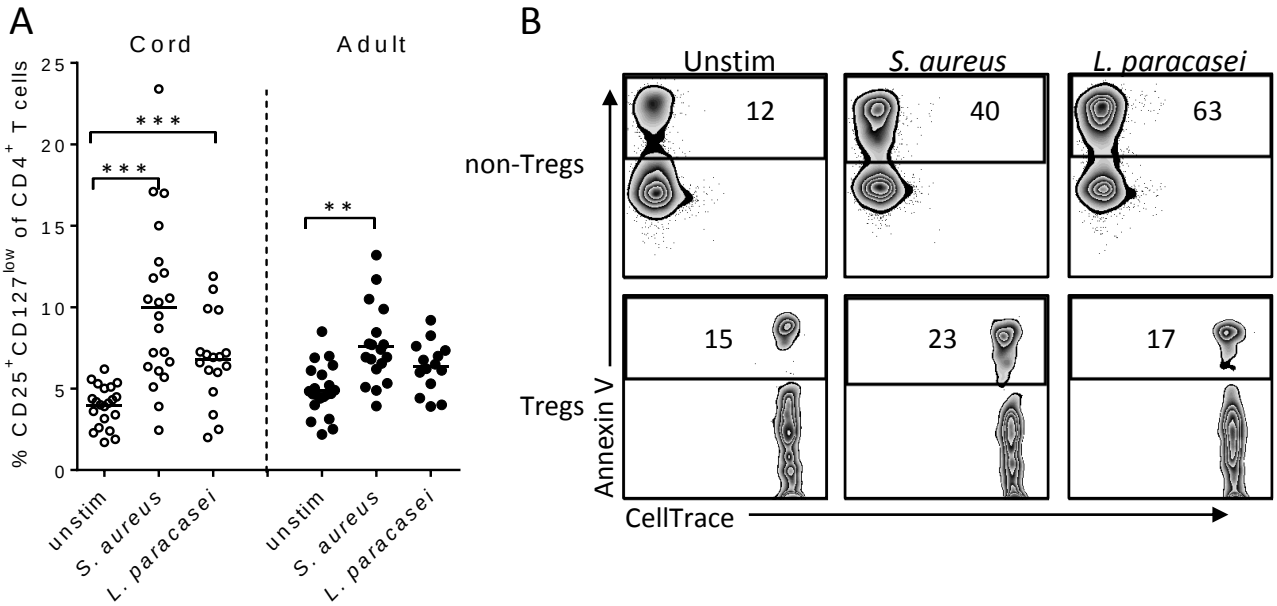

Figure 9. (A) The proportion of $CD25^{+}CD125^{low}$ T cells within the $CD4^{+}$ T cell population after *S. aureus*- or *L. paracasei*-stimulation of mononuclear cells from cord blood or peripheral blood from adults for 3 days. (B) The proportion of Annexin V^{+} cells within the pre-existing Tregs and conventional $CD4^{+}$ T cells (non-Tregs) after stimulation with *S. aureus* or *L. paracasei* for 3 days. ** $P \leq 0.01$ and *** $P \leq 0.005$ (Kruskal-Wallis test followed by Dunn's multiple comparison test)

Thus, to examine if bacterial stimulation indeed could generate $CD25^{+}CD127^{low}$ T cells, we next tracked the conventional $CD4^{+}$ T cells with CellTrace violet during stimulation. As shown in Figure 10A, stimulation with *S. aureus* resulted in *de novo* generation of $FOXP3^{+}CD25^{+}CD127^{low}$ T cells within the CellTrace $violet^{+}$ non-Treg population in cultures from children but not adults. Stimulation with *L. paracasei* did not induce $FOXP3^{+}CD25^{+}CD127^{low}$ T cells to the same extent as stimulation with *S. aureus*. Others have shown that stimulation with *L. acidophilus* is able to induce functional $FOXP3^{+}CD25^{+}$ Tregs in cell cultures with cells from adults [135]. However, it was also shown that different strains of lactobacilli differed in their ability to induce Tregs [135], which might explain why we only observed a moderate induction of $CD25^{+}CD127^{low}$ T cells after stimulation with *L. paracasei*.

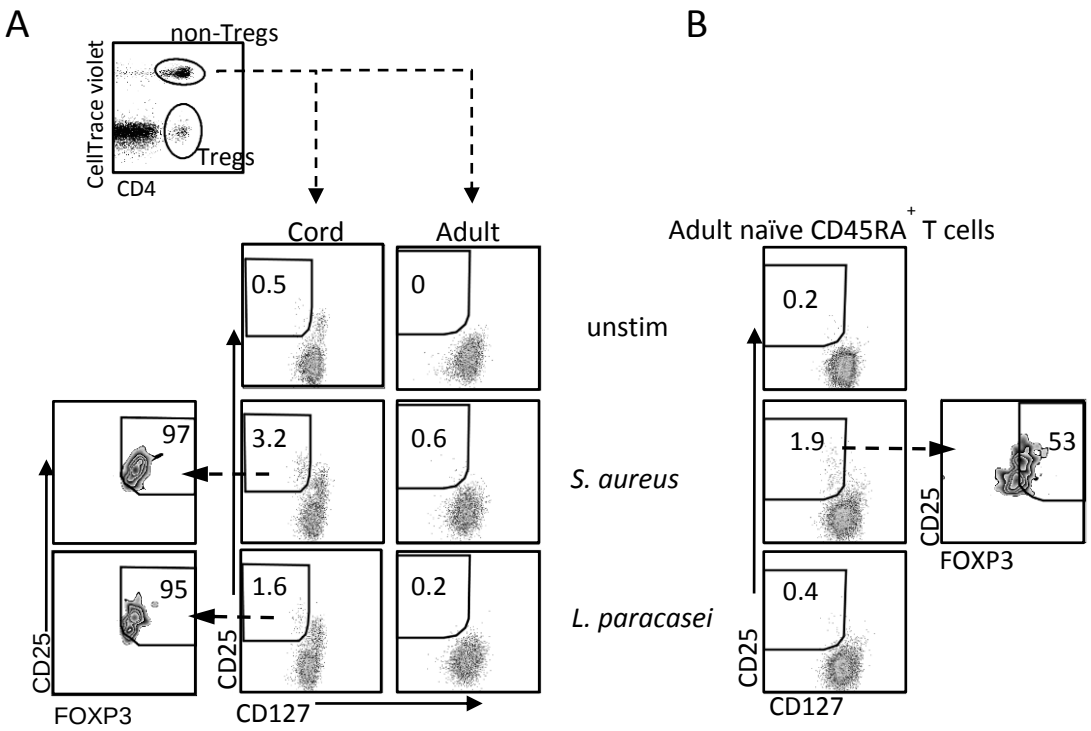

Figure 10. (A) The proportion of induced CD25$^+$CD127^{low} T cells within the CellTrace violet$^+$ non-Treg population and the expression of FOXP3 within the induced CD25$^+$CD127^{low} T cells after bacterial stimulation of CellTrace violet stained non-Tregs, pre-existing Tregs and remaining mononuclear cells from cord or peripheral blood from adults for 4 days. (B) The proportion of induced CD25$^+$CD127^{low} T cells within the CellTrace violet$^+$ T cell population and the expression of FOXP3 within the induced CD25$^+$CD127^{low} T cells after bacterial stimulation of CellTrace violet stained naïve CD45RA$^+$ T cells, pre-existing Tregs and remaining mononuclear cells from peripheral blood from adults for 4 days.

As newborn children have a higher proportion of naïve T cells than adults, the lack of *de novo* generation of FOXP3$^+$CD25$^+$CD127^{low} T cell in cultures from adults could be due to a lower fraction of naïve T cells. To examine if this was true, we sorted CellTrace violet stained-naïve CD45RA$^+$ T cells from adults that were co-cultured with autologous pre-existing Tregs and remaining PBMC and stimulated with *S. aureus* or *L. paracasei* for 4 days. Interestingly, stimulation with *S. aureus*, but not *L. paracasei*, did convert naïve adult CD45RA$^+$ T cells into CD25$^+$CD127^{low} T cells (Figure 10B). However, induced CD25$^+$CD127^{low} T cells from adults expressed FOXP3 to a lower extent (53% and 42% in two experiments, respectively) than those induced in newborns (median 84%). These results suggest that it is the naïve T cells that differentiate into CD25$^+$CD127^{low} T cells, but that the induction of FOXP3$^+$ putative Tregs occur primarily after stimulation of neonatal T cells.

8.1.1 The suppressive function of the *S. aureus*-induced CD25^{+}CD127^{low} T cells

It has been shown that conventional CD4^{+} T cells upregulate CD25 as well as FOXP3 and downregulate CD127 upon stimulation. Thus, to elucidate whether the *de novo* generated CD25^{+}CD127^{low} T cells were functional Tregs, we tested if they were able to suppress proliferation of CD4^{+}CD25^{neg} responder T cells. However, the low induction of CD25^{+}CD127^{low} T cells after stimulation with *L. paracasei* made it impossible to sort sufficient numbers of cells to perform suppression assays.

In initial suppression experiments, non-Tregs and remaining mononuclear cells from cord blood were stimulated with *S. aureus* in the absence of pre-existing Tregs (Figure 5A in material and methods). The induced CD25^{+}CD127^{low} T cells were thereafter sorted and co-cultured with CellTrace violet-stained CD4^{+} responder cells that were stimulated with anti-CD3 and anti-CD28. We found that *S. aureus*-induced CD25^{+}CD127^{low} T cells from these experiments were not able to suppress the CD4^{+} responder cells, but rather increased their proliferation (52% vs. 24%) (Figure 11A).

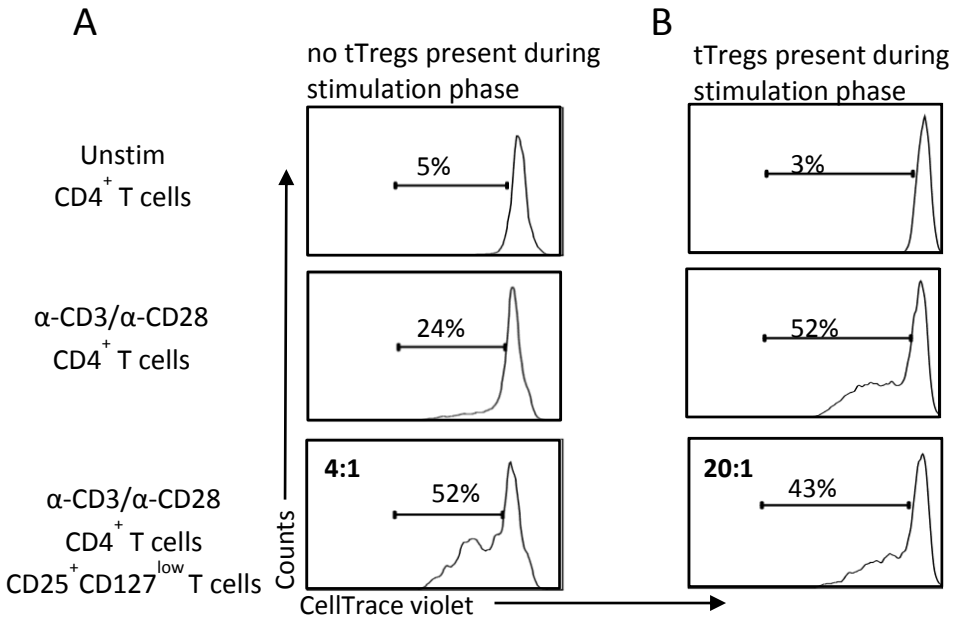

Figure 11. (A-B) The suppressive function of *S. aureus* induced CD25^{+}CD127^{low} T cells that were induced in the (A) absence (n=3) or (B) presence of pre-existing Tregs (tTregs).

These results prompted us to ask if pre-existing Tregs might be needed for the generation of induced Tregs. To test this theory, pre-existing Tregs needed to be CellTrace violet-stained before co-culture with non-Tregs and remaining mononuclear cells and *S. aureus* (Figure 5B in material and methods). It is hard to obtain the required numbers of induced CD25^{+}CD127^{low} T cells from human cord blood to perform such a suppression assay. We have therefore only been able to execute one such experiment. We found that *de novo* generated CD25^{+}CD127^{low} T cells in the presence of pre-existing Tregs were indeed

suppressive (43% vs. 52%), despite the fact that the responder cells and the induced $CD25^+CD27^{low}$ T cell were cultured in a ratio of 20 to 1 (Figure 11B). Taken together, these results suggest that *S. aureus* possess an ability to convert conventional $CD4^+$ T cells into functional $FOXP3^+CD25^+CD127^{low}$ Tregs, and that the conversion into functional suppressor cells requires the presence of pre-existing Tregs.

8.1.2 The involvement of PD-1 and PD-L1 in the conversion of conventional $CD4^+$ T cells into $CD25^+CD127^{low}$ T cells after stimulation with *S. aureus*

The expression of the inhibitory molecule programmed cell death ligand 1 (PD-L1) on APCs has been linked to their ability to inhibit T cell activation [136, 137] and to induce $FOXP3^+$ Tregs [61]. To investigate if the PD-1/PD-L1 axis was involved in the induction of $CD25^+CD127^{low}$ T cells, the PD-L1 expression on APCs, i.e. B cells, was studied after *S. aureus*-stimulation. *S. aureus* increased the proportion of $PD\text{-}L1^+$ B cells in cultures from both newborn children and adults, but to a higher extent in neonatal B cells (Figure 12A). Furthermore, lymphocytes other than $CD20^+$ B cells did not upregulate PD-L1 to the same extent (Figure 12B). These results suggest that APCs may upregulate PD-L1 after stimulation with *S. aureus*. Accordingly, it has previously been shown that monocytes in blood from adults upregulate PD-L1 after stimulation with *S. aureus* [138]. Unfortunately, we were not able to study the expression of PD-L1 on monocytes (in the mixed cell cultures) after bacterial stimulation as they down-regulated their specific identification markers and became highly autofluorescent. This made it impossible to properly gate the monocyte population and subsequently PD-L1 on these cells.

