Adaptive immune maturation in relation to allergic disease and vaccine responses in children

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ABSTRACT

Adaptive immune maturation in children is likely the result of a complex interplay between both intrinsic and environmental factors, but surprisingly little is known about how early life immune maturation is related to immune responses and subsequent development of allergic disease. The aim of the FARMFLORA birth-cohort study, including farmers' and non-farmers' children, was to visualize longitudinal patterns of adaptive immune maturation in relation to allergic sensitization and disease, vaccine-induced antibody responses, as well as to certain environmental factors in childhood.

By the use of multivariate factor analyses, we show that higher proportions of circulating neonatal regulatory T cells was strongly associated with sensitization in early childhood, and that a sustained higher fraction of these cells related to allergic disease at school age. Allergic disease at this age was also associated with higher proportions of naïve CD45RA$^+$ T cells in infancy and with higher proportions of immature/naïve CD5$^+$ B cells from birth to 8 years of age. These results indicate that allergic disease in childhood is preceded by a heightened immaturity in the adaptive immune system. Further, growing up on a dairy farm was associated with a higher degree of adaptive immune maturation, which may in part explain the lower incidence of allergic disease among farmers' children.

We further found that higher antibody levels induced by the non-live vaccine against diphtheria, tetanus and pertussis was associated with increased baseline immune maturation prior to vaccination. In contrast, higher antibody levels induced by the live attenuated vaccine against measles, mumps and rubella were generally associated with a lower degree of baseline adaptive immune maturation. Differences in the formulations of these vaccines and their respective way to induce immune responses in the host may be a possible explanation for these diverging association patterns.

Keywords: Adaptive immune maturation, children, allergic disease, farm, vaccine responses, prospective birth-cohort, multivariate factor analysis
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POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunsystem finns till för att försvara oss mot infektioner orsakade av mikroorganismer, till exempel bakterier och virus. Immunsystemet består av många olika typer av vita blodkroppar, så kallade immunceller, som var och en fyller en viktig funktion. **I den här avhandlingen** har jag framförallt studerat två typer av immunceller, B-celler och T-celler. Dessa celler har en enastående förmåga att känna igen och komma ihåg eventuella inkräktare. B-cellernas huvuduppgift är att producera ett sorts målsökande protein, så kallade antikroppar, som binder till ytan av mikroorganismer och signalerar till immunsystemets övriga celler att dessa ska förstöras. T-cellerna producerar istället budbärarmolekyler, så kallade cytokiner, som är viktiga för att styra och koordinera immunsystemet så att en eventuell inkräktare kan oskadliggöras effektivt. Den T- eller B-cell som vid en infektion känner igen en inkräktare, har förutsättning att bli aktiverad och då påbörjas även en mognadsprocess i cellen. Ju fler bakterier och virus immunsystemet stöter på, desto mer ”moget” blir det. Detta innebär att immunsystemet hos en vuxen individ generellt sett är betydligt mer moget och effektivt än immunsystemet hos ett litet barn. Man har dock sett att ”mognadsgraden” av immunsystemet inte bara är kopplat till ålder, utan också till vilken miljö man lever i. Man vet till exempel att barn i utvecklingsländer, där det generellt sett är en högre exponering för olika mikroorganismer, har ett mer moget immunsystem än jämnåriga barn i industrialiserade länder.

Under senare hälften av 1900-talet ökade förekomsten av **allergier** dramatiskt i Sverige och i andra industrialiserade länder. Man vet inte exakt varför vissa barn drabbas, men mycket tyder på att uppväxtmiljön utgör en viktig faktor. Flera vetenskapliga studier har visat att barn som växer upp på bondgård med mjölkkor har en betydligt lägre risk att utveckla allergi än andra barn. Allergi är en immunologisk sjukdom och enligt den så kallade hygienhypotesen beror den ökade allergiförekomsten på att små barns immunsystem idag inte exponeras för tillräckligt mycket mikroorganismer. Därmed får immuncellerna inte heller tillräcklig stimulans för att utbildas och mogna på ett korrekt sätt. Istället börjar dessa celler överreagera vid kontakt med helt ofarliga ämnen, som t.ex. födoämnen, pollen och kvalster, vilket kan utlösa allergiska reaktioner. Men trots att hygienhypotesen lanserades för snart 30 år sedan vet man ännu inte hur immunsystemets mognad är relaterat till allergiutveckling hos barn.

För att få svar på detta startades den så kallade BONDGÅRDSFLORA-studien år 2005. I studien inkluderades 65 barn från Västra Götalands-regionen. Ungefär hälften av barnen bodde på små mjölkgårdar och hälften bodde i samma geografiska område men inte på gårdar. Sedan starten har barnen följts med regelbundna blodprovstagningar för immunologiska analyser samt med läkarundersökningar för att påvisa eventuell allergi-utveckling. **I arbete I och IV** i denna avhandling har vi undersökt kopplingar mellan uppväxt-miljö, mognadsgrad av barnens immunsystem och risken att utveckla allergi. Barnen på bondgård hade lägre förekomst av allergi och en högre mognads-grad av T- och B-celler i blodet jämfört med övriga barn. I hela gruppen av barn fann vi dessutom att de som var allergiska vid 8 års ålder generellt sett hade en lägre mognadsgrad av sina T- och B-celler i blodet under uppväxten än de övriga barnen. Sammantaget fann vi att immunsystemets mognad hos det lilla barnet är relaterat till allergiutveckling senare i barndomen, vilket kan vara en viktig pusselbit för att kunna utveckla strategier för att förhindra allergiutveckling hos barn.

Efter att immunförsvaret har lärt sig att känna igen sjukdomsorsakande mikroorganismer kan det vid en ny kontakt stoppa dessa tidigt utan att några infektionssymtom uppstår. Detta fenomen utnyttjas vid vaccinationer. Genom att injicera en liten dos av en försvagad version av den mikro-organism som man vill skydda sig emot undviks infektionssymtom, men immunförsvaret lär sig ändå att känna igen mikroorganismen och producerar i de flesta fall skyddande antikroppar. Antikroppsnivåerna i blodet efter vaccinering varierar kraftigt mellan olika individer och i olika länder, men de underliggande orsakerna bakom denna variation är fortfarande okänd. I **arbete II och III**, som också är baserade på BONDGÅRDSFLORA-studien, har vi undersökt om mognaden av immunsystemet avgör hur effektivt barnens immunceller svarar med antikroppsproduktion vid vaccinering. Vi fann att nivåerna av de vaccinspecifika antikropparna i blodet var starkt sammankopplat med hur moget deras immunsystem var vid vaccinationstillfället. Intressant nog tyder våra resultat på att ett mer moget immunsystem gynnade svaret mot vissa typer av vaccin (inaktiverade) medan det missgynnade svaret mot andra (levande försvagade). Våra resultat understryker vikten av att studera samband mellan immunsystemets utveckling och vaccinationssvar hos barn. En bättre förståelse av detta samband kan vara grunden till att kunna förbättra vaccinationsstrategier i olika delar av världen och för att utveckla ännu mer effektiva vacciner än vad som finns idag.

PAPERS INCLUDED IN THE THESIS

I. **Strömbeck A**, Rabe H, Lundell A-C, Andersson K, Johansen S, Adlerberth I, Wold AE, Hesselmar B, Rudin A. *High proportions of FOXP3^{+}CD25^{high} T cells in neonates are positively associated with allergic sensitization later in childhood.* Clinical & Experimental Allergy, 2014;44:940-52.

II. **Strömbeck A**, Lundell A-C, Nordström I, Andersson K, Adlerberth I, Wold AE, Rudin A. *Earlier infantile immune maturation is related to higher DTP-vaccine responses in children.* Clinical & Translational Immunology. 2016 Mar 11;5(3):e65

III. **Strömbeck A**, Lundell A-C, Nordström I, Andersson K, Adlerberth I, Wold AE, Rudin A. *Delayed adaptive immunity is related to higher MMR vaccine-induced antibody titers in children.* Clinical & Translational Immunology. 2016 Apr 29;5(4):e75

IV. **Strömbeck A**, Nordström I, Andersson K, Andersson H, Johansen S, Maglio C, Rabe H, Adlerberth I, Wold AE, Hesselmar B, Rudin A, Lundell A-C. *Allergic disease in 8-year old children is preceded by delayed B-cell maturation.* Submitted manuscript.

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The following manuscript is also referred to in the text:

APPENDIX

Rabe H, Strömbeck A, Ljung A, Lundell A-C, Nordström I, Andersson K, Wold A E, Adlerberth I, Rudin A. *The infantile gut flora is related to the capacity to produce cytokines but not to the proportions of circulating FOXP3^{+}CD25^{high} T cells later in childhood.* Manuscript in preparation.