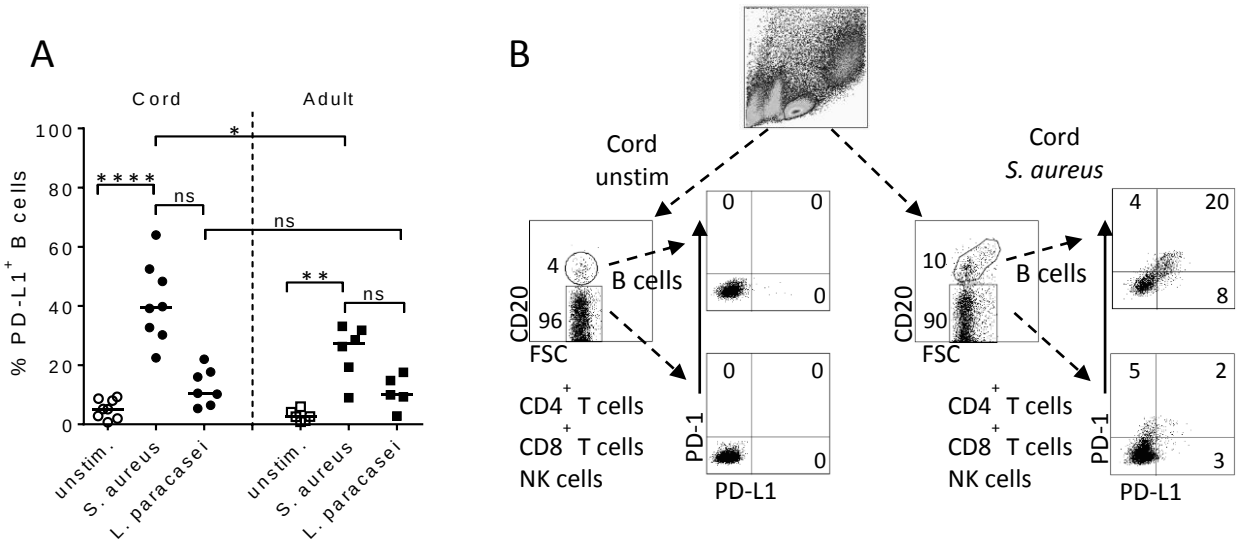

Figure 12. (A) Proportion of $PD\text{-}L1^+$ B cells after stimulation of mononuclear cells from cord or peripheral blood from adults with *S. aureus* or *L. paracasei* for 3 days. (B) The expression of PD-L1 on neonatal B cells and remaining lymphocytes in unstimulated and *S. aureus* stimulated cultures. * $P \leq 0.05$, ** $P \leq 0.01$ and **** $P \leq 0.0001$ (Kruskal-Wallis test followed by Dunn's multiple comparison test)

Next, we blocked PD-L1 with the use of antibodies during stimulation with *S. aureus*. As shown in Figure 13, we found a reduced conversion of conventional CD4+ T cells into CD25+CD127low cells when PD-L1 was blocked.

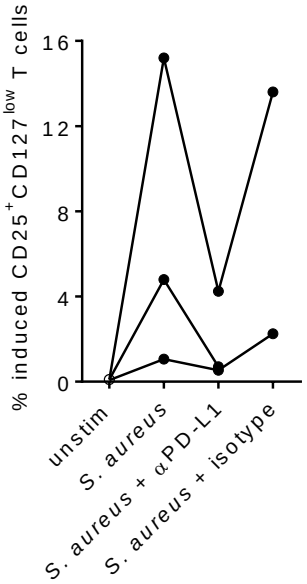

Figure 13. The proportion of induced CD25+CD127low T cells whitin the CellTrace violet+ non-Treg population after stimulation with *S. aureus* in the absence or prescence of anti-PD-L1 antibodies or isotype control for 4 days.

Thus, conversion of conventional CD4+ T cells into CD25+CD127low T cells after stimulation with *S. aureus* seems to occur via the PD-1/PD-L1 axis. As this axis has been shown to be involved in the induction of regulatory T cells by others [60-62], our findings further suggest that the *de novo* generated FOXP3+CD25+CD127low T cells in response to *S. aureus* stimulation are Tregs rather than activated T cells.

8.2 The development of memory T cells and cytokine responses during childhood (Papers II and III)

During early childhood the proportions of peripheral CD45RO+ memory CD4+ T cells as well as the cytokine-producing capacity by lymphocytes increase in an age dependent manner [139]. In the FARMFLORA cohort study, we had the unique opportunity to study the T cell development and effector function during early childhood and factors that may be associated with this development. In accordance with previous studies, we found that both the proportion of CD45RO+ memory cells within the CD4+ T cell population and the capacity of mononuclear cells to produce cytokines in response to PHA-stimulation increased with age (Table VII).

We also examined the proportion of putative FOXP3+CD25high Tregs, i.e. the CD4+ T cell subset that express the highest level of CD25 and FOXP3, during the first 3 years of life [24]. However, since several studies define human Tregs as FOXP3+CD25+ we also analyzed the proportion of these cells. CTLA-4+CD25+ was also analyzed within CD4+ T cell population as CTLA-4 is highly expressed by Tregs as well as by activated T cells and it has been

shown to be a immunoregulatory molecule in both T cell subsets. As shown in Table VII, the proportions of both FOXP3$^+$CD25^{high} and FOXP3$^+$CD25$^+$ cells increased during the first days of life, while the fraction of CTLA-4$^+$CD25$^+$ cells within the CD4$^+$ T cell population did not differ during the first 3 years of life.

Table VII. The proportions of CD45RO$^+$, FOXP3$^+$ and CTLA-4$^+$ T cells within the CD4$^+$ T cell population and the cytokine levels in the supernatants of PHA-stimulated mononuclear cells from birth up to 3 years of age

	Marker/ cytokine		**Cord**	**3-5 days**	**1 month**	**4 months**	**18 months**	**36 months**
Memory T cells	CD45RO	%	3	4	7	7	12	17
Proinflammatory	IL-1β	pg/ml				2	18	93
	IL-6	pg/ml				106	39	128
	TNF	pg/ml				130	245	942
Th1-related	IFN-γ	pg/ml				1	42	133
Th2-related	IL-5	pg/ml				0	36	55
	IL-13	pg/ml				24	106	175
FOXP3$^+$CD25^{high} T cells		%	1	2	2	2	1	1
FOXP3$^+$CD25$^+$ T cells		%	3	5	6	5	5	5
CTLA-4$^+$CD25$^+$ T cells		%	5	4	5	5	4	4

All numbers denote median values

We next wanted to elucidate if the proportion of memory T cells (Y-variable) was associated with cytokine responses after stimulation with PHA (X-variables). In OPLS analyses, we found that the bars representing PHA-induced TNF, IL-13, IL-5 and IFN-γ production were positioned in the same direction as the bar representing the proportion of CD45RO$^+$ cells within the CD4$^+$ T cell population (Figure 14A-B), which demonstrates a positive association between these variables. Univariate analysis confirmed that children with higher proportions of CD45RO$^+$CD4$^+$ T cells at 4 and 36 months of age had mononuclear cells with higher capacity to produce TNF, IL-13, IL-5 and IFN-γ at 18 months and IL-13 and IL-5 at 36 months of age, respectively (denoted with asterisks in the OPLS plots, Figure 14A-B). Thus, infants with higher proportions of memory T cells also have higher capacity to produce both proinflammatory as well as Th2-related cytokines in response to mitogen stimulation than children with lower proportion of memory T cells. There are several factors that might contribute to the variation in the T cell development between children. Early bacterial colonization of the intestine might be such a factor.

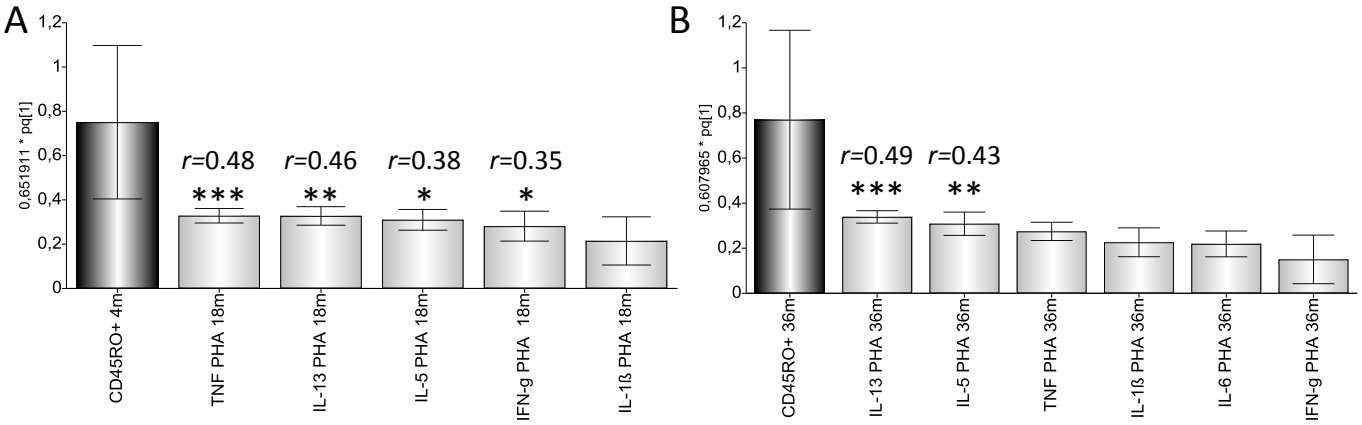

Figure 14. (A-B) Orthogonal projection to latent structures by means of partial squares (OPLS) column loading plots that depicts the association between the proportion of CD45RO$^+$ cells within the CD4$^+$ T cell population at (A) 4 or (B) 36 months of age (Y-variables) and the cytokine responses after PHA stimulation of mononuclear cells at (A) 18 and (B) 36 months of age. Statistical significant differences between Y- and X-variables are denoted with asterisks in the OPLS column plots. *$P\leq 0.05$, **$P\leq 0.01$ and ***$P\leq 0.005$ (Spearman's rank correlation test)

8.3 The gut microbiota and T cell development during childhood (Paper II)

During the last 40 years the gut colonization pattern has changed in the western world as colonization by *E. coli* and *Bacteroides* is delayed and instead *S. aureus* is one of the first colonizers of the intestine [89]. As germ-free animal models have demonstrated that the colonization of the gut is a strong stimulus for the developing immune system [96, 97], we here wanted to examine if the bacterial colonization of the human gut early in life could influence postnatal T cell development. To study this, PCA analysis was performed to get an overview of how the early gut bacterial colonization pattern was associated with the proportion of CD4$^+$ T cells that were memory CD45RO$^+$ cells, putative FOXP3$^+$CD25^{high} Tregs or CTLA-4$^+$CD25$^+$ cells. We also studied the association of the gut flora with the mononuclear cytokine responses after stimulation with PHA at different ages.

Variables that represent colonization by *E. coli*, bifidobacteria and *Bacteroides* were projected on the right side of the Y-axis (orange dots) together with variables representing PHA-induced cytokine responses by mononuclear cells and proportions of CD45RO$^+$CD4$^+$ T cells at all time points examined (yellow dots, Figure 15), which suggests a positive association between these variables. In contrast, the variables representing colonization by enterococci, enterobacteria other than *E. coli*, *S. aureus* and clostridia were projected on the left side of the Y-axis (green dots, Figure 15) and were therefore inversely related to PHA-induced cytokine responses and memory T cells. Yeasts and lactobacilli (green dots) together with the proportions of FOXP3$^+$CD25^{high} and CTLA-4$^+$CD25$^+$ T cells within the CD4$^+$ T cell population were mainly projected close to origo at all time points (blue dots, Figure 15),

indicating that these X-variables are not associated to the early gut bacterial colonization pattern.

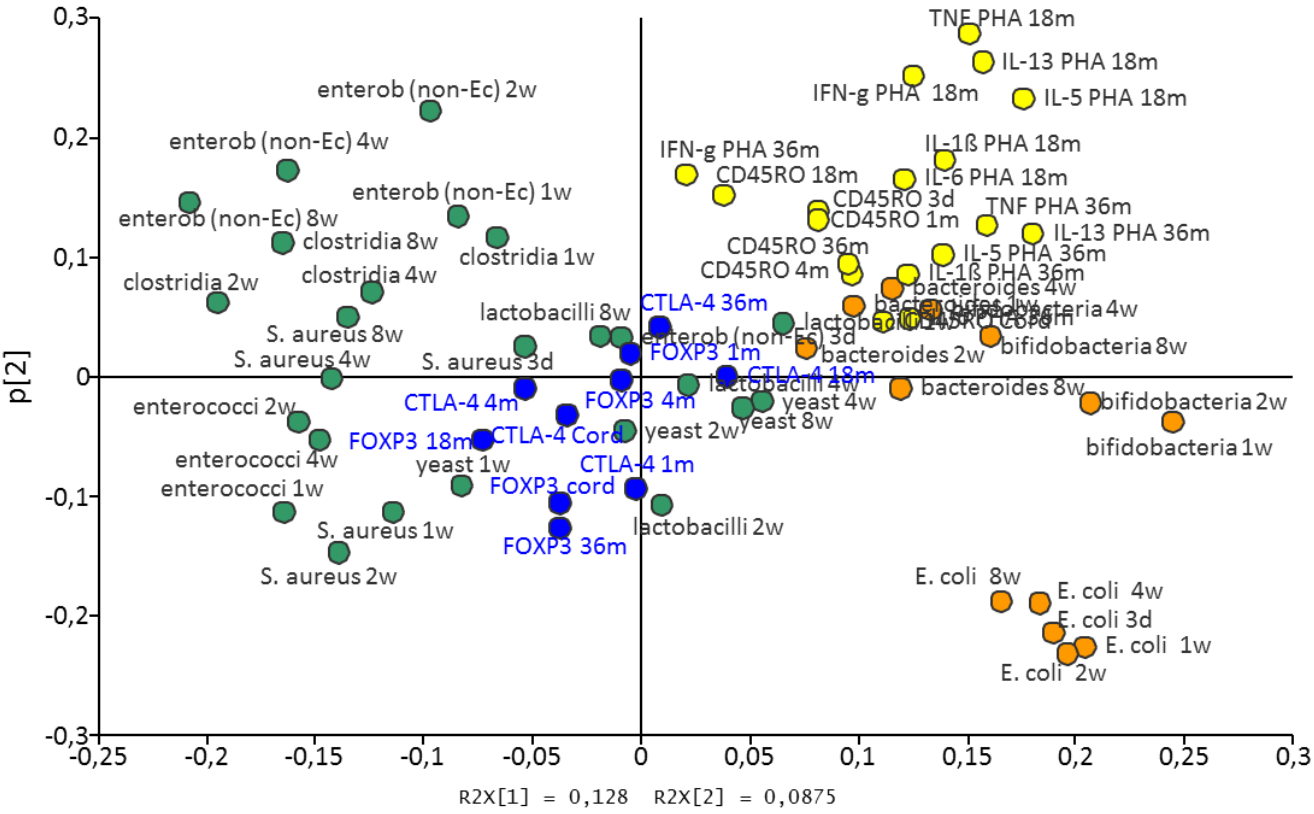

Figure 15. Principal component analysis score scatter plot that depicts the association between gut colonization by various bacteria in the first 8 weeks of life and PHA-induced cytokine responses at 18 and 36 months of age, as well as the proportions of FOXP3^{+}CD25^{high}, CTLA-4^{+}CD25^{+} or CD45RO^{+} of the CD4^{+} T cell population at birth, 1, 4, 18 and 36 months of age.

OPLS analyses verified that there were no association between the proportions of CD4^{+} T cells that were FOXP3^{+}CD25^{high} or CTLA-4^{+}CD25^{+} (Y-variables) and the early gut microbiota (X-variables) (data not shown). Thus, higher capacity to produce cytokines in response to mitogen stimulation and higher proportion of memory T cells were positively associated with early colonization by *E. coli*, bifidobacteria and *Bacteroides*, but inversely related to colonization by enterobacteria other than *E. coli, S. aureus*, enterococci and clostridia. The proportions of FOXP3^{+}CD25^{high} and CTLA-4^{+}CD25^{+} T cells, on the other hand, were not associated with the early gut bacterial colonization pattern.

8.3.1 The early gut bacterial colonization pattern and mononuclear cytokine responses upon mitogen stimulation during childhood

To study how specific cytokine responses were related with the early bacterial colonization of the intestine, OPLS analyses were performed. The score scatter plot in Figure 16 depicts the relation between the Y-variables; PHA-induced TNF and IL-13 responses by

mononuclear cells at 36 months of age (Y-axis and X-axis, respectively) and the different bacteria that colonize the gut (X-variables). Early colonization by bifidobacteria and *E. coli* was positively associated with both TNF and IL-13 responses, as they were projected in the upper right quadrant in the score scatter plot (Figure 16). Colonization by *S. aureus*, enterococci, yeasts or clostridia was inversely related to TNF and IL-13 responses as they were projected in the lower left quadrant in the score scatter plot (Figure 16). X-variables projected close to origo were unrelated to both TNF and IL-13 responses after PHA stimulation (Figure 16).

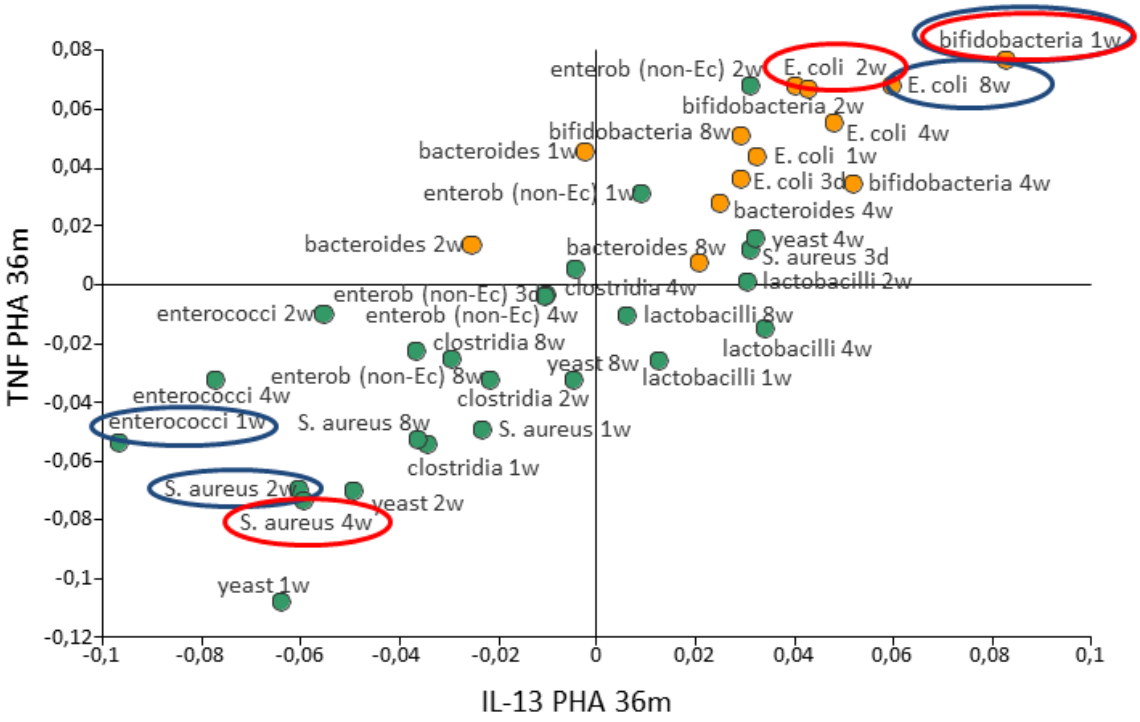

Figure 16. OPLS score scatter plot that depicts the association between the Y-variables, i.e. TNF and IL-13 (Y-axis and X-axis, respectively), and bacteria colonizing the gut at different time points (X-variables). Red circles depict statistical significant differences (Mann-Whitney U test) between the TNF responses from colonized or non-colonized children, whereas blue circles depict statistical differences between the IL-13 responses in colonized or non-colonized children.

Univariate analyses confirmed the associations observed in the OPLS analysis. Infants colonized with bifidobacteria at 1 and 2 weeks, or *E. coli* at 2 and 8 weeks of age had mononuclear cells with higher capacity to produce TNF as well as IL-13 upon PHA stimulation at 36 months of age, compared to non-colonized children (Figure 17). In contrast, children colonized by *S. aureus* at 2 or 4 weeks of life displayed lower PHA-induced TNF or IL-13 responses than children not colonized by these bacteria (p=0.04 and p=0.05, respectively). Furthermore, children colonized by enterococci by 1 week of age had mononuclear cells with lower capacity to produce IL-13 in response to PHA stimulation than non-colonized children (p=0.007). Similar association patterns were observed

regarding IL-1β, IL-6 and IL-5 responses and the early gut bacterial colonization (as shown in paper II).

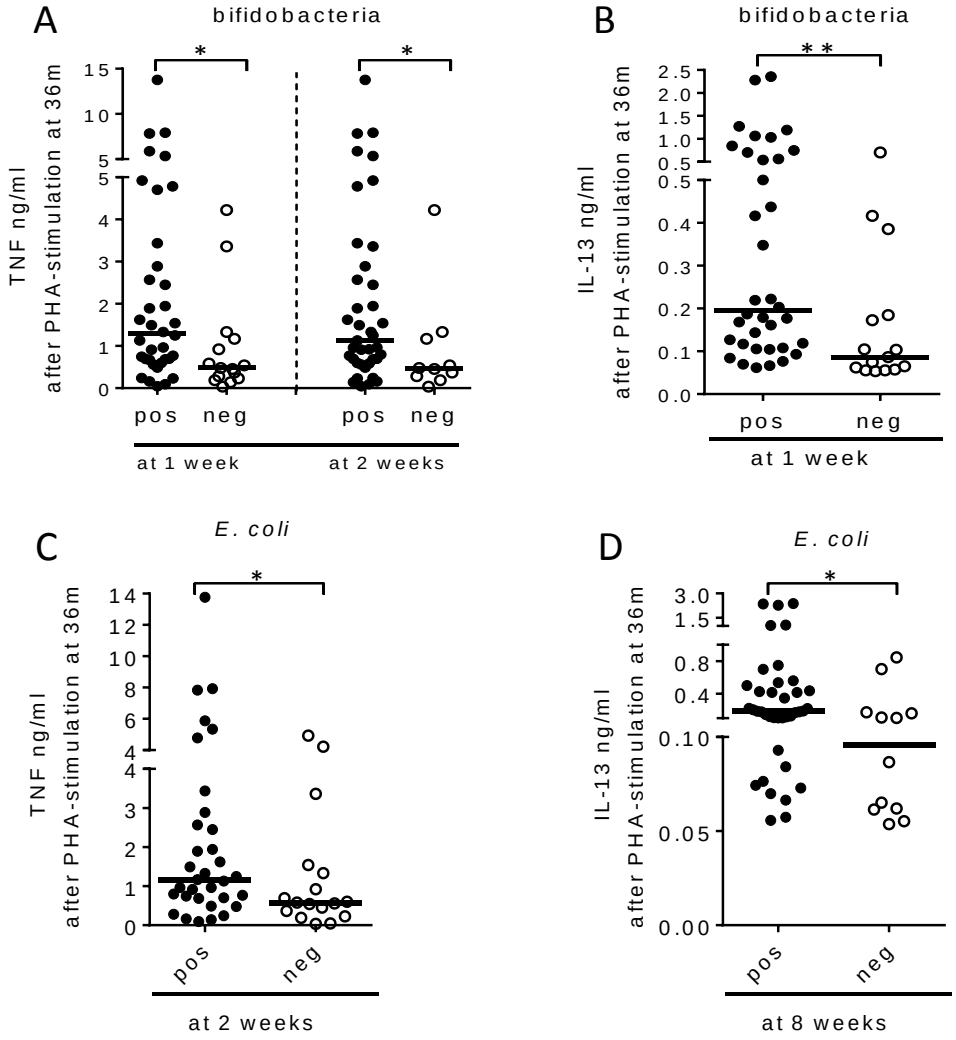

Figure 17. The levels of PHA-induced (A and C) TNF or (B and D) IL-13 by mononuclear cells at 36 months of age in children colonized or not by (A-B) bifidobacteria or (C-D) *E. coli* at 1, 2 or 8 weeks of life. Each dot represents an individual and horizontal bars indicate the median **P*≤ 0.05 and ***P*≤ 0.01 (Mann-Whitney U test)

Similar association patterns were also observed in OPLS analyses comparing the proportion of $CD45RO^+$ $CD4^+$ T cells at 1, 4 and 36 months of age and the gut microbiota (paper II). We found no relation between the early colonization pattern of the gut and the production of IFN-γ in response to PHA (data not shown). Moreover, neither delivery mode (vaginal or caesarean section), having siblings nor being raised on a farm were confounding factors for the observed associations (paper II).

Thus, infants colonized by bifidobacteria or *E. coli* early in life have mononuclear cells with higher capacity to produce both proinflammatory and Th2-related cytokines in response to PHA stimulation later in childhood. In contrast, children colonized by a gut microbiota including *S. aureus* or enterococci have mononuclear cells with lower capacity to produce these cytokines.

8.4 Farming environment and T cell development during childhood (Paper IV)

There is a lower prevalence of allergy among farmers' compared to non-farmers' children [118]. Indeed, in the FARMFLORA study we found that children who lived on farms had a significantly lower prevalence of allergy compared to non-farmers' children (Figure 18).