TABLE OF CONTENTS

ABBREVIATIONS

APC	Antigen-presenting cell
BAFF	B cell activating factor
DAMP	Damage-associated molecular pattern
DTP-vaccine	Vaccine against diphtheria, tetanus and pertussis
GALT	Gut-associated lymphoid tissue
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MMR-vaccine	Vaccine against measles, mumps and rubella
NLR	NOD-like receptor
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PRR	Pathogen recognition receptor
T_{FH} cell	T follicular helper cell
T_{FR} cell	T follicular regulatory cells
T_H cell	T helper cell
TLR	Toll-like receptors
Tregs	Regulatory T cells

1 THE IMMUNE SYSTEM – AN OVERVIEW

Our immune system has evolved to protect us from harmful pathogens. However, while keeping up an effective defense against pathogenic intruders, the immune system must also avoid aggressive immune responses to harmless antigens in the environment as well as to structures of our own body.

The immune system consists of several different cell types, which all act in concert to direct and tune the immune responses. The **innate immune system** represents our first line of defense and is responsible for rapid recognition and eradication of intruding pathogens, but also for clearance of dead cells and initiation of repair of damaged tissue. If the pathogen is not rapidly cleared by the innate immune system, our second line of defense, i.e. **the adaptive immune system**, will get involved. The key mediators of adaptive immunity are the lymphocytes, i.e. T cells and B cells. Unlike cells of the innate immune system, lymphocytes are equipped with **antigen-specific receptors**.

T cells are responsible for cell-mediated immunity and their **T cell receptors** recognize peptides from protein antigens presented on MHC class I (MHC-I) and MHC class II (MHC-II) molecules. MHC-I molecules are present on the surface of all nucleated cells in the body and MHC-II molecules are mainly present on **antigen-presenting cells** (APCs), such as dendritic cells, macrophages and B cells. **B cells** recognize antigens of many diverse structures through their **B cell receptors**, which are surface-bound forms of antibodies. Activation of B cells leads to secretion of **antibodies** with the same antigen-specificity as their surface receptor that recognized the antigen. Unlike the innate immune system that provide immediate protection against intruding pathogens, the adaptive arm of the immune system requires **expansion** and **differentiation** of antigen-specific lymphocytes before it can provide an effective defense. However, once activated, the adaptive immune system targets the pathogen with much greater precision and it also provides us with **immunological memory**.

2 THE INNATE IMMUNE SYSTEM

Most pathogens enter the body through the skin and mucosal surfaces, such as the gastrointestinal and the respiratory tract. **The epithelial lining** of these surfaces are included as an important component of the innate immune system since they form a mechanical barrier against pathogens and also produce antibacterial substances, and thus literally constitute our first line of defense against intruding pathogens. However, if pathogens succeed in crossing the epithelial barrier, the invaders are in most cases rapidly recognized by tissue resident phagocytic innate immune cells, such as **macrophages** and **dendritic cells**. Upon pathogen-recognition, these cells become activated and react by secreting a range of cytokines and chemokines that act as alarm signals to induce local inflammation. In response to proinflammatory cytokines, surrounding blood vessels dilate and endothelial cells becomes activated and upregulate adhesion molecules. In combination with secreted chemokines, these changes drastically increase the entry of circulating innate immune cells, such as **neutrophils**, **natural killer cells** and **monocytes**, and also plasma proteins from the **complement system** to the infected tissue, which further promote inflammatory responses and eradication of the pathogens.

Dendritic cells play a pivotal role in the orchestration of immune responses by linking innate and adaptive immunity through **antigen presentation** to T cells. Dendritic cells capture antigens and internalize them through phagocytosis or receptor-mediated endocytosis. Upon antigen encounter in this inflammatory microenvironment, dendritic cells become activated and upregulate specific surface co-receptors that are necessary for full activation of T cells. In addition, the activated dendritic cells also upregulate the homing receptor CCR7, which enables them to migrate into draining lymph nodes, where induction of adaptive immune responses occur (see section 3.2).

2.1 Danger recognition by innate immune cells

Cells of the innate immune system express several different classes of receptors that trigger danger signal dependent activation. Collectively, these receptors are referred to as pattern recognition receptors (**PRRs**), which allow the cells to recognize and respond to pathogen-associated structures (pathogen-associated molecular patterns, **PAMPs**) and to danger signals released from damaged or necrotic hosts cells (damage-associated molecular patterns, **DAMPs**). Engagement of specific PRRs trigger different signaling pathways, which lead to innate immune responses that are tailored for eradication of the particular type of microbe encountered. Two major receptor families included in PRRs are the Toll-like receptors (**TLRs**) and the **NOD-like receptors** (**NLRs**). Humans express 10 functional members of the TLR family, i.e. TLR1 to TLR10, distributed on various innate immune cells, and each member specifically recognizes one or more PAMP. TLRs that recognize molecular components on the surface of pathogens are located on the cell surface, whereas TLRs that recognize pathogenic nucleic acids are found in the membranes of endosomes where ingested microbes are digested [1]. Engagement of TLRs leads to activation of the transcription factors nuclear factor-κβ (NF-κβ) and interferon-regulatory factors, which induce production of pro-inflammatory cytokines and type I interferons (IFNs), respectively. The NLRs are a large family of cytosolic receptors, including NOD-receptors and NLRP receptors, which sense PAMPs and DAMPs in the cytoplasm. NOD-1 and 2 are specific for bacterial peptidoglycans and, similarly to TLRs, activation of these receptors leads to induction of NF-κβ and subsequent inflammatory responses. NLRP3 is an important sensor for cellular stress. Upon activation, NLPR3 oligomerizes to form a complex, known as the inflammasome, which generates IL-1 secretion that induces acute inflammation and fever.

2.2 The concept of 'trained immunity'

The established view of immunological memory to be confined exclusively to the adaptive immune system has recently been challenged through the concept of 'trained immunity', which suggest that also cells of the innate immune system, such as macrophages and NK cells, develop memory-like responses [2]. In this model, innate immune activation by pathogens through various PRRs leads to **epigenetic modifications**, which preserves a **heightened activation state** in the cells for weeks or months. Upon

subsequent pathogen encounters, the 'trained' cells show an improved responsiveness with increased production of inflammatory mediators and an enhanced capacity to eliminate infection [2]. The increased innate responsiveness conferred by trained innate immunity is **not pathogen-specific**, and increased responses may be mediated through re-stimulation of both the same and different PRRs.

3 $CD4^+$ T CELLS

3.1 Development and selection of T cells

T cells arise from hematopoetic stem cells in the bone marrow. T cell precursors migrate from the bone marrow to the thymus, where they undergo several quality control checkpoints and develop into either $CD4^+$ or $CD8^+$ T cells. Developing T cells in the thymus are referred to as thymocytes. The T cell receptor complex consists of an α-chain, a β-chain, as well as a CD3 co-receptor. Each α and ß-chain contains one constant region as well as one variable region, of which the latter recognize and bind to the antigen. During the development in the thymus, the gene segments coding for the α and ß-chain will undergo random somatic recombination, which will dramatically increase the diversity of the receptors expressed by the T cell compartment.

The most immature T cell precursors in the thymus do not express any of the proteins required for the T cell receptor complex, and since they also lack the expression of CD4 and CD8, they are referred to as **double-negative thymocytes**. In the next step of development, the thymocytes express both CD4 and CD8, as well as low levels of the complete T cell receptor and are therefore called **double-positive thymocytes**. These cells interact with thymic epithelial cells that express MHC-I and MHC-II. Thymic epithelial cells also express the transcription factor **AIRE**, which enable expression of proteins that are normally only present in peripheral tissues. Thus, thymic epithelial cells have the capacity to present a wide range of self-proteins to the thymocytes, which is an important part of the quality control of the developing T cells.

Thymocytes that recognize self-peptides on a MHC-molecule with low or moderate affinity are **positively selected** to survive and thereby increase the expression of their T cell receptor, whereas T cells that do not recognize MHC-molecules in the thymus are considered as useless and die by apoptosis. Thymocytes with T cell receptors that recognize peptides bound to MHC-I molecules downregulate their expression of CD4, while recognition of

peptide:MHC-II complexes induces downregulation of CD8. This process thus leads to generation of **single-positive CD8$^+$** or **CD4$^+$ T cells**.