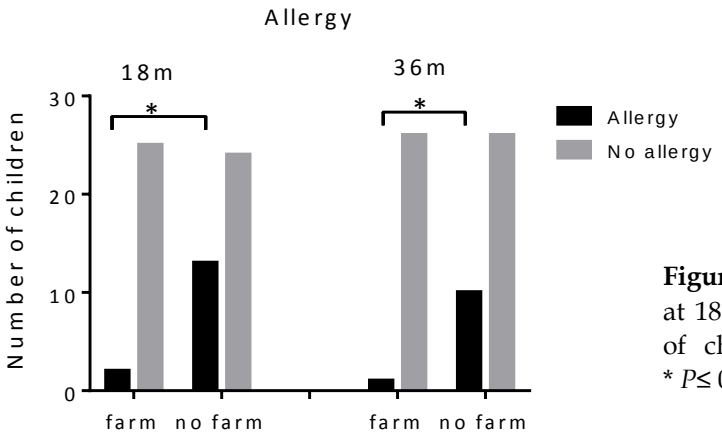

Figure 18. (A) The number of allergic children at 18 and 36 months of age within the group of children living on dairy farms or not. * $P \leq 0.05$ (Fisher's exact test)

The lower prevalence of allergic children among farmers' children might be due to environmental factors that stimulates the immune system. To test this hypothesis, we examined the development of T cells during the first 3 years of life in children living on a dairy farm or not. Thus, growing up on a farm or not (Y-variables) was related to the proportions of $CD4^+$ T cells that were $\alpha4\beta7^+$, HLA-DR^+, $CCR4^+$, $CD45RO^+$, $FOXP3^+CD25^{high}$ and CTLA-4^+CD25^+, as well as to the capacity of mononuclear cells to produce cytokines in response to PHA-, OVA- or birch allergen stimulation (X-variables).

As shown in the OPLS plot in Figure 19, there was a positive association between living on a farm (Y-axis) and higher proportions of $CD4^+$ T cells that were $CCR4^+$ or HLA-DR^+ at 18 months and $CD45RO^+$ at 1, 4 and 36 months as well as higher PHA-induced IL-1β, and IFN-γ levels at 18 months of age (projected in the upper left quadrant). In contrast, higher proportions of $FOXP3^+CD25^{high}$ of $CD4^+$ T cells at 3 days and 36 months of age were inversely related with living on a farm (projected on the lower right quadrant in Figure 19).

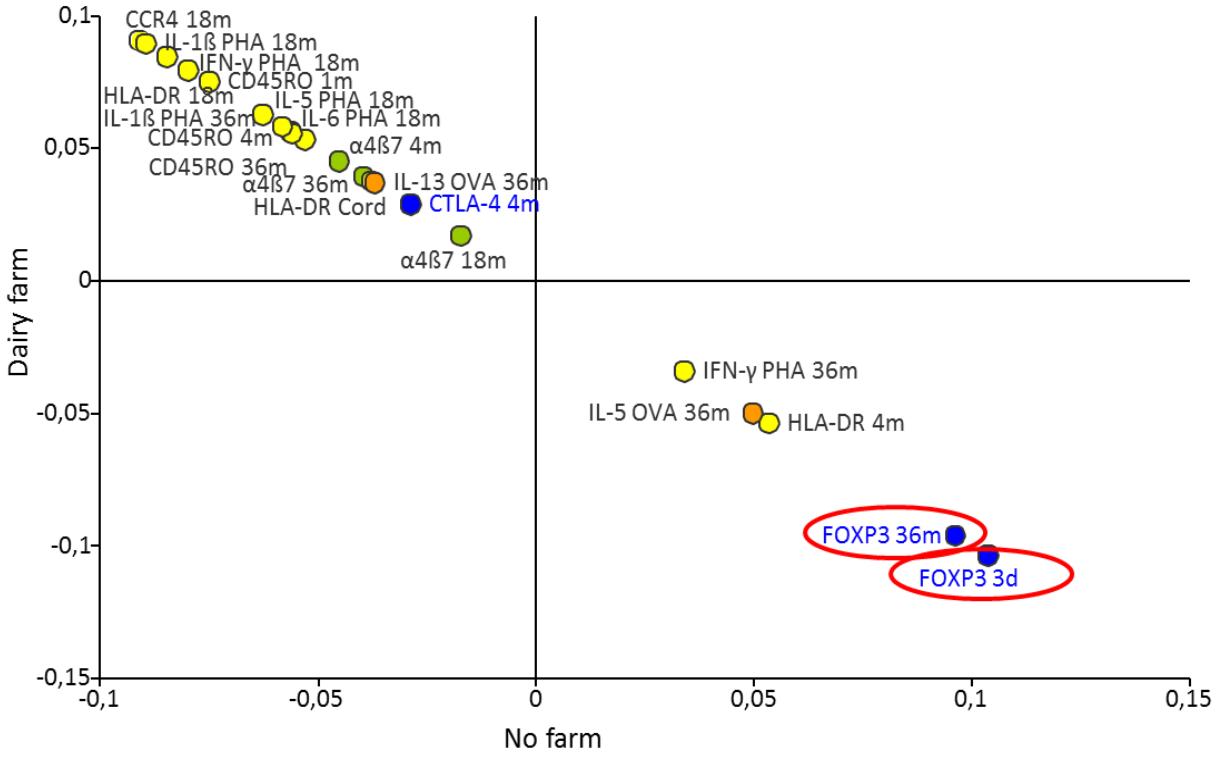

Figure 19. OPLS score scatter plot that depicts the association between the Y-variables, i.e. living on a dairy farm or not (Y-axis and X- axis, respectively), and the proportion of $CD4^+$ T cells that were $\alpha4\beta7^+$, $HLA\text{-}DR^+$, $CCR4^+$, $CD45RO^+$, $FOXP3^+CD25^{high}$ and $CTLA\text{-}4^+CD25^+$ as well as the capacity of mononuclear cells to produce cytokines in response to PHA-, OVA- or birch allergen-stimulation (X- variables). The red circles depict statistical significant differences (Mann-Whitney U test) between the X-variables in children raised on farms or not.

Univariate analyses confirmed that infants who lived on a farm had lower proportions of $FOXP3^+CD25^{high}$ cells within the $CD4^+$ T cell population at 3 days and 36 months of age than children not raised on a farm (p=0.007 and p=0.04, respectively). However, none of the other associations observed in the OPLS analysis were statistically significant in the univariate analyses (data not shown). Furthermore, the proportion of $FOXP3^+CD25^+$ cells within the $CD4^+$ T cell population at 1 month of age, but no other time point, was also significantly lower in children who lived on farms compared to those that did not live on farms (p=0.05). When we excluded children with allergy in the OPLS analysis and univariate analysis there was still a lower proportion of $FOXP3^+CD25^{high}$ cells within the $CD4^+$ T cell population at 3 days and 36 months of age in farmers' compared to non-farmers' children. Thus, allergy was not a confounding factor for the observed associations.

Taken together, our results imply that there is a difference in the T cell development among children raised on dairy farms compared to children who lived in the same geographic area, but not on a farm. In specific, farmers' children had lower proportions of putative $FOXP3^{+}CD25^{high}$ Tregs early in infancy than non-farmers' children.

8.5 Allergy and T cell development (Paper IV)

According to the hygiene hypothesis, the immune system needs to be exposed to bacteria and viruses to develop properly [117]. Reduced and/or altered microbial stimulation may result in poor maturation of the child's immune system, failure in the immune tolerance and as a consequence of this allergy development. $FOXP3^{+}$ Tregs are also thought to play an important role in the protection against allergic disorders, as children with a dysfunctional *foxp3* gene develop severe dermatitis, high levels of IgE and sometimes eosinophilia [40]. Thus, we here questioned if allergic and non-allergic children would differ in T cell development and effector function during childhood.

OPLS analyses were used to examine the relation between being allergic at 18 and/or 36 months of age (Y-variables) and the proportions of $CD4^{+}$ T cells that were $\alpha4\beta7^{+}$, $HLA\text{-}DR^{+}$, $CCR4^{+}$, $CD45RO^{+}$, $FOXP3^{+}CD25^{high}$ or $CTLA\text{-}4^{+}CD25^{+}$, as well as the capacity of mononuclear cells to produce cytokines in response to PHA-, OVA- or birch allergen stimulation (X-variables).

As shown in the OPLS plot, we found a positive association between being allergic at both 18 and 36 months of age and a higher capacity of mononuclear cells to produce the Th2-related cytokine IL-5 after PHA-stimulation (upper right quadrant in Figure 20). An allergy diagnosis at both 18 and 36 months was also positively associated with higher proportions of $CD4^{+}$ T cells that were $FOXP3^{+}CD25^{high}$ at 36 months, $HLA\text{-}DR^{+}$ or $CCR4^{+}$ at 4 months of age (Figure 20). In contrast, a higher capacity to produce IFN-γ in response to birch allergen and higher proportions of $CD4^{+}$ T cells that were $CTLA\text{-}4^{+}CD25^{+}$ at 36 months and $\alpha4\beta7^{+}$ at 4 months of age were inversely related to a clinical diagnosis of allergy at both ages (lower left quadrant in Figure 20).

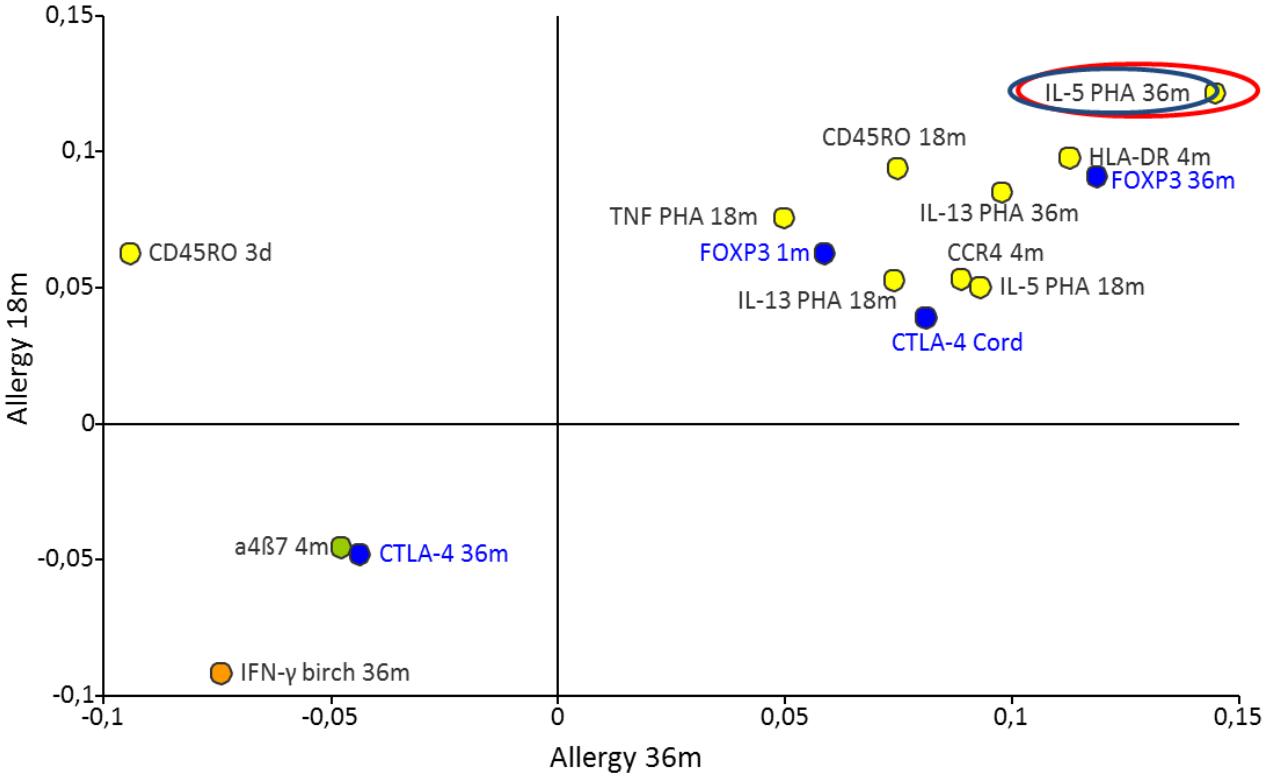

Figure 20. OPLS score scatter plot that depicts the association between the Y-variables, i.e. allergy at 18 and 36 months of age (X-axis and Y-axis, respectively), and the proportion of $CD4^+$ T cells that were $\alpha4\beta7^+$, HLA-DR^+, $CCR4^+$, $CD45RO^+$, $FOXP3^+CD25^{high}$ and $CTLA\text{-}4^+CD25^+$, as well as the capacity of mononuclear cells to produce cytokines in response to PHA-, OVA- or birch allergen-stimulation (X-variables). Statistical significant differences (Mann-Whitney U test) between the X-variables in allergic children at 18 or 36 months compared to non-allergic children are depicted with blue and red circles, respectively.

Univariate analysis confirmed that children with allergy at 18 or 36 months of age had mononuclear cells with significantly higher capacity to produce IL-5 in response to PHA at 36 months of age (Figure 21). None of the other associations observed in the OPLS analyses (Figure 20), were statistical significantly in univariate analyses (data not shown). Moreover, children with allergy at 36 months, but not 18 months of age, also had significantly higher proportions of $CD4^+$ cells that were $FOXP3^+CD25^+$ at 18 months of age than non-allergic children (p=0.03). Excluding children raised on farms from the OPLS analysis did not alter the associations observed between immune parameters and allergy (paper IV). Thus, a farming environment *per se* was not likely to be a confounding factor for these findings.

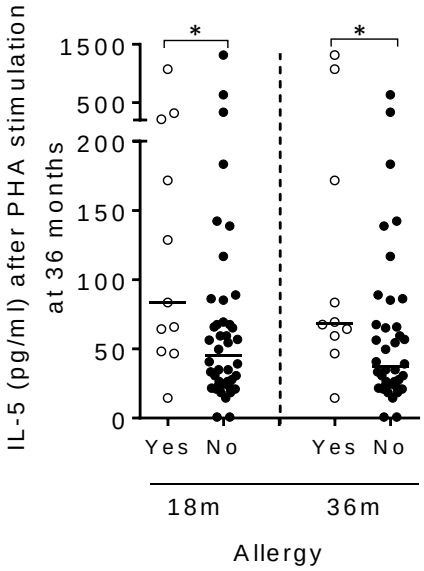

Figure 21. The levels of IL-5 after PHA-stimulation of mononuclear cells at 36 months in children with or without an allergy diagnosis at 36 months of age. Each dot represents an individual and horizontal bars indicate the median *$P \leq 0.05$ (Mann-Whitney U test)

Taken together, our results suggest that allergic children at 18 and 36 months of age have mononuclear cells with higher capacity to produce IL-5 after polyclonal stimulation than non-allergic children. Since IL-5 is a Th2-related cytokine and allergy is considered to be a Th2 driven disorder, the higher capacity of mononuclear cells from allergic children to produce this cytokine in response to PHA is not surprising. Although, allergy was not associated with the proportions of putative $FOXP3^{+}CD25^{high}$ Tregs, allergy was positively association with the proportions of $FOXP3^{+}CD25^{+}$ cells within the $CD4^{+}$ T cell population right before onset of allergic disorder. This might be a result of an increase of activated T cells within the $FOXP3^{+}CD25^{+}$ T cell subset in response to the allergic inflammation.

8.6 Allergic sensitization and T cell development (Paper IV)

Allergic sensitization occurs when an individual develops IgE antibodies towards allergens. Although not all individuals with allergic symptoms are sensitized, children are more prone to develop symptoms of wheezing, asthma and rhinitis at 5 years if they are sensitized to any allergen at 18 months of age [114]. Thus, we next wanted to elucidate if there was a difference in the T cell development between sensitized and non-sensitized children. The association between sensitization at 18 or 36 months of age and the T cell variables described above were compared with the use of OPLS analyses.

Interestingly, there was a positive association between sensitization at both 18 and 36 months of age and higher proportions of $FOXP3^{+}CD25^{high}$ cells within the $CD4^{+}$ T cell population at birth and 3 days of life (upper right quadrant in Figure 22). In contrast, sensitization at both ages was inversely related to higher proportions of $CD4^{+}$ T cells that were $\alpha4\beta7^{+}$ at birth or $CCR4^{+}$ at 36 months of age (lower left quadrant in Figure 22). Also, a higher capacity of mononuclear cells to produce IFN-γ, IL-6, TNF or IL-β after PHA-stimulation at 36 months was inversely related to sensitization at 18 and 36 months of age (lower left quadrant in Figure 22).

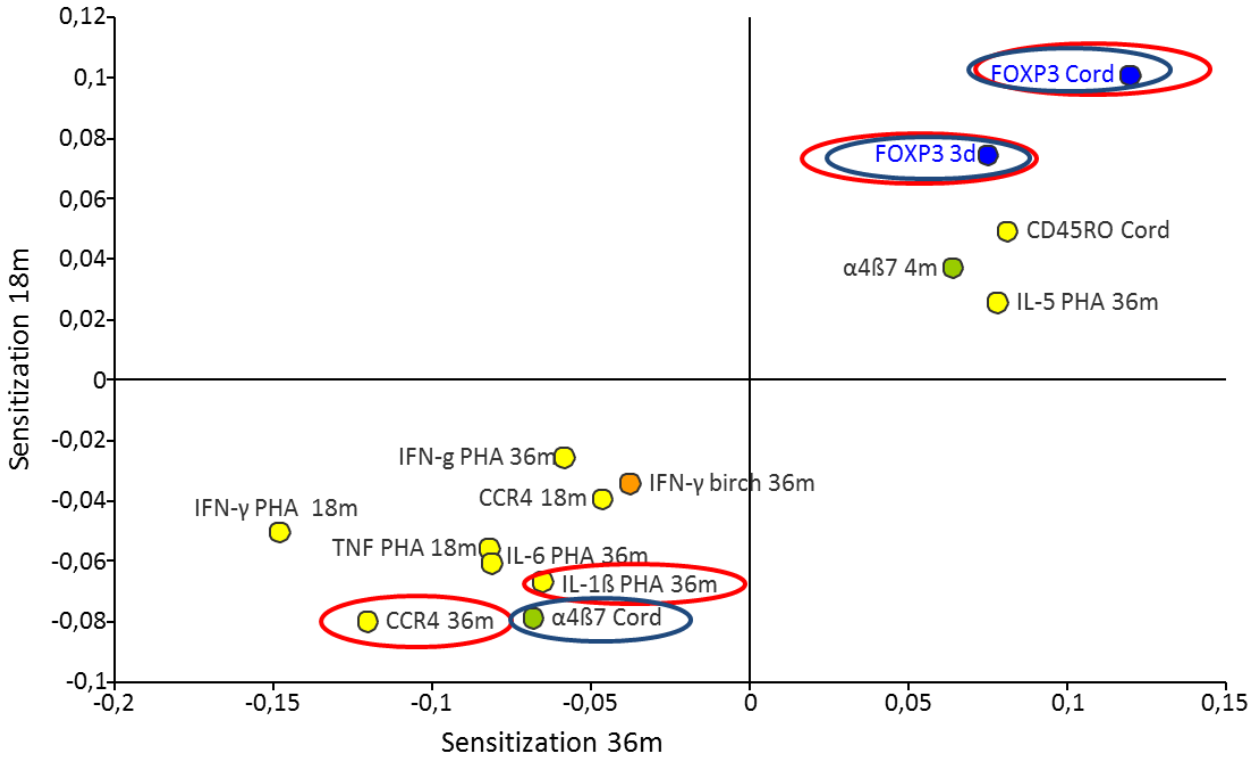

Figure 22. OPLS score scatter plot that depicts the association between the Y-variables, i.e. sensitization at 18 and 36 months of age (Y-axis and X-axis, respectively), and the proportion of CD4$^+$ T cells that were α4β7$^+$, HLA-DR$^+$, CCR4$^+$, CD45RO$^+$, FOXP3$^+$CD25^{high} and CTLA-4$^+$CD25$^+$ as well as the capacity of mononuclear cells to produce cytokines in response to PHA-, OVA- or birch allergen-stimulation (X-variables). Statistical significant differences (Mann-Whitney U test) between the X-variables in allergic children at 18 or 36 months compared to non-allergic children are depicted with blue and red circles, respectively.

These, results were verified by univariate analyses, which showed that children who were sensitized at 18 and 36 months of age had higher proportions of FOXP3$^+$CD25^{high} cells within the CD4$^+$ T cell population at birth and 3 days of age (Figure 23A and B). We also found that children who were sensitized at 18 months of age had lower proportions of CD4$^+$ T cells that were α4β7$^+$ at birth than non-sensitized children (p=0.02). Sensitized children at 36 months had lower proportions of CCR4$^+$ cells within the CD4$^+$ T cell population and mononuclear cells with lower capacity to produce IL-1β in response to PHA at 36 months of age compared to non-sensitized children (p=0.04 and p=0.05, respectively). Regarding FOXP3$^+$CD25$^+$ T cells, we found that sensitized children at 18 months, but not 36 months of age, had higher proportion of these cells within the CD4$^+$ T cell population at birth than non-sensitized children (p=0.0005).

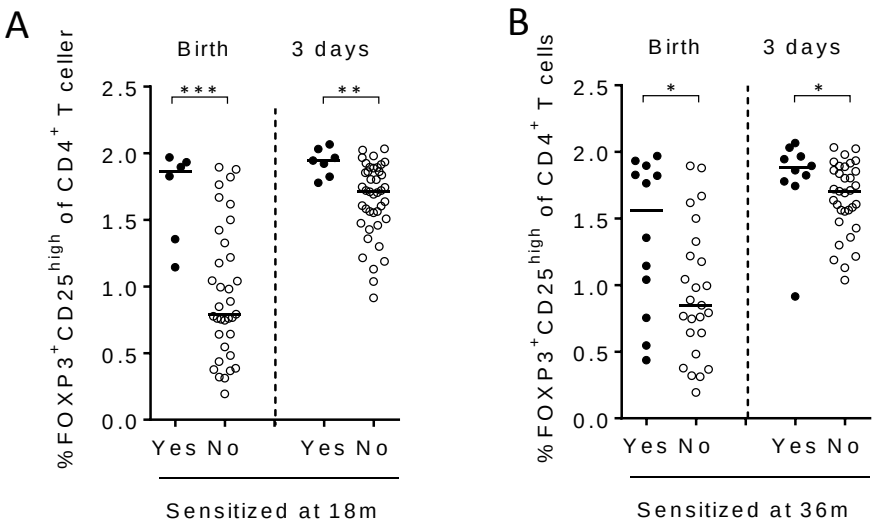

Figure 23. The the proportions of CD4+ T cells that were FOXP3+CD25high at birth and 3 days of life in children that were sensitized or not at (A) 18 and (B) 36 months of age. Each dot represents an individual and horizontal bars indicate the median *$P \leq 0.05$, **$P \leq 0.01$ or ***$P \leq 0.005$ (Mann-Whitney U test)

Thus, a higher proportion of FOXP3+CD25high T cells within the CD4+ T cell population in early infancy does not seem to prevent atopic disorders, but is rather associated with sensitization later in childhood. Furthermore, as the expression of CCR4 on CD4+ T cells is linked to the conversion of naïve T cells into CD45RO+ memory cells [24], these results also points to that sensitized children had lower proportions of activated T cells compared to children who remained non-sensitized.

8.7 The relation between Tregs and T cell memory conversion and cytokine responses (Papers II and III)

As we found that high proportions of putative FOXP3+CD25high Tregs early in life was associated with both sensitization and a non-farming environment, we wanted to elucidate if high proportions of T cells with regulatory function early in life might affect the postnatal T cell development. Thus, OPLS analyses were performed to study the relation between the proportion of FOXP3+CD25high, FOXP3+CD25+ or CTLA-4+CD25+ cells within the CD4+ T cell population (Y-variables) and the proportion of CD45RA+, α4β7+, CD62L+, CD45RO+, HLA-DR+ and CCR4+ cells within the CD4+ T cell population and the cytokine responses by mononuclear cells after stimulation with PHA at 18 and 36 months of age (X-variables).

We found that higher proportions of FOXP3+CD25high or CTLA-4+CD25+ cells within the CD4+ T cell population at birth were positively associated to higher fractions of naïve CD45RA+ T cells, α4β7+ and CD62L+ T cells within the CD4+ T cell population, but inversely related to the proportions of CD45RO+ memory T cells and PHA-induced cytokine responses later in childhood (Figure 24A-B, respectively). Similar multivariate

association patterns were observed for FOXP3^{+}CD25^{high} and CTLA-4^{+}CD25^{+}, as well as FOXP3^{+}CD25^{+} cells within the CD4^{+} T cell population and memory T cell conversion and cytokine production at all time points examined (data not shown). These results suggest that a higher proportion of FOXP3^{+}CD25^{high}, FOXP3^{+}CD25^{+} or CTLA-4^{+}CD25^{+} cells within the CD4^{+} T cell population early in infancy may impede the T cell memory conversion and consequently the effector cell function.

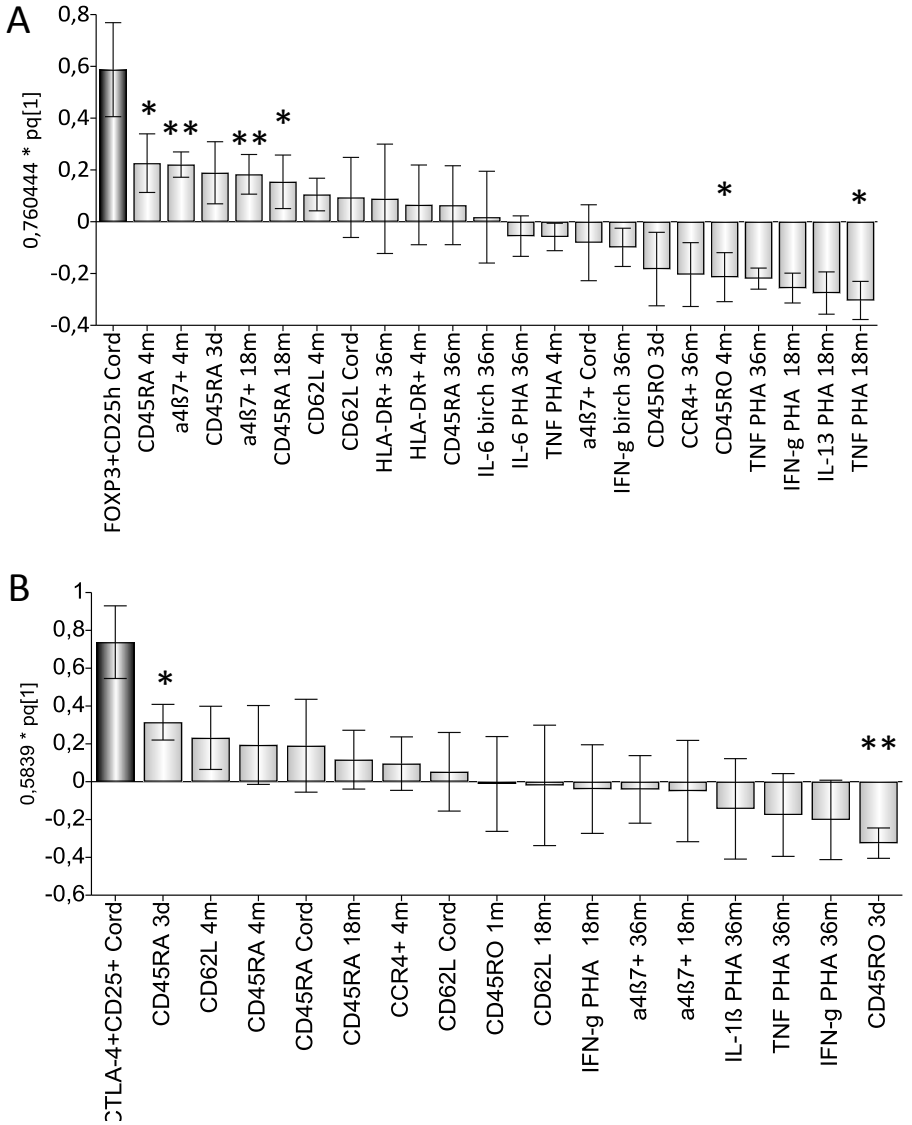

Figure 24. (A-B) Orthogonal projection to latent structures by means of partial least squares (OPLS) column loading plots that depict the association between the proportion of (A) FOXP3^{+}CD25^{high} and (B) CTLA-4^{+}CD25^{+} cells within the CD4^{+} T cell population at birth (Y-variables) and the proportion of CD4^{+} T cells that express CD45RA, α4β7, CD62L, CD45RO, HLA-DR or CCR4 during the first 36 months of life as well as the cytokine responses by mononuclear cells after PHA stimulation at 18 and 36 months of age (X-variables). Statistical significant differences between Y- and X-variables are denoted with asterisks in the OPLS column plots. * $P \leq 0.05$ and ** $P \leq 0.01$ (Spearman's rank correlation test).