Thymocytes that recognize the self-peptide:MHC-complex with a high affinity are negatively selected to undergo apoptosis. This **negative selection** serves to eliminate self-reactive T cells and is a major mechanism of **central tolerance**. However, some of the thymocytes that bind to self-peptide:MHC-complex with high affinity do not undergo apoptosis, but instead develop into CD4$^+$ **regulatory T cells** (**Tregs**) that migrate to peripheral tissues. Why some of the self-reactive thymocytes die while others develop into Tregs is not yet known. The process of negative selection is, however, imperfect and some self-reactive T cells also egress into the periphery; the ability to induce tolerance also in the periphery thus serves as an important back-up system to prevent autoimmunity by these escaping self-reactive T cells. **Peripheral tolerance** is also crucial to prevent T cell responses to self-peptides that are not presented in the thymus as well as to harmless environmental allergens, as described further in chapter 9. It is estimated that around 98% of all thymocytes that develop in the thymus also die in the thymus, which reflects the intensive quality control the cells undergo for the ability to recognize self-peptide:MHC complexes and for self-tolerance.

3.2 T cell activation

After maturation into CD4$^+$ T cells in the thymus, **naïve T cells**, which express the RA isoform of the CD45 molecule (CD45RA$^+$), enter the circulation. The migration of CD45RA$^+$ T cells between the circulation and peripheral lymphoid organs depends on interactions between the lymphocytes and tissue-specific chemokines in combination with endothelial adhesion molecules. Naïve CD45RA$^+$ T cells express the homing receptor L-selectin (CD62L) and the chemokine receptor CCR7, which enable migration into secondary lymphoid organs, such as lymph nodes, where they may become activated by APCs [3, 4].

When naïve CD4$^+$ T cells enter the lymph node, they migrate to the **T cell zone** in the paracortex to search for their cognate antigen peptides presented by APCs. Lymph nodes are highly organized organs that provide a structure that is optimized to facilitate T cell interaction with APCs. If the T cell receptor and the co-receptor CD4 together identify peptide antigens

presented on MHC-II molecules on APCs, the T cell initiates its activation program. For a full activation to occur, the T cells need to receive **co-stimulatory signals from APCs**, such as interactions of CD28 and CD40 ligand on T cells with CD80, CD86 and CD40, respectively, on activated APCs (figure 1). In addition, adhesion molecules on T cells recognize their ligands on APCs, which enables the T cell receptor and peptide-MHC complex to engage for a sufficiently long period for an activating signal to be transmitted into the T cell (e.g. LFA-1 on T cells and ICAM on APCs) [5]. Antigen-recognition without adequate co-stimulation results in T cell anergy, i.e. unresponsive T cells, or death by apoptosis.

Upon activation, the antigen-specific $CD4^+$ T cells rapidly start to secrete cytokines, such as **IL-2**. Activated T cells upregulate their expression of high-affinity IL-2 receptors (CD25), thus enhancing the ability of the T cell to bind and respond to IL-2. This autocrine signaling stimulates survival and proliferation of the T cell, resulting in a robust expansion of **antigen-specific clones**. These newly activated T cells then differentiate into either effector T cells or memory T cells.

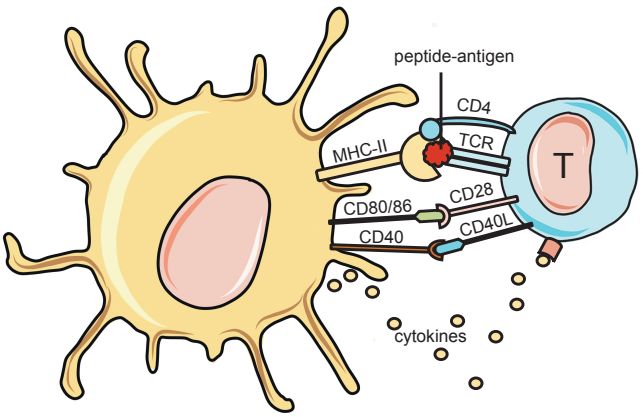


Figure 1. Activation of a naïve $CD4^+$ T cell by an antigen-presenting cell (APC). Activation of naïve $CD4^+$ T cells requires T cell receptor (TCR) recognition of a peptide-antigen presented on MHC-II on APCs in combination with co-stimulatory receptors signaling and stimulatory cytokines.

3.3 Effector $CD4^+$ T cells

Following activation, the T cells are instructed by the APC and the surrounding cytokine milieu to differentiate into functionally distinct effector subsets, which are distinguished on the basis of the specific cytokines they produce and which transcription factors they express. The classical subsets of $CD4^+$ effector T cells include T helper (T_H) 1, T_H2, T_H17, T follicular helper (T_{FH}) cells, as well as peripherally induced Tregs (figure 2). Although the functional specialization of these effector cell subsets is coordinated by distinct genetic programs, there appear to be a certain degree of cellular plasticity and flexibility between these programs [6].

T_H1 cells play a critical role in the defense against intracellular pathogens and produce cytokines, such as IFN-γ, IL-2 and TNF. IFN-γ is a potent activator of macrophages and trigger these cells to enhance their ability to kill phagocytosed microbes. Differentiation into T_H1 cells is primarily stimulated by IL-12, produced by macrophages and dendritic cells, but also by monocytes, neutrophils, and B cells [7]. T_H1 differentiation can also be induced by other cytokines, such as IFN-γ from macrophages, dendritic cells and NK cells. These cytokines activate the transcription factors STAT4, STAT1 and T-bet [8].

In contrast to T_H1 cells, **T_H2 cells** mediate immune responses to extracellular parasites through the production of IL-4, IL-5 and IL-13. Differentiation into T_H2 cells is driven by IL-4, which may be produced by many cell types, such as mast cells, basophils, and by the T_H2 cells themselves. IL-4 activates the transcription factors STAT6 and GATA-3 [8]. In some individuals, T_H2 responses are induced in allergic reactions to innocuous environmental allergens, as further described in chapter 9.

T_H17 cells mediate responses against fungi and extracellular bacteria, but are also associated with autoimmune diseases and chronic inflammation. As implied by the name, T_H17 cells produce IL-17. This cytokine stimulates chemokine production by other cells, which in turn induces inflammation by recruitment of neutrophils and monocytes. T_H17 cells also produce IL-22, which helps to maintain the epithelial barrier functions and promotes repair of damaged tissues. T_H17 development is promoted by TGF-β, IL-6 and IL-1 and is dependent on the transcription factor RORC [9, 10]. Stimulation with TGF-β alone, however, promote naïve $CD4^+$ T cells to differentiate into

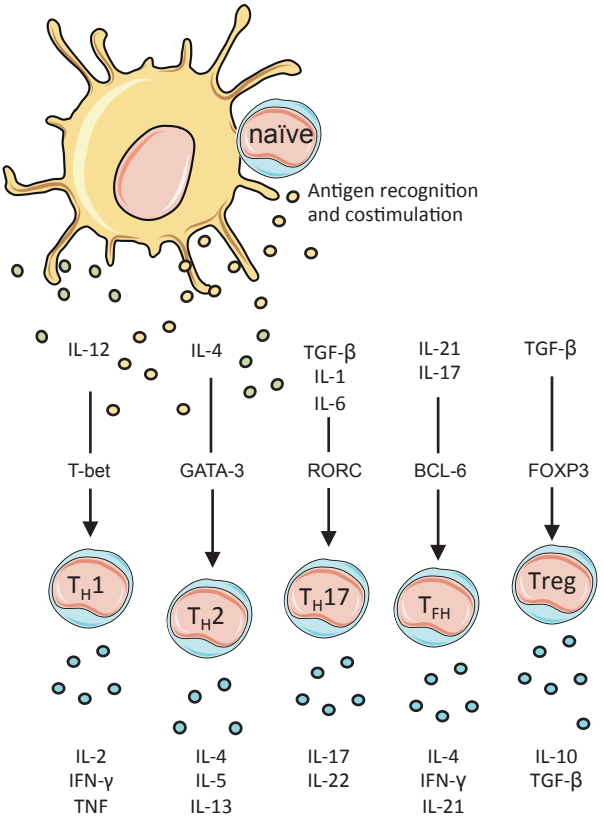


Figure 2. Development of effector T cells. Differentiation of naïve $CD4^+$ T cells into the classical effector T cell subsets and the cytokines and transcription factors involved in the process, as well as the different T effector signature cytokines

$FOXP3^+$ **Tregs**. These peripherally induced Tregs will be further described in section 3.5.