8.8 The relationship between FOXP3⁺CD25⁺ T cells and CTLA-4⁺CD25⁺ T cells during the three first years of life (Paper III)

CTLA-4 is highly expressed by FOXP3⁺ Tregs, but also by newly activated T cells. There are few studies that have analyzed the intracellular expression of FOXP3 and CTLA-4 in the same cells. In the FARMFLORA study FOXP3 and CTLA-4 were intracellularly stained in separate tubes and could therefore not be studied in the same cells. We found that the proportion of FOXP3⁺CD25^{high} or FOXP3⁺CD25⁺ and CTLA-4⁺CD25⁺ cells within the CD4⁺ T cell population displayed different association patterns with respect to allergy and sensitization. Thus, we questioned whether FOXP3 and CTLA-4 were expressed by the same cells and therefore correlated the CD4⁺ T cells that were FOXP3⁺CD25⁺ to those that were CTLA-4⁺CD25⁺ at birth, 3 days, 1, 4, 18 and 36 months of age.

As shown in figure 25, we found that the proportions of FOXP3⁺ and CTLA-4⁺ T cells correlated at 1 and 4 months of age, but at no other time point examined (figure 25). Moreover, there were no correlations between FOXP3⁺CD25^{high} and CTLA-4⁺CD25^{high} cells within the CD4⁺ T cell population during the three first years of life (data not shown).

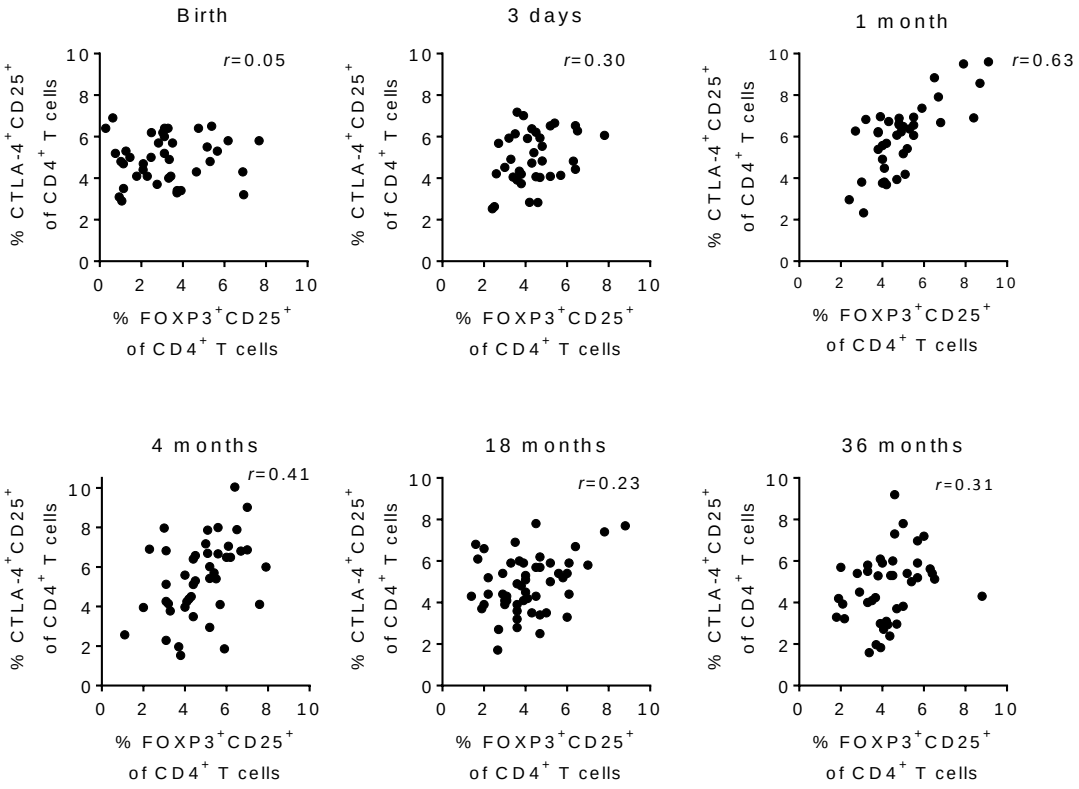

Figure 25. Correlations between the fractions of circulating FOXP3⁺CD25⁺ T cells and CTLA-4⁺CD25⁺ T cells within the CD4⁺ T cell population at birth, 3 days, 1, 4, 18 and 36 months of age. (Spearman's rank correlation test).

These results suggest that $CD4^+$ T cells that express FOXP3 may not necessarily express CTLA-4 and vice versa during childhood, which could explain why we did not observed the same association patterns between $FOXP3^+CD25^+$ T cells and $CTLA\text{-}4^+CD25^+$ cells within the $CD4^+$ T cell population in the different multivariate factor analyses performed. Thus, our observations indicate that it is the proportion of the putative $FOXP3^+$ Treg subset *per se,* and not the expression of the regulatory molecule CTLA-4, that were inversely related to being raised on a farm but positively associated with allergic sensitization.

9. GENERAL DISCUSSION

9.1 Introduction

The hygiene hypothesis states that reduced exposure to microbes leads to failed immune maturation and the development of allergy [117]. Thus, in this thesis I examined if gut commensal bacteria could induce $FOXP3^+CD25^+CD127^{low}$ Tregs *in vitro*. I also investigated whether there is a relation between the infantile gut microbiota and postnatal T cell development *in vivo*, as well as studied how the T cell development was associated with allergy and allergic sensitization during childhood.

9.1.1 Induction of regulatory T cells *in vitro*

In mice the gut microbiota has been shown be important for the induction and function of Tregs, as germ-free mice have lower proportion and less functional Tregs than wild type mice [98-100]. However, colonization with either a mixture of bacteria, different clostridia species or monocolonization with *Bacteroides fragilis* resulted in *de novo* generation of $FOXP3^+$ Tregs in the intestine [98-100]. In paper I, we found that stimulation of neonatal mononuclear cells with the gut commensal *S. aureus* was able to induce $FOXP3^+CD25^+CD127^{low}$ T cells *in vitro,* and that these cells were suppressive. Although stimulation with *L paracasei* also resulted in induction of putative Tregs, the *de novo* generation of $FOXP3^+CD25^+CD127^{low}$ T cells was not as obvious as in *S. aureus* stimulated cultures. The discrepancy in capacity of bacteria to induce Tregs may be dependent on the bacterial species as it has been shown that stimulation of mononuclear cell cultures from adult blood with *L. acidophilius* or *B. fragilis* generated higher proportions of $FOXP3^+CD25^+$ Tregs than *E. coli* [135].

Bacteria consist of several components that may influence the innate and adaptive immune system. Peptidoglycan, which is extensively expressed on the cell wall of Gram-positive bacteria such as *S. aureus* and *L. paracasei,* is a potent activator of innate immune cells that express the Toll-like receptor 2 (TLR2) or nucleotide-binding oligomerization-domain protein 2 (NOD2). Interestingly, peptidoglycan derived from lactobacilli has been shown to stimulate the induction of regulatory $CD103^+$ DCs and $FOXP3^+$ Tregs in the gut of mice with induced colitis [140]. In our study, however, *S. aureus* was a more potent generator of $FOXP3^+CD25^+CD127^{low}$ T cells compared to *L. paracasei,* which suggests that it was probably not peptidoglycan that stimulated *de novo* generation of $FOXP3^+CD25^+CD127^{low}$ T cells. Instead, *S. aureus* may possess other stimulatory functions not present in lactobacilli. For instance, certain *S. aureus* strains produce enterotoxins, i.e. superantigens, that exhibit a unique T cell stimulatory capacity, since they can activate as many as 10-30% of all T cells compared to <0.1% for conventional antigens [141]. In addition, it has been shown that that *S. aureus* enterotoxin induce $FOXP3^+$CTLA-4^+ T cells that displayed suppressor abilities *in vitro* [142], and neonatal exposure to staphylococcal enterotoxin has been shown to improve the induction of oral tolerance in a mouse model of airway allergy [143]. Here, we used an UV-killed *S. aureus* strain that was not able to produce enterotoxin in the cell culture. However, toxin molecules might still be present in the bacteria or on the bacterial cell wall,

as UV-killed *S. aureus* that have previously been able to produce enterotoxins are more potent in stimulating IL-17 production by mononuclear cells than UV-killed *S. aureus* strains that lacked the capacity to produce classical superantigens [144]. Furthermore, *S. aureus* possess a range of virulence factors, such as protein A, that could also be involved in the induction of Tregs.

We also found that certain conditions were needed to generate FOXP3$^+$CD25$^+$CD127^{low} T cells following stimulation with *S. aureus*. First, APCs were pivotal since stimulation of neonatal mononuclear cells in the absence of APCs did not result in induction of CD25$^+$CD127^{low} T cells (Figure 26A). Second, mainly the naïve CD4$^+$ T cells were converted into FOXP3$^+$CD25$^+$CD127^{low} T cells, as CD25$^+$CD127^{low} T cells were only induced in cultures from adults if effector T cells were depleted before stimulation in the presence of APCs and pre-existing Tregs. Third, the presence of pre-existing Tregs during *S. aureus*-stimulation of neonatal mononuclear cells seemed to be required, as the absence of pre-existing Tregs resulted in induction of activated CD25$^+$CD127^{low} T cells that increased the proliferation of responder cells rather than suppressed them (Figure 26B). In contrast, FOXP3$^+$CD25$^+$CD127^{low} T cells induced in the presence of pre-existing Tregs were functional. However, as it was difficult to obtain sufficient numbers of cord blood T cells to generate adequate numbers of *S. aureus*-induced FOXP3$^+$CD25$^+$CD127^{low} T cells to perform suppression assays, we were only able to perform one such experiment in which pre-existing Treg were present during the induction phase. Thus, our results are limited by the fact that we have not been able to reproduce the latter suppression assay. Still our findings are supported by the fact that CD25$^+$CD4$^+$ tTregs are able to convey their suppressive function to conventional CD4$^+$ T cells that start to secret IL-10 and TGF-β, which is referred to as infectious tolerance [145, 146]. Moreover, Tregs are able to downregulate the maturation markers HLA-DR, CD80 and CD86 on DCs that results in tolerogenic APCs that secrete IL-10 [147]. As antigen stimulation of CD25^{neg}CD4$^+$ T cells in the presence of TGF-β induces FOXP3$^+$CD25$^+$CD4$^+$ T cells [53, 54], it is possible that tTregs are required to generate a regulatory cytokine milieu that facilitate conversion of CD4$^+$ T cells into functional FOXP3$^+$ Tregs during *S. aureus* stimulation.

The last requirement for the conversion of CD4$^+$ T cells into FOXP3$^+$CD25$^+$CD127^{low} T cells was the activation of the PD-1/PD-L1 axis. The major role of PD-1 and PD-L1 interaction is to limit T cell activation [136, 137], but the axis has also been shown to be important in the induction of Tregs [60-62, 64]. Accordingly, interaction between PD-L1 and PD-1 during activation of TBET$^+$Th1 cells convert these cells into FOXP3$^+$ Tregs [63, 64]. Furthermore, stimulation with *S. aureus* has been shown to increase the expression of PD-L1 on monocytes from adult blood [138]. However, we were not able to elucidate if this was the case in neonatal monocytes as these cells down-regulated typical identification markers and became highly autofluorescent after three days of culture, which made it impossible to distinguish them from other activated mononuclear cells. Another limitation of the study was that we were not able to study the expression of PD-L1 on DCs, due to their low number in the circulation. However, we did observe an upregulation of PD-L1 on B cells,

which are also able to act as APCs. Also, the induced Tregs expressed PD-1 to a higher extent than conventional CD4+ T cells that did not convert into Tregs after stimulation with *S. aureus*. This might suggest that stimulation with *S. aureus* may facilitate the binding between PD-1/PD-L1 by augmenting the PD-L1 expression on APCs, thus increasing the opportunity for these two markers to interact. Interestingly, blocking PD-L1 reduced or completely inhibited *de novo* generation of FOXP3+CD25+CD127low T cells following *S. aureus* stimulation (Figure 26C, upper row). We therefore suggest that PD-1/PD-L1 axis could be one of the mechanisms by which APCs induce FOXP3+CD25+CD127low T cells upon *S. aureus* stimulation (Figure 26C, lower row).

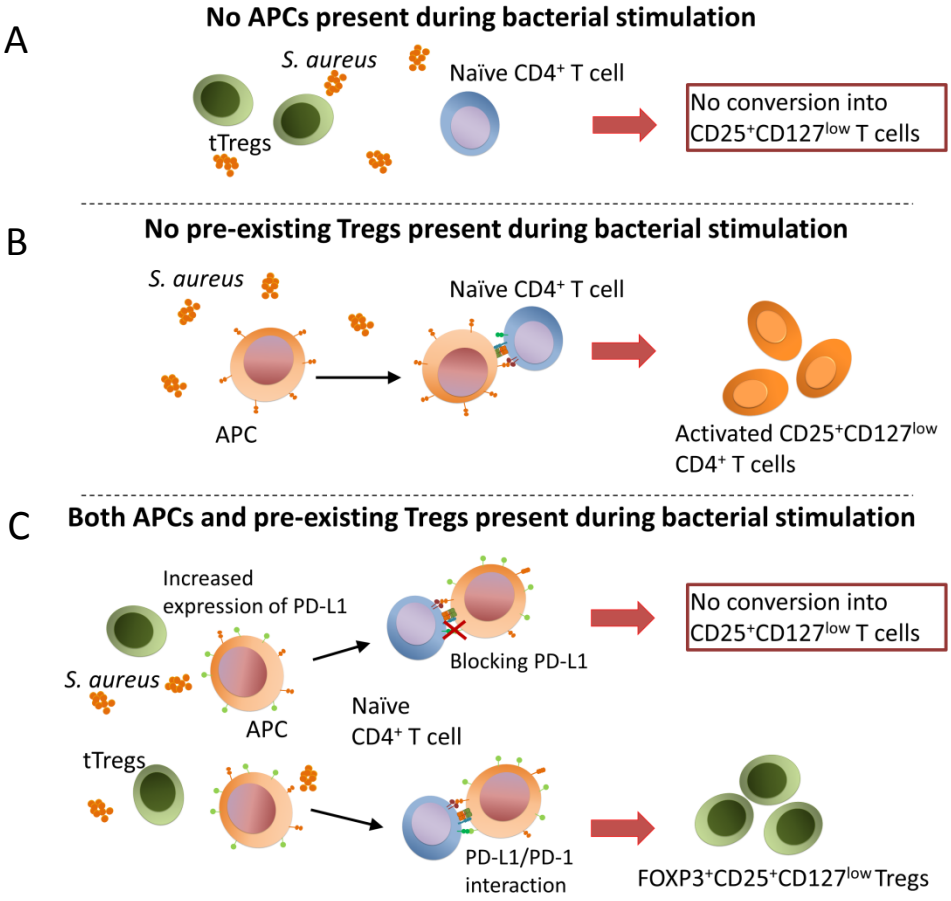

Figure 26. The different culture conditions and outcome during stimulation of mononuclear cells with *S. aureus*. (A) Conventional CD4+ T cells were stimulated with *S. aureus* in the presence of pre-existing Tregs but no APCs, which resulted in lack of induction of CD25+CD127low T cells. (B) Conventional CD4+ T cells were stimulated with *S. aureus* in the presence of APCs but no pre-existing Tregs, which lead to activation of conventional CD4+ T cells expressing CD25+CD127low. (C) Conventional CD4+ T cells were stimulated with *S. aureus* in the presence of both APCs and pre-existing Tregs, which leads to upregulation of PD-L1 on the APCs. In the upper row PD-L1 was blocked and there was no induction of CD25+CD127low T cells. In the lower row interaction between PD-L1/PD-1 was allowed and the naïve CD4+ T cells converted into suppressive FOXP3+CD25+CD127low T cells.

9.1.2 The early bacterial colonization of the gut and T cell development

As we found that the gut commensal *S. aureus* was able to convert neonatal $CD4^+$ T cells into putative $FOXP3^+CD25^+CD127^{low}$ Tregs *in vitro*, we questioned if there was an association between the early bacterial gut colonization pattern and the proportion of circulating Tregs *in vivo* during early childhood. To our knowledge this is the first prospective cohort study that has followed the early gut colonization pattern and development of the adaptive immune system prospectively. Here we found that was no association between the early gut bacterial colonization and the proportions of circulating $FOXP3^+CD25^{high}$, $FOXP3^+CD25^+$ or $CTLA\text{-}4^+CD25^+$ T cells during early childhood. These results may seem contradictory to what has been shown in mice in which colonization by a mixture of different clostridia species increased the proportion of Tregs in the colon to a higher degree than colonization by lactobacilli or *Bacteroides* [98]. Others have shown that monocolonization by *B. fragilis* increases the proportion of IL-10 producing $FOXP3^+$ Tregs in the colon of mice to a higher degree than wild type mice [100]. However, our findings are limited by the fact that we have studied the association of circulating putative Tregs and not the Tregs isolated from the gut mucosa. It is also possible that other bacteria not detected in our analyses because they cannot be cultured may be associated with the proportions of circulating $FOXP3^+CD25^{high}$, $FOXP3^+CD25^+$ or $CTLA\text{-}4^+CD25^+$ T cells.

In paper II we also studied whether the early bacterial colonization of the intestine was associated with T cell memory conversion and effector function. Interestingly, we found that early colonization by bifidobacteria or *E. coli* was associated with mononuclear cells with higher capacity to produce both proinflammatory and Th2-related cytokines. Others have found that early colonization by bifidobacteria at 2 months of age do not correlate with the IL-6 or TNF production by mononuclear cells following stimulation with LPS [148]. Our results and those by Sjögren et al are hard to compare due to several differences in the study procedures. First, Sjögren et al studied the relative percentage of bifidobacteria in the feces compared to the levels of cytokine production, whereas we have studied the presence of different bacteria, i.e. yes or no at various time points. Secondly, we stimulated mononuclear cells from the blood with PHA that is a potent stimulator of T cells, while Sjögren et al used LPS, which mainly stimulates the innate immune cells. Finally, we found that colonization by bifidobacteria had to occur as early as at 1 or 2 weeks of age to associate with cytokine responses. Sjögren et al studied the relation between cytokine production and colonization of bifidobacteria at 1 and 2 months of age, when we no longer found any association between bifidobacteria colonization of the gut and cytokine responses [148]. However, in line with the present findings our group has shown that children who are colonized by bifidobacteria and *E. coli* have higher numbers of circulating memory B cells than children not colonized by bifidobacteria or *E. coli* [101]. These two bacteria are strict fecal bacteria that are among the first colonizers of the infantile gut [84, 149], and are most often obtained from the mothers gut flora during vaginal delivery. Accordingly, infants delivered by caesarean section have a delayed acquisition of these bacteria [82, 150]. Colonization by bifidobacteria and *E. coli* in our study may therefore also

represent acquisition of a broad range of fecal bacteria that might activate the adaptive immune response early in childhood.

In contrast, we found that early colonization by *S. aureus,* as well as enterococci, clostridia and yeasts was associated with a lower capacity of mononuclear cells to produce cytokines later in childhood. *S. aureus* is a skin commensal that is more likely to colonize the gut only when there is low competition from bacteria better adapted to the gut milieu. In accordance, colonization by this bacterium has increased in parallel with delayed acquisition of *E. coli* and *Bacteroides* [89, 90]. Although *S. aureus* is the most potent inducer of TNF from both cord and peripheral blood mononuclear cells [151], the presence of *S. aureus* in the gut flora may reflect a gut microbiota with low diversity that is unable to suppress the growth of staphylococci. The low cytokine responses in these children could thus be due to reduced and/or low turnover rate of other bacteria, which might lead to limited stimulation of the immune system.

It has been hypothesized that a high microbial exposure early in life would drive the immune system in a proinflammatory/Th1 direction, dominated by IFN-γ, and thereby suppress Th2 responses and subsequent Th2-mediated disorders [117, 152]. For example children supplemented with lactobacilli (*L. reuteri*) prenatally and during the first year of life has been shown to have mononuclear cells with lower IL-5 and IL-13 responses at 1 and 2 years of age compared to children in the placebo group [153]. The children who received lactobacilli supplementation were also shown to have lower prevalence of IgE mediated eczema at 2 years of age [123], but not respiratory allergies later in childhood [124]. However, powerful Th2 responses are also associated with lower allergy prevalence as allergic diseases are low in areas with high helminth load [154]. Moreover, in parallel with reduction in the incidence of infectious diseases the prevalence of both Th1- and Th2-mediated disorders has increased in the Western world during the last decades [115], which also argues against a Th2 deviation. In the FARMFLORA cohort we found that early gut colonization by bifidobacteria was associated with higher PHA-induced TNF, IL-6, IL-1β, IL-5 as well as IL-13 responses, while the IFN-γ levels were unrelated to the early bacterial colonization pattern. Thus, we found no support for the hypothesis that exposure to bacteria would drive the immune system in a proinflammatory/Th1 direction. In fact, no bacteria seemed to be associated specifically with either Th1- or Th2-induced cytokines.

We also found that higher proportions of $FOXP3^{+}CD25^{high}$ and $CTLA\text{-}4^{+}CD25^{+}$ cells within $CD4^{+}$ T cell population in early infancy were related to impaired immune effector functions, i.e. lower PHA-induced cytokine levels, and memory T cell proportions later in childhood. It is possible that higher proportion of Tregs may inhibit T cell activation and consequently memory conversion in countries in which children are less exposed to microbes and acquire a gut flora including *S. aureus*, enterococci or clostridia (Figure 27, left column). In contrast, early acquisition of a gut microbiota including bifidobacteria and *E. coli* might direct enhance T cell activation and cytokine production and silence the suppression from Tregs (Figure 27, right column). Indeed, it has been shown that the

regulatory function of Tregs is reduced during inflammatory conditions [155]. For instance, TNF reduces the function of Tregs [156] and IL-6 impedes the conversion of conventional $CD4^+$ T cells into Tregs and in combination with TGF-β induce differentiation of inflammatory Th17 cells [157]. Furthermore, Tregs from birch pollen allergic adults suppress birch-allergen induced IL-5 and IL-13 responses outside but not during birch-pollen season [128]. Tregs may therefore not only be hampered during a Th1-driven inflammation but also during a Th2-driven inflammation. In the present study, the early bacterial gut colonization pattern was not associated with the proportion of putative $FOXP3^+CD25^{high}$ Tregs. However, early colonization by bifidobacteria or *E. coli* resulted in higher cytokine responses of both proinflammatory and Th2-cytokines, which might have hampered the suppressive function of Tregs and their effect on T cell activation and memory conversion.

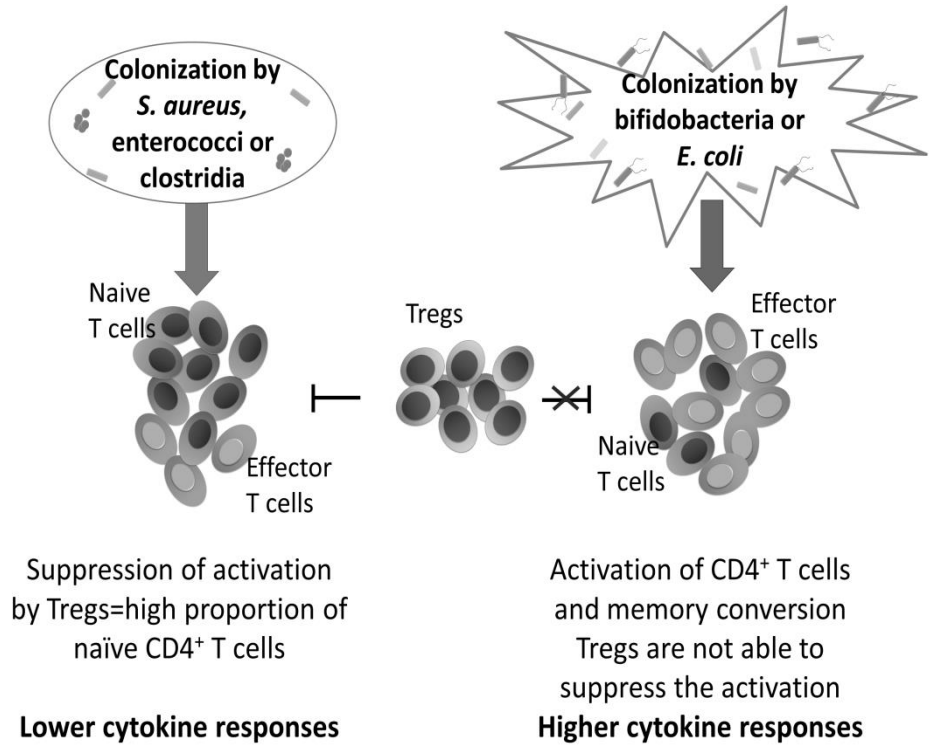

Figure 27. Hypothesis for the influence of a less complex and a diverse gut flora on the developing immune system. In a system were the host has a gut flora including *S. aureus*, enterococci or clostridia, the Tregs may be able to impede T cell activation and memory conversion as illustrated in the left column. However, in children that acquire a gut flora, including bifidobacteria and *E. coli*, the T cells are activated and Tregs are not able to suppress the activation to the same degree as in children with a less diverse gut flora (right column), which results in mononuclear cells with higher capacity to produce cytokines in response to polyclonal stimulation.