Most differentiated effector T cells leave the lymphoid organs where they were activated and migrate to the site of infection/inflammation. Upon re-encounter with their cognate antigen, the effector cells respond in ways that serve to eradicate the pathogen. **T_{FH} cells**, however, remain in the lymphoid organs and migrate into lymphoid follicles where they stimulate the stepwise activation of antigen-specific B cells [11, 12]. It was recently demonstrated that not only the B cells, but also the T_{FH} cells undergo progressive activation

during this interaction, and that these changes are critical for the concurrent B cell response [12]. T_{FH} cells are induced by the cytokines IL-21 and IL-17 and they produce IL-4, IFN-γ and IL-21. The interaction between T_{FH} cells and B cells in the lymphoid follicles will be further described in section 4.3. Effector T cells are short-lived and die as the pathogen is eliminated, but long-lived memory cells of the same subset and specificity are formed, which are ready to be activated upon re-encounter with the same antigen.

3.4 Memory T cells

The acquisition of long-lived antigen-experienced memory T cells may provide life-long protection against a pathogen. Memory T cells are functionally inactive, but may be rapidly induced to produce cytokines upon a secondary encounter with the antigen that once induced their development. Activation of memory T cells require much less costimulatory signals than activation of naïve T cells. In humans, these cells can be distinguished by the expression of **CD45RO**, as the longer CD45RA molecule is spliced during activation of the naïve T cell [13]. Human blood comprise three major sub-populations of $CD4^+CD45RO^+$ memory T cells; **central memory T cells** and **stem cell memory T cells**, which circulate and migrate to lymphoid tissues, and **effector memory T cells** that have the capacity to traffic to mucosal and other peripheral tissues [14]. In addition, non-circulating **tissue resident memory T cells** predominate in mucosal and peripheral tissues [14, 15].

3.5 Regulatory T cells

In contrast to $CD4^+$ effector T cell subsets that promote pro-inflammatory responses, Tregs are specialized in **suppressing immune responses** toward self- and environmental antigens [16, 17]. Tregs do not only suppress the development and functions of other T cells, but also suppress the functions of B cells and innate immune cells such as macrophages and dendritic cells. During the development in the thymus, T cells that have a T cell receptor with high affinity to self-peptides are induced to develop into Tregs through the mechanism of central tolerance, as described in section 3.1 [18]. In addition to the thymic development of Tregs, antigen stimulation of naïve $CD4^+$ T cells in the periphery can also induce Treg differentiation; *in*

vitro, this conversion is triggered by antigen-stimulation in the presence of TGF-β [17, 19]. A subset of Tregs, called follicular Tregs (**T_{FR} cells**) migrate to the B cell follicle in peripheral lymphoid organs and induce suppression of the T_{FH} cells. This leads to inhibition of the reaction by which B cells develop into antibody-producing plasma cells [20].

3.5.1 Characterization of Tregs

Tregs specifically express **FOXP3**, a transcription factor essential for their development and function [16, 17]. In mice, forced FOXP3 expression converts naïve $CD4^+$ T cells towards a T cell phenotype functionally similar to naturally occurring Tregs [21, 22]. Children born with loss of function-mutations in the *FOXP3* gene develop IPEX syndrome (immune dysregulation polyendocrinopathy enteropathy X-linked syndrome), which is a fatal disorder that often presents in the first year of life. Characteristic symptoms of IPEX syndrome includes severe enteropathy, thyroid abnormalities, early onset of type 1 diabetes, eczema, food allergy, hyper-IgE as well as autoimmune hematologic disorders [23-25].

$FOXP3^+$ Tregs are found within the $CD4^+CD25^+$ T cell population. The fraction of $FOXP3^+$ Tregs is highest within the **$CD25^{high}$** subset, which constitutes approximately the top 2% of the total $CD25^+$ T cell subset [26, 27]. Since not only Tregs, but also newly activated $CD4^+$ T cells express CD25 and FOXP3 [28], analysis of the $FOXP3^+CD25^{high}$ T cell subsets results in a lower contamination of the Tregs by activated non-regulatory T cells [29]. More recently, it been shown that human Tregs express little or no IL-7 receptor (**CD127**); and expression of CD127 in combination with CD25 can therefore distinguish between human regulatory T cells and conventional T cells [30, 31]. Indeed, the proportion of $CD25^+CD127^{low}$ T cells correlate strongly with the proportion of $CD25^+FOXP3^+$ Tregs [30]. Since CD127, unlike FOXP3, is expressed on the cell surface, this allows identification of viable Tregs for functional studies. Figure 3 shows examples of different gating strategies for Tregs.

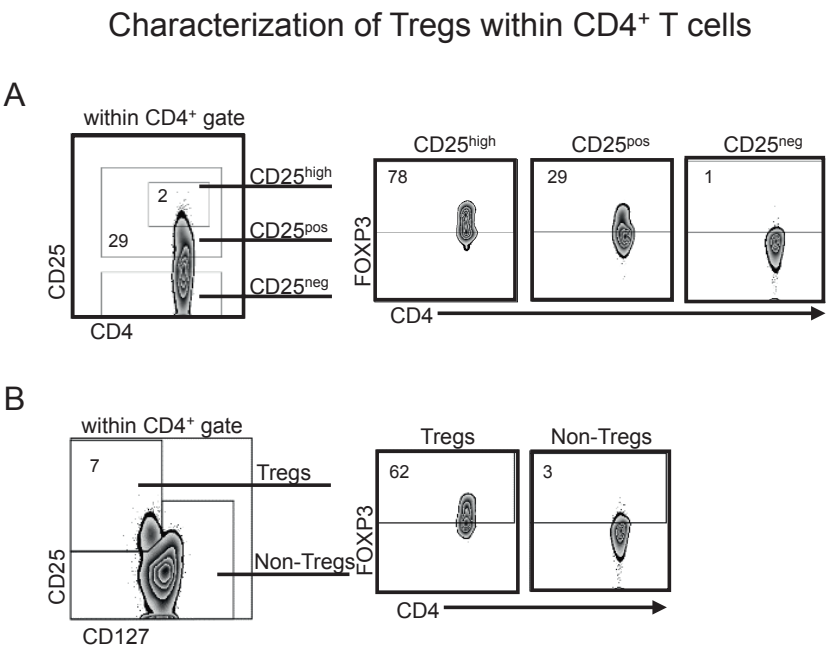


Figure 3. Selected examples of gating strategies for regulatory T cells (Tregs). A) Identification of proportions of FOXP3$^+$ cells that are CD25^{high} or CD25$^+$ within the CD4$^+$ T cell population. The FOXP3 gate is set based on lack of expression within the CD25^{neg} subset. B) Identification of proportions of CD25$^+$CD127$^{lo/neg}$ Tregs within the CD4$^+$ T cell population and proportions of FOXP3$^+$ cells among Tregs and non-Tregs (adapted from paper IV in this thesis).

Similar to conventional CD4$^+$ T cells, naïve human Tregs express the RA isoform of CD45, whereas activated/memory cells express CD45RO [32, 33]. Recent work in mouse models have identified long-lived antigen-specific Tregs with potent immunosuppressive capacities after antigen elimination, i.e. memory Tregs [34, 35], and there is now emerging evidence for memory Tregs also in humans [35].

3.5.2 Treg-mediated suppression

Several mechanisms of Treg-mediated suppression have been proposed, and these include both contact-dependent mechanisms and secretion of immunosuppressive cytokines, such as **IL-10** and **TGF-β**. In adults, FOXP3$^+$ Tregs constitutively express high quantities of **CTLA-4**, which is essential for Tregs to regulate activation and proliferation of other T cells [32, 36, 37]. CTLA-4 binds to its ligand CD80/CD86 on APCs with a higher affinity than the co-stimulatory molecule CD28 on CD4$^+$ T cells. A blockade of CD80/CD86 will make the APCs incapable of providing co-stimulation via CD28, which is crucial for T-cell activation [38, 39]. CTLA-4 may also downregulate CD80 and/or CD86 on APCs by binding and removing these ligands by transendocytosis [40]. As mentioned above, Tregs express high levels of CD25, which is a component of the receptor for the essential T cell growth factor IL-2. Consequently, Tregs may bind and consume large amounts of IL-2, thus reducing its availability for other T cells.

4 B CELLS

4.1 Peripheral B cell maturation

As mentioned in chapter 1, B cells are key players in the humoral immune response, and they mediate their main function through antigen-specific antibodies. Similarly to T cells, B cells develop in the bone marrow from hematopoetic stem cells. When immature B cells leave the bone marrow and enter the blood stream and peripheral lymphoid organs, they undergo several transitional developmental stages before they are fully mature. These stages are defined based on expression of different cell surface antigens, and the current model involves **five major consecutive stages**: immature transitional B cells that have just left the bone marrow, mature naïve B cells that have not yet encountered antigen, actively engaged germinal center B cells, memory B cells and antibody-secreting plasma cells [41] (figure 4).

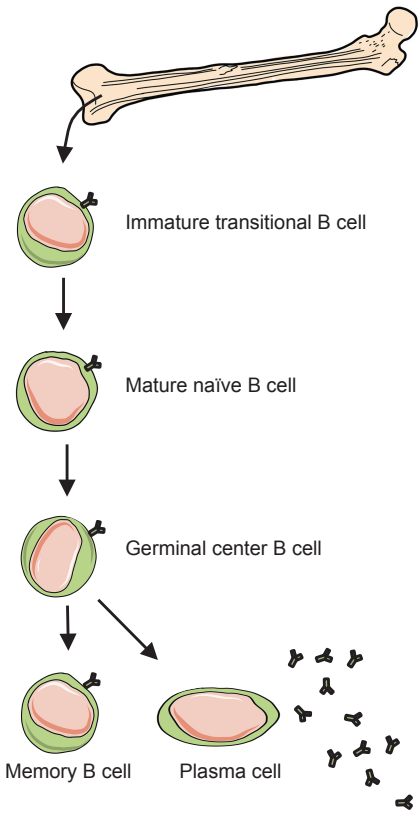


Figure 4. Peripheral B cell maturation.

4.2 T cell mediated activation of B cells

B cells leave the bone marrow and enter the circulation as **$CD24^{hi}CD38^{hi}$ immature transitional B cells** [42]. The expression of CD24 and CD38 gradually decrease as the B cells develop from the immature transitional stage toward a more mature naïve phenotype, and based on this, human transitional B cells can be further subdivided into T1, T2 and T3 B cells, T1 being the most immature [43]. These B cells constantly recirculate between the blood and peripheral lymphoid organs, i.e. lymph nodes, the spleen and mucosal lymphoid tissues. B cell activating factor (BAFF) is a cytokine produced by both innate immune cells and non-hematopoietic cells, such as lymph node stromal cells [44-46], and BAFF has been shown to be pivotal for differentiation of immature transitional cells into mature naïve B cells [47, 48].

When B cells enter a lymph node, they migrate into **follicles** (B cell zone), located in the cortex of the lymph node. **Mature naïve $CD24^{int}CD38^{int}$ B cells** that recognize their specific antigen through their B cell receptor will internalize and process the antigen and then present the peptides on surface MHC-II molecules. Antigen-activated B cells transiently increase their expression of CCR7 and migrate to the border of the B cell follicle and the T cell zone. At this location, the B cells may interact with **$CD4^+$ T_H cells** that recognize the cognate antigen and receive additional signals, e.g. from binding of CD40 to CD40 ligand, which leads to proliferative **expansion of the antigen-specific B cells**. In T cell dependent B cell responses, the antigen-activated B cell can subsequently follow three different pathways [49]:

1. differentiation into extrafollicular **short-lived plasma cells** that typically secretes low levels of antibodies with low affinity
2. differentiation into **germinal center-independent memory B cells**,
3. or **formation of germinal centers** that result in the generation of affinity-mature memory B cells and long-lived plasma cells.

4.3 The germinal center reaction

During the germinal center reaction, the rapidly proliferating B cells acquire random mutations in the antigen-binding region of the B cell receptor to enhance the affinity for the antigen, i.e. **somatic hypermutations**. The affinity-matured B cells may then relocate to the light zone of the germinal center where affinity selection takes place through interaction with antigen-presenting follicular dendritic cells and antigen-specific T_{FH} cells. B cells with disadvantageous mutations will undergo apoptosis, while cells that obtained a higher affinity will survive and undergo **isotype class switch** of the constant region from IgM to IgG, IgE or IgA, which alter the effector function of the antibody. After class switch, the B cells may differentiate into either germinal center-dependent **memory B cells** or into plasmablasts that subsequently mature into **long-lived antibody producing plasma cells**, as also mentioned in section 4.2.

4.4 Plasma cells

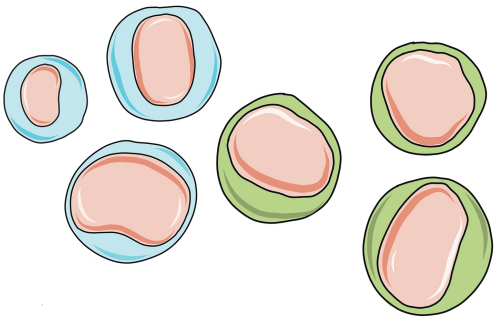
After the germinal center reaction, the plasmablasts tend to migrate to the bone marrow or mucosal tissues where they mature into long-lived plasma cells, which produce **high-affinity antibodies** that may clear the primary pathogen. Plasma cells beneath mucosal surfaces, e.g. in the gut and respiratory organs, produce IgA antibodies that are transported across the epithelia into the lumen of the organ, where it may bind to and neutralize microbes. Plasma cells do not express a surface-bound antigen receptor, but instead release high-affinity antibodies at a constant rate also in the absence of the antigen [50]. Preexisting circulating antibodies function as an immediate protection upon re-encounter with the specific pathogen. Specific serum antibody titers can have half-life in the range of 50-200 years [51].

4.5 Memory B cells

As mentioned above, a fraction of the B cells that have gone through the germinal center reaction develop into isotype-switched memory cells with high affinity for the previous encountered antigen. These cells circulate in the blood and reside in mucosal and other tissues and may survive for extended periods of time, even in the absence of antigen exposure. Indeed, vaccine-specific memory B cells have been detected more than 50 years after smallpox vaccination in humans [52]. In contrast to plasma cells, memory cells express surface bound antigen receptors, and antigen recognition is necessary to trigger a memory response. Upon a re-infection, memory B cells rapidly differentiate into plasmablasts that produce large amounts of high-affinity class-switched antibodies capable of clearing the pathogen [49]. Essentially all B cells that have undergone class switch and somatic hypermutations express **CD27**, which has consequently been considered as a general marker for memory B cells in humans [53]. Memory B cells can also be distinguished by the **CD24^{high}CD38^{neg}** phenotype, and most of these cells do express CD27 [54].

In addition to isotype-switched memory B cells, the existence of **IgM memory B cells**, also called natural memory B cells, have been described [55, 56]. IgM memory B cells are suggested to develop in the spleen upon TLR-stimulation in a T cell independent manner and in the absence of germinal centers [56]. As the name implies, IgM memory cells are non-switched and they produce antibodies of the IgM isotype with fewer somatic mutations as compared to switched memory B cells [56]. It has been suggested that IgM memory B cells are key players in T cell independent immune responses and that lack of these cells are associated with an inability to respond to polysaccharide antigens and increased susceptibility to certain bacterial infections [57].

Thus, the immune system can memorize previously encountered antigens by continued production of antigen-specific antibodies and by memory B- and T cells. These capabilities are fundamental for successful vaccinations, which will be further described in chapter 7.



5 ADAPTIVE IMMUNITY COMES WITH AGE

Most of the global mortality in children under 5 years of age is caused by infections, and the highest mortality rate is found among newborns and infants. This vulnerability slowly decreases as the immune system gradually matures during infancy and childhood, after encounter with an increasing number of pathogens. Due to the low exposure to external antigens *in utero*, the adaptive immune system in newborns is mainly composed of naïve T and B cells. Since these naïve cells require at least 1-2 weeks after antigen encounter to provide effective immune responses, the newborn baby has to rely on the innate immune system to provide protection. However, during this critical period in life the baby is also provided with protective maternal IgG antibodies transferred to the baby during pregnancy and also with maternal IgA if the baby is breastfed (described further in section 5.2).

However, although designated the name "innate", numerous components of this arm of the immune system are also characterized by immaturity and impaired function in neonates as compared to adults [58]. For instance, dendritic cells from newborns express lower levels of MHC-II as well as of the co-stimulatory receptors CD80, CD86 and CD40 as compared to adults [59, 60], and neonatal neutrophils show impaired functions, such as a reduced ability to migrate from the circulation to sites of infection [61]. Furthermore, although the expression of TLRs on innate cells in early childhood appear to be comparable to adult levels, stimulation of TLRs and the inflammasome/IL-1 pathway fail to induce potent proinflammatory responses [62, 63]. Thus, not only the adaptive but also the innate immune system seems to be impaired at birth, which makes the newborn extra vulnerable to infections.