9.1.3 Farming environment and the T cell development

Children living on farms are protected against allergies compared to children who do not live on farms [118]. This may to be due to a higher exposure to microbes or other environmental factors such as farm milk or farm animals that might stimulate the adaptive

immune response making it less susceptible to allergens in farmers' children than non-farmers' children [158]. Children raised on farms have been shown to have lower levels of the allergy-related antibodies IgE against grass and cat allergens in school age compared to non-farmers' children [159], but few studies have ivenstigated on how the farming environment influences the development of the immune system. Furthermore, mononuclear cells from farmers' children responded with higher proportions of activated $CD25^{+}CD4^{+}$ T cells upon stimulation with ionomycin or LPS compared to children not raised on farms [160]. In the present study, we found that being raised on a farm was positively associated with higher capacity of mononuclear cells to produce both proinflammatory and Th2-related cytokines in response to PHA at 18 months of age. Taken together, these results might support the idea that children raised on farms have a more antigen-experienced adaptive immune system. However, in the present study the associations between living on a farm and higher cytokine responses by mononuclear cells were not statistically significant in univariate analyses. It is possible that the associations observed between living on a farm and the cytokine responses would be stronger if our cohort would have been larger.

It has been suggested that the strongest protective effect of farm exposure occurs prenatally [161]. For example, cord blood mononuclear cells from farmers' children produced higher levels of IFN-γ and TNF after stimulation with a combination of phorbol 12-myristate 13-acetate, ionomycin, LPS and staphylococcal enterotoxin B compared to mononuclear cells from non-farmers' children [162]. Furthermore, in the PAULCHEN study the proportion of Tregs defined as $CD25^{high}CD4^{+}$ within total mononuclear cells was significantly higher both in the unstimulated and PHA-stimulated cultures of children from farming mothers compared to non-farmers' children [163]. The neonatal Tregs from farmers' children were also more potent in suppressing proliferation of $CD4^{+}$ responder cells [163]. In the present study, however, a farming environment did not seem to affect the fetal fraction of putative $FOXP3^{+}CD25^{high}$ Tregs as we did not find any difference in the proportion of these cells at birth between farmers' and non-farmers' children. The discrepancy between the proportion of Tregs between newborn children from farming families in the present cohort and the PAULCHEN study might be due to the fact that we study the $FOXP3^{+}CD25^{high}$ cells within the $CD4^{+}$ T cell population *in vivo* in the circulation whereas the proportion of neonatal Tregs in the PAULCHEN study was investigated after *in vitro* culture for 3 days.

Interestingly, we also found that children who lived on farms had lower proportions of $FOXP3^{+}CD25^{high}$ cells within the $CD4^{+}$ T cell population at 3 days and 36 months of age compared to children who did not live on a farm. It is possible that the combination of lower exposure to microorganisms, animals or farm milk and higher proportions of putative $FOXP3^{+}CD25^{high}$ Tregs in non-farmers children hinders activation of conventional $CD4^{+}$ T cells in children who are not raised on farms (Figure 28, left column). On the other hand, children who are raised on farms have lower proportions of $FOXP3^{+}$ Tregs early in life and are more exposed to environmental factors that are able to activate T cells, which results in an increased effector function that are able to silence the suppression of Tregs

(Figure 28). However, future studies are needed to elucidate if children living on farms have a different gut colonization pattern than non-farmers' children, and if this could influence the associations observed between living on a farm and having a higher T cell effector function.

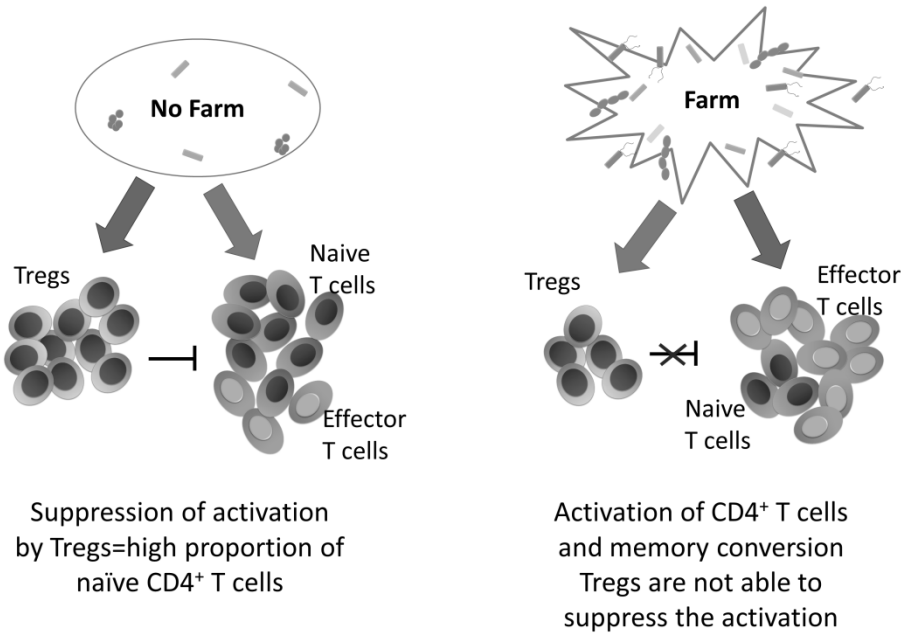

Figure 28. Hypothesis for how farming environment may influence the developing immune system. Children who are not raised on farms have higher proportions of Tregs that may impede T cell activation and memory conversion as illustrated in the left column. However, children who are raised on farms have lower proportions of $FOXP3^{+}CD25^{high}$ Tregs early in life and might be more exposed to microorganisms, or other stimulatory factors, which might lead to activation and memory conversion of T cells (right column).

9.1.4 Allergy, allergic sensitization and T cell development

According to Strachan's hygiene hypothesis, exposure to microorganisms stimulates the immune system and makes it less susceptible to allergic sensitization, whereas reduced microbial exposure may lead to allergy development [117]. This notion has been supported by several epidemiological studies that demonstrated protective effects for growing up in a farming environment, early attendance at a day-care facility and a history of gastro-intestinal infections [118, 119, 164, 165]. However, these studies did not investigate the immune maturation before onset of the disease to elucidate if the allergic and healthy children differed in this regard. Actually, there is only one other study, except for ours, that has compared the postnatal T cell development among allergic or sensitized children and healthy children before allergy onset. They found that children with eczema at 2 years of age had higher levels of the Th2-related chemokines CCL17, CCL18 and CCL22, but lower levels of the Th1-related chemokine CXCL10 in the serum at 1 year of age compared to healthy children [166]. This might suggest that children who develop eczema have a

Th2 dominated immune response before onset of disease. We found similar results as there was a positive association between allergy at 18 and 36 months and the Th2-related cytokine IL-5 responses at 36 months of age.

We also found that there was a positive association between allergy and higher proportions of $FOXP3^+CD25^+$ cells, but not putative $FOXP3^+CD25^{high}$ Tregs, within the $CD4^+$ T cell population just before onset of the disease. Accordingly, it has been shown that children with eczema at 2 years of age have higher proportion of $FOXP3^+CD25^+CD4^+$ T cells within the $CD3^+$ T cell population compared to non-allergic children at the same time point [132]. It is possible that higher proportions of $FOXP3^+CD25^+$, but not $FOXP3^+CD25^{high}$, T cells within the $CD4^+$ T cell population just before or during established allergic disease might be a result of an increase in activated cells within the $FOXP3^+CD25^+$ T cell subset in response to the allergic inflammation.

In the present study we also found that sensitized children had higher proportions of $FOXP3^+CD25^{high}$ cells within the $CD4^+$ T cell population at birth and 3 days of life than non-sensitized children. These associations were also true for the proportions of $FOXP3^+CD25^+$ T cells at birth. However, it has been shown that the fraction of $FOXP3^+CD25^+$ T cells at birth was unrelated to sensitization at both 1- and 2 years of age [132]. A possible explanation for this discrepancy may be that we have examined the proportions of $FOXP3^+CD25^{high}$ and $FOXP3^+CD25^+$ cells within the $CD4^+$ T cell population, whereas the other study examined the fraction of $FOXP3^+CD25^+CD4^+$ cells within the $CD3^+$ T cell population, which includes both $CD4^+$ and $CD8^+$ T cells [132]. Furthermore, our cohort included children from rural areas of whom only one third had allergic parents, the study by McLoughlin et al examined inner-city children with a parental history of allergic disease or asthma [132].

The homing receptor CCR4 is linked to the $CD45RO^+$ memory T cell phenotype in children, as the differentiation from naïve T cells into memory T cells coincides with homing receptor switch, i.e. from being $\alpha4\beta7^+$ to be $CCR4^+$ [24]. In adults, newly activated T cells upregulate CCR4 [167], which suggest that the expression of the homing receptor CCR4 may be used as a differentiation marker in $CD4^+$ T cells. In the present study we found that sensitized children had lower proportions of $CCR4^+$ $CD4^+$ T cells and mononuclear cells with lower capacity to produce IL-1β in response to polyclonal stimulation. Our results therefore imply that sensitized children have lower T cell differentiation and effector function compared to non-sensitized children. Thus, it is possible that a lower proportion of $FOXP3^+CD25^{high}$ cells within the $CD4^+$ T cell population early in life of non-sensitized children may allow a postnatal T cell activation (Figure 29, left column), whereas higher proportion of these cells and low T cell activation and function observed in sensitized children may reflect a defective immune regulation and immune immaturity (Figure 29 right column). Still, future studies are needed to elucidate if allergic and sensitized children have different bacterial gut colonization pattern than healthy children, and if this could influence the T cell memory conversion and effector function.

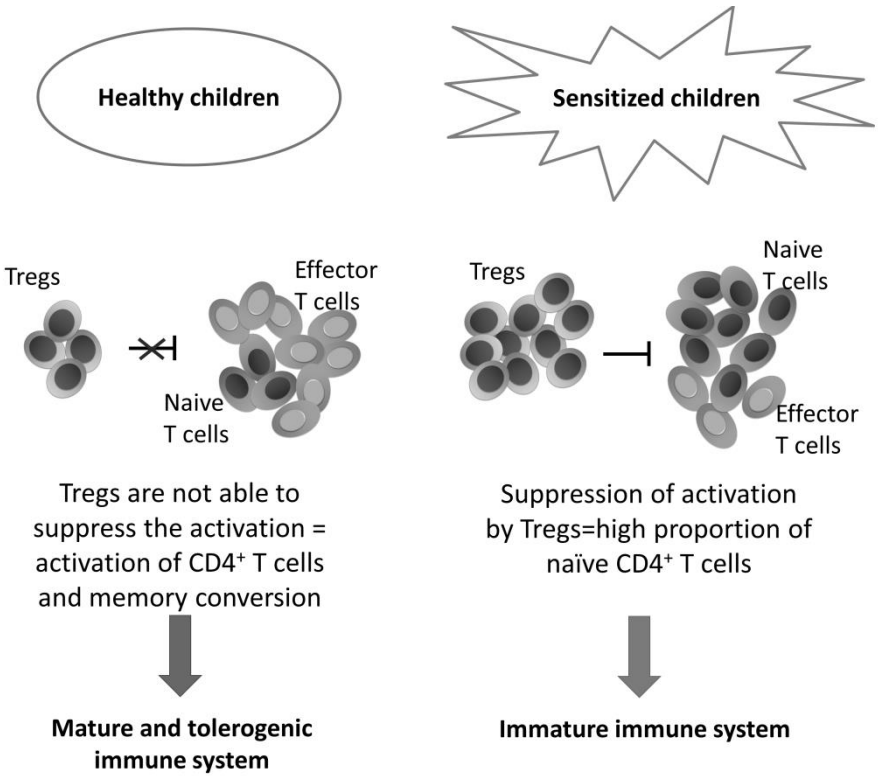

Figure 29. The immune maturation of sensitized and healthy children. Lower proportions of Tregs early in life will allow activation of the developing immune system and increase the memory T cell conversion and effector function, which will lead to a mature immune system. Consequently, higher proportions of putative FOXP3^{+}CD25^{high} Tregs early in life may hinder activation of conventional CD4^{+} T cells, which results in a higher proportions of naïve T cells and consequently an immature immune system.

9.2 Conclusions

In conclusion, the gut commensal *S. aureus* has the ability to convert neonatal CD4^{+} T cells into FOXP3^{+}CD25^{+}CD127^{low} T cells *in vitro*, which is dependent on the presence of both thymic derived Tregs and of APCs that express PD-L1. However, if colonization by *S. aureus* might increase the Treg population in the infantile intestine still needs to be elucidated.

Furthermore, as infants who were sensitized had higher proportion of FOXP3^{+}CD25^{high} cells within the CD4^{+} T cell population early in life compared to healthy children, higher proportions of Tregs early in life may not be protective against allergic disease. Indeed, it is possible that high proportions of putative FOXP3^{+}CD25^{high} Tregs within the CD4^{+} T cell population early in infancy may modulate the effector T cell development in a way that could even predispose to allergic sensitization. However, early gut colonization with a gut microbiota including bifidobacteria and *E. coli,* or being exposed to a farming environment early in life, might instead enhance the effector T cell development.

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