5.1 Peripheral T cell maturation in children

At birth, naïve CD45RA$^+$ T cells constitute approximately 90% of all circulating CD4$^+$ T cells [64, 65]. However, as children grow older and encounter new antigens, the proportion of naïve T cells declines while proportions of CD45RO$^+$ memory T cells increase. The fraction of CD45RA$^+$ T cells drops significantly already in the first month of life, and at three years of age these cells constitute around 70% of all CD4$^+$ T cells in the circulation [64, 65]. The proportions of CD45RA$^+$ T cells continue to decrease throughout childhood, adolescence and adulthood, and at 90 years of age only around 20% of all circulating CD4$^+$ T cells are of a CD45RA$^+$ T cell phenotype [66].

In accordance, the proportions of CD45RO$^+$ memory T cells in peripheral blood increase gradually from approximately 5% of all peripheral T cells at birth to just below 20% at three years of age [14, 64, 66]. At the age of three, there is a large individual variation in the proportions of these cells, ranging from 10% up to almost 40%, which might reflect differences in antigen exposure between children [64, 66]. The proportion of circulating memory T cells continues to increase gradually with age, and constitutes around 80% of all CD4$^+$ T cells at 90 years of age [66]. Consistent with memory T cells in blood, the proportions of memory T cells in peripheral tissues, including lymphoid tissues, gut, lungs, and skin, also increase with age [14, 15]. The adaptive immune maturation progress in childhood is accompanied by an enhanced capacity to produce cytokines. For instance, the production of TNF, IFN-γ, IL-4, IL-5 and IL-10 upon polyclonal stimulation of blood cells increases in the first year of life [67, 68]; and the capacity to produce IFN-γ, IL-4 and IL-5 has been shown to continue to increase in an age-dependent manner at least until adolescence [69].

It is well known that T cells express a broad **homing receptor repertoire**. The expression of certain homing receptors differ based on the activation status of the T cells. For example, naïve CD4$^+$ T cells express the homing receptor CD62L and CCR7, which enable migration into secondary lymphoid organs, as also mentioned in section 3.2. Upon activation, these homing receptors are downregulated and the T cell acquires new homing receptors, which direct the cell to the target tissue where its effector functions are needed [3, 4]. Thus, T cells activated in lymph nodes draining the skin or the lung start to express the homing receptor CCR4, while T cells activated in the small intestine start to express CCR9 [70-72]. Naïve T cells also

express the **homing receptor α4β7**, which interacts with the adhesion molecule MAdCAM-1. In adults, MAdCAM-1 is expressed primarily in the gut-associated lymphoid tissue (GALT), but also in the lactating mammary gland [73, 74]. However, during fetal development and early childhood MAdCAM-1 is also expressed in peripheral lymph nodes [75]. In our prospective birth cohort study, we have previously shown that $CD4^+$ T cells undergo a homing receptor switch, from being $\alpha4\beta7^+$ to $CCR4^+$ in parallel with their differentiation from a naïve $CD45RA^+$ to an activated/memory $CD45RO^+$ T cell phenotype [26]. Indeed, the proportion of $\alpha4\beta7^+$ cells of $CD4^+$ T cells decrease from around 90% in cord blood to 70% at three years of age, similar to the proportions of $CD45RA^+$ T cells, while the proportions of $CCR4^+$ T cells increase [64]. Is thus seems like the expression of α4β7 may be used as a differentiation marker for $CD4^+$ T cells [26].

5.1.1 Regulatory T cells in children

Similar to conventional $CD4^+$ T cells, naïve human Tregs express CD45RA whereas activated cells express CD45RO [32, 33]. The highest fraction of $CD45RA^+$ Tregs is found in cord blood and the proportions of these cells gradually decline with age, whereas the proportions of $CD45RO^+$ Tregs increase [33]. Naïve Tregs also express the homing receptor α4β7, and we have previously shown that a homing receptor switch to CCR4 is associated with memory conversion of Tregs [26].

In line with others, we have shown that the proportion of Tregs among circulating $CD4^+$ T cells increases rapidly during the first days after birth [33, 76]. After this rapid increase, the fraction of Tregs remains relatively constant throughout childhood and adulthood [33]. Interestingly, the proportions of Tregs among $CD4^+$ T cells in cord blood display a remarkably large inter-individual variation, which is considerably reduced already 3 days after birth [76]. Since the variation between children exists already at birth, this suggests that proportions of Tregs in neonates are influenced by the environment *in utero*, genetic factors, or a combination of both.

Interestingly, higher proportions of $FOXP3^+CD25^+$ or $CTLA\text{-}4^+$ Tregs in early infancy have been shown to be associated with lower fractions of $CD45RO^+$ memory T cells and $CCR4^+$ T cells later in childhood [64]. Since Tregs possess potent immunoregulatory properties already at birth [30, 77], a high proportion of these cells in infancy could reduce activation and tissue

trafficking of T cells and thus modulate peripheral T cell maturation during childhood, which may be an explanation for the observed associations.

5.2 Peripheral B cell maturation in children

Almost 95% of all circulating B cells in the first months of life are of a naïve $CD27^{neg}$ phenotype [78]. Approximately 50% of all B cells in newborn children are further characterized as phenotypically immature $CD24^{hi}CD38^{hi}$ transitional cells, and the proportion of these cells decrease in an age-dependent manner until early teenage years, when proportions are comparable with those in adults (~5%) [79]. Similarly, after hematopoetic stem cell transplantation in adults, immature transitional B cells are the first B cells detected in peripheral blood [79], and the proportion of these cells gradually decreases with time, while the proportion of mature naïve $CD24^{int}CD38^{int}$ B cells increases to around 80% 9 months after transplantation [79]. As for memory T cells, memory B cells survive for long periods of time in the absence of antigen, and the proportion of these cells hence increase with age. The proportions of circulating CD27 expressing memory B cells in children are below 5% during the first months in life, increase significantly between 4 and 18 months, and then continue to increase in an age-dependent fashion to approximately 20% in young adults [78, 80]. Figure 5 shows a schematic overview of age-related B cell maturation.

We and others have shown that the vast majority of the circulating transitional B cells also express CD5, both in newborns and in adults [54, 79, 81]. In mice, expression of CD5 identifies a specific B cell lineage, referred to as B-1a cells, which are primarily found in the peritoneal cavity [82]. In humans however, expression of CD5 does not define a specific B cell subset; instead, it likely represents immature naïve B cells. Indeed, CD5 expression decreases gradually as the B cells develop from the immature transitional stage via mature naïve to memory B cells [43, 54, 79]. We have shown that the proportions of circulating $CD5^+$ B cells increase significantly from birth (~40%) up to one month of age (~70%); thereafter, the proportions of these cells decease in an age-dependent manner, similar to transitional B cells [80, 83]. Furthermore, approximately 90% of the peripheral $CD5^+$ B cell population in both children and adults are of a naïve $CD24^{hi/int}CD38^{hi/int}$ phenotype [81].

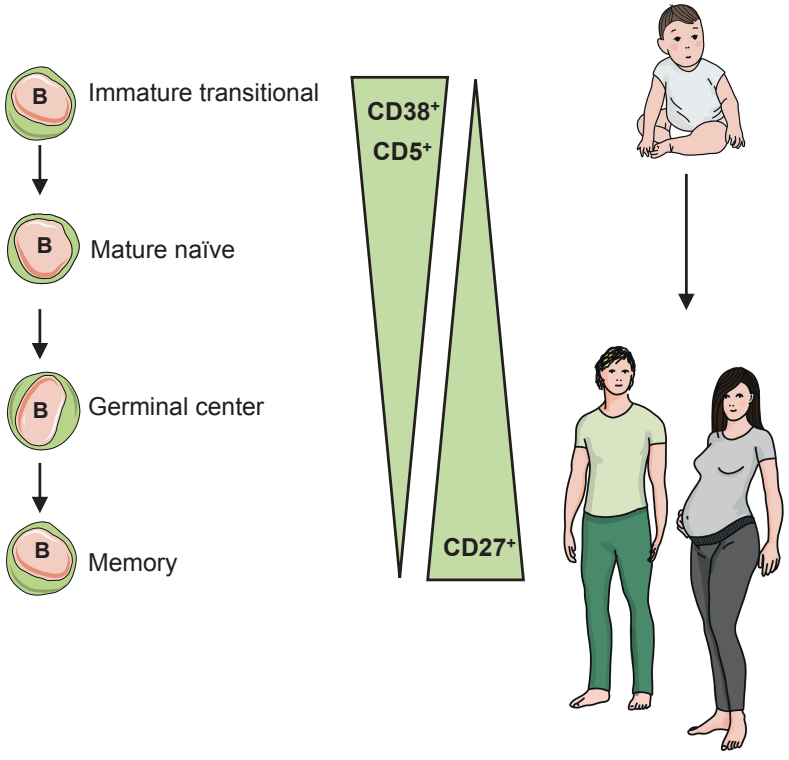


Figure 5. Age-related B cell maturation. Selected examples of phenotypical cell surface markers that distinguish different peripheral B cell maturation stages as well as their proportional changes in blood with age.

A general immaturity in the early life B cell compartment is also accompanied by **blunted antibody production** as compared with adults. However, in parallel with postnatal B cell maturation, the antibody-producing capacity is enhanced and there is an age-related increase in IgG- and IgA-expressing B cells in the circulation [84, 85]. Numerous different factors have been suggested to account for impaired neonatal B cell responses [86]. For instance, neonatal B cells and dendritic cells have lower surface expression of the co-receptors CD40, CD80 and CD86 compared with cells from adults [60]. Additionally, neonatal T cells have a lower gene expression

of *CD40 ligand* as compared to adults [60]. Since the interaction between CD40 and CD40 ligand along with CD80/86 and CD28 is crucial for T cell activation, and the interaction between CD40 and CD40 ligand is central for proliferative expansion of antigen-specific B cells, hampered interactions between these molecules in neonates most probably contribute to decreased B cell responses in neonates. Additionally, a reduced capacity of neonatal T_{FH} cell expansion and defective localization of these cells within the lymph nodes have been suggested as other key limiting factors for effective B cell responses in early life [87].

To counterbalance these deficiencies in neonatal immunity, **maternal IgG** antibodies pass through the placenta to the blood stream of the baby during pregnancy. These passively transferred antibodies will protect the infant during the first months of life from infections already experienced by the mother. If the baby is breastfed, it will also receive **maternal IgA** antibodies through the breast milk. These antibodies are the product of maternal immune responses to pathogens encountered in the gut and in the airways, and will be passively transferred to the gut of the infant to provide protection. Thus, in addition to transplacental transfer of maternal IgG, a breastfed infant will also be provided with local protection against gastrointestinal pathogens that are common in the environment where the mother lives [88].

5.3 Absolute numbers of T and B cells

The absolute numbers of circulating $CD4^+$ T cells and B cells peak at around 4 months in life, and thereafter gradually decrease with age [89, 90]. In our prospective birth-cohort, we demonstrate that higher total numbers of $CD4^+$ T cells during infancy is associated with higher proportions of naïve $\alpha4\beta7^+$ T cells, but with lower proportions of $CD45RO^+$ memory T cells in early childhood as well as in school-aged children [89]. Similarly, high numbers of B cells in infancy are associated with higher proportions of naïve $\alpha4\beta7^+$ T cells and lower proportions of $CD45RO^+$ memory T cells in childhood [89]. Important to emphasize is that these results do not demonstrate any causal relationship between low infantile lymphocyte counts and immune maturation and activation. Instead, our results indicate that higher total numbers of $CD4^+$ T cells and B cells in infancy may reflect a more immature/naïve adaptive immune system in general.

6 WHAT MAY INFLUENCE ADAPTIVE IMMUNE MATURATION IN CHILDREN?

6.1 Geographical differences

It has been demonstrated that cord blood lymphocyte maturation and activation status differ notably between babies born in areas with a more traditional lifestyle with high microbial exposure, and babies born in areas with lower microbial exposure. For example, neonates from a semi-urban area in Gabon present with significantly lower proportions of naïve $CD5^+$ B cells at birth compared to babies born in an urban area in Austria [91]. In addition, the expression of the co-stimulatory molecule CD28 on $CD4^+$ T cells is significantly reduced in cord blood from Gabonese children [91]. Since CD28 have been shown to be down-regulated on antigen-experienced T cells [92], these results suggest that neonates in Gabon have a more mature T cell compartment as compared to neonates in Austria.

The same tendency also applies at older ages, as adolescents and adults from rural or semi urban parts of Malawi and Ethiopia, respectively, present with significantly higher proportions of antigen-experienced memory $CD4^+$ T cells than their age-matched counterparts in the UK and in the Netherlands [93, 94]. Furthermore, adults from the Netherlands present with higher total numbers of circulating $CD4^+$ T cells compared to the Ethiopian group [93]. This supports our finding that higher numbers of $CD4^+$ T cells may reflect a more naïve/immature adaptive immune system [95]. Interestingly, in a study comprising young adults from rural areas of Senegal, urban areas of Senegal, and urban areas of the Netherlands, the proportions of memory T and B cells show a rural to urban gradient [96]. Thus, young adults from rural areas of Senegal presented with highest proportions of both $CD45RO^+$ memory T cells and $CD27^+$ memory B cells, while individuals from urban areas of the Netherlands presented with the lowest [96].

Regarding Tregs, it has been shown that the proportion of circulating $CD4^+$ T cells that are $CD25^{hi}$ is significantly lower in cord blood from Gabonese compared to Austrian neonates [91]. Also, the expression of FOXP3 and CTLA-4 by $CD4^+CD25^{hi}$ T cells is significantly lower in cord blood from the Gabonese infants [91]. In line with this, cord blood from newborn babies in rural areas of Papua New Guinea constitute significantly lower proportions of Tregs compared to cord blood from babies in urban parts of Australia [97]. The finding that environments with higher microbial exposure seem to be associated with lower proportions of blood Tregs in children, will be further discussed in section 6.2.2. Although some of the geographical differences reviewed above may owe to genetic variability, these results point to that the environment, both pre- and postnatally, impacts adaptive immune maturation.

6.2 The farming environment

The traditional farming environment with many different animal species provides an environment with a high microbial antigen diversity that have few equals in western affluent countries today. The airborne dust in animal sheds contains particles from numerous species of bacteria and molds, which are transported into the dwelling house. Indeed, farming environments are associated with increased exposure to various microbial products, and **higher levels of endotoxins**, i.e. lipoplysacharide (**LPS**), and mold β(1,3)-glucans as well as fungal extracellular polysaccharides have been measured in house dust of farming families [98-100]. Interestingly, a recent study shows that endotoxin levels in airborne dust were strikingly higher in homes of Amish, who practice traditional farming on single-family farms, compared to homes of Hutterites, who live on larger and more industrialized farms [101]. Thus, since there seem to be a gradient of endotoxin exposure related to farming practices, this should be considered when comparing results from separate studies investigating associations between farming environment and immune maturation.

6.2.1 The farming environment and innate immunity

It has been shown that blood cells from farmers' children have a significantly higher spontaneous production of IL-12 and IL-10, and that this production correlates with the number of specific farm exposures [102]. Additionally, farmers' children have a significantly higher gene expression of *TLR2*, *TLR4*, and *CD14* in peripheral blood compared to non-farmers' children at school age [103-105]. Both TLR2 and TLR4 are membrane-bound receptors that recognize various PAMPs on pathogen surfaces, while CD14 interacts with several TLR ligands and enhances their ability to activate TLRs. The authors suggest that the difference in expression is related to increased environmental exposure to microbial compounds in the farming environment.

Interestingly, the increased gene expression among farmers' children was not associated with the current exposure of the child, but rather to maternal exposure during pregnancy [104]; and upregulation of gene expression in farmers' children increased along with increasing number of farm animal species encountered by the mother during pregnancy [104]. Since TLR2 and TLR4 are receptors for different types of microbial compounds, the authors speculate that the dose-dependent upregulation of these receptors may be due to exposure to both increased levels and diversified microbial compounds [104]. Thus, maternal exposure to the rich microbial environment of traditional farms during pregnancy may induce prenatal immuno-programming with long-lasting upregulation of innate immunity gene expression.

Long-term exposure to high endotoxin levels may, however, also induce a tolerogenic mechanism referred to as **LPS-tolerance** [106]. This phenomenon may explain why school-aged children who were exposed to higher levels of endotoxins in childhood have peripheral blood cells with a lower capacity to produce TNF, IL-12, and IL-10 in response to LPS-stimulation *in vitro* [100]. Similarly, lower LPS-induced TNF production by peripheral blood mononuclear cells (PBMCs) among farmers' children at 4 years of age has been reported [102]. Also, in the study of school-aged Amish and Hutterite children mentioned above, blood cells from Amish children produced significantly lower levels of several cytokines after LPS-stimulation *in vitro,* as compared with Hutterites [101]. In addition, Amish children had increased proportions of neutrophils and eosinophils, but similar proportions of monocytes as compared with Hutterites [101].

6.2.2 The farming environment and adaptive immunity

The farming environment and $CD4^+$ T cells – prospective studies

Longitudinal data that demonstrate the influence of a farming environment on adaptive immune maturation in children have been lacking. To the best of our knowledge, we are the first to present detailed prospective data that demonstrate how farmers' and non-farmers' children from the same rural area differ in regard to peripheral T cell maturation throughout childhood. In the FARMFLORA birth-cohort study, we demonstrate that growing up in a dairy farming environment is associated with lower proportions of Tregs, defined as $FOXP3^+CD25^{hi}$ of $CD4^+$ T cells, but with a more pronounced T cell memory conversion and PBMCs with higher PHA-induced production of IFN-γ and IL-1β in the first three years in life [76]. Although these findings need to be confirmed in additional larger prospective birth-cohort studies, they suggest that growing up in a farming environment is associated with a more rapid acquisition of adaptive immunity in childhood. This may partly be explained by exposure to higher levels and diversity of microbial compounds that trigger innate and adaptive immune responses among farmers' children.

The farming environment and $CD4^+$ T cells - cross sectional observations

Few studies have investigated whether proportions of Tregs differ between farmers' and non-farmers' children and results are conflicting. This inconsistency may in part confer to differences in phenotypic characterization of Tregs in the studies. A study in rural parts of Germany has shown that farmers' children present with higher proportions of $CD4^+CD25^{hi}$ cells of total cord blood mononuclear cells compared to non-farmers' children [107]. This observation was not confirmed in our Swedish cohort, where no difference in proportions of $FOXP3^+CD25^{hi}$ of $CD4^+$ T cells in cord blood was observed between farmers and non-farmers children [76]. At three days of life, however, farmers' children in Sweden presented with significantly lower proportions of these putative Tregs in the circulation than non-farmers' children [76].

The Swedish farm children had significantly lower proportions of $FOXP3^+CD25^{hi}$ of $CD4^+$ T cells also at 3 years of age as compared to non-farmers' children [76]. A study from central Europe shows that the proportions of Tregs, defined as the upper 20% $CD4^+CD25^+$ T cells that were $FOXP3^+$ among total lymphocytes, did not differ between farmers' and non-farmers' children at 4 years of age [108]. However, when they, in the

same study, identified Tregs as $CD4^{+}CD25^{hi}CD127^{-}$ of total lymphocytes, they found that the proportions of these cells were significantly increased among farmers' children [108]. Since differences in phenotypic characterization of Tregs from a single study resulted in dissimilar outcomes, this also illustrates difficulties when comparing the outcomes from several separate studies. Although significant differences were observed regarding endotoxin exposure and innate immunity gene expression between Amish and Hutterites, the proportions of Tregs, defined as $CD4^{+}FOXP3^{+}CD127^{-}$, did not differ between these two groups of children [101].

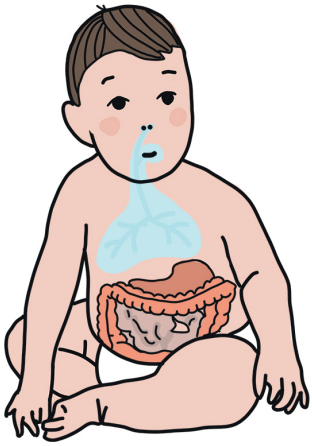
In addition to differences in phenotypic characterization of Tregs, another explanation for discrepancies in results between studies are putative differences in farming practices, as reviewed in section 6.2 . In Sweden, cows are kept in a barn separated from the dwelling house during the winter period. In many of the studies from central Europe, however, the animal sheds are generally located closer to the dwelling house, not seldom even in the same building. One more important parameter for inconsistent results is, of course, the differences in life style between the farming families and the control families.

It has further been shown that mitogen-stimulated blood cells from children born by mothers who live on a farm have increased capacity to produce IFN-γ at birth [109] and at 3 months of age [110], compared to non-farmers' children. In our prospective cohort, a farming environment was associated with higher cytokine responses upon mitogen-stimulation of PBMCs at 18 months of age [76]. In addition, the spontaneous production of IL-10, IL-12 and IFN-γ by PBMCs is higher among farmers' children at 4 years of age, and this production correlated with the number of specific farm exposures [102].

The farming environment and B cells

Studies investigating longitudinal associations between farming environments and peripheral B cell maturation are lacking. However, we demonstrate that farmers' children have significantly higher proportions of memory B cells, defined as $CD27^{+}$ or $CD24^{hi}CD38^{lo/neg}$, but lower proportions of $CD24^{int}CD38^{int}$ mature naïve B cells at 8 years of age, as compared to non-farmers' children in the same rural area (Strömbeck et al *in manuscript*: paper IV in this thesis). As mentioned in section 4.1, the cytokine BAFF is crucial for differentiation of transitional B cells into mature naïve cells, and we have shown that higher BAFF levels at birth are associated with a higher degree of

peripheral B cell maturation in the first three years in life [111]. There is a striking inter-individual difference in cord blood BAFF levels, which suggests that BAFF levels may be influenced by prenatal immuno-programming. Indeed, babies born by mothers who live on dairy farms have significantly higher BAFF levels in cord blood compared to babies born by non-farming mothers [111]. Thus, the increased degree of maturation of the circulating B cell compartment in farmers' children may be initiated already before birth by prenatal immunoprogramming due to maternal farm exposure.



6.3 The gut microbiota

The gut microbiota may stimulate adaptive immunity in children by several means. Lymphocytes are found throughout the intestinal tract, both in organized tissues, such as **Peyer's patches** and isolated lymphoid follicles, and scattered close to the mucosal epithelium and in the underlying lamina propria. These organized secondary lymphoid structures in the gut, together with the draining **mesenteric lymph nodes**, comprise the gut associated lymphoid tissue (**GALT**).

The intestines are generally designed to protect us from entry of harmful pathogens. However, GALT has an epithelial structure that facilitates antigen entry. Epithelial **M cells**, localized over Peyer's patches in the small intestine, are specialized for sampling and transepithelial transport of antigens [112]. These antigens are then captured by dendritic cells and macrophages within

the Peyer's patches and presented to the lymphocytes [113]. Tissue-resident dendritic cells are also able to open tight junctions between adjacent luminal epithelial cells and stretch dendrites between the epithelial cells into the intestinal lumen to directly take up bacteria [114]. Early in life, bacteria can also cross the gut epithelium via a process termed **translocation** and thereby get in contact with the underlying lymphoid tissues and tissue resident dendritic cells and macrophages. Later in childhood, however, production of **secretory IgA** antibodies together with a **protective mucus layer** will block translocation. Thus, acquisition of a diverse gut microbiota with a broad spectra of bacterial antigens early in infancy, may provide important stimuli for the developing immune system in children.

Numerous **experimental studies** in various animal species have emphasized the importance of bacterial gut colonization for normal maturation of the immune system. For example, mice raised under germ-free conditions present with fewer and smaller Peyer's patches with reduced numbers of T cells as well as with mesenteric lymph nodes that lack distinct germinal centers [115, 116]. Germ-free mice also have lower proportions of Tregs within the $CD4^+$ T cell population in the colon lamina propria compared to colonized mice [117, 118]. It has also been shown that serum antibody levels during the first weeks of life differ significantly between piglets that were colonized shortly after birth and piglets that were maintained uncolonized [119]. At 2 weeks of age, serum IgM levels were 30-fold higher in colonized compared to non-colonized piglets and at 6 weeks, the IgG levels were more than 10-fold higher [119]. Thus, it is clear that the gut microbiota provides critical stimuli for adaptive immune maturation; however, the impact of infantile gut bacterial colonization pattern on long-term adaptive immune maturation in children has not yet been established.

The infantile gut colonization pattern has changed over the last decades in Sweden and other affluent countries, possibly due to the parallel improvement of sanitary conditions at maternity wards and in the home environment [120]. *Escherichia coli (**E. coli**),* **bifidobacteria** and ***Bacteroides*** are recognized as early gut colonizers in classical studies of the infantile gut colonization pattern, and at least the acquisition of *E. coli* is often delayed in modern western societies [120-122]. Instead, early colonization by typical skin bacteria, such as *Staphylococcus aureus* (***S. aureus***) and other staphylococci has become more common in western infants, possibly due to a reduced competition from more "traditional" gut colonizers [120].

As mentioned above, IgA is an important contributor to gut barrier function, and IgA-secreting plasma cells of the gut are predominantly found within the lamina propria. Studies of infant gut biopsies show that no plasma cells are present in the lamina propria at birth [123, 124], which is yet another contributing factor for the enhanced susceptibility to infections among newborns. The first plasma cells observed in the infant gut are IgM plasma cells, and the first IgA plasma cells appears around 12 days of life [123]. Both IgM and IgA plasma cells increase quantitatively during the first weeks of life; but IgA plasma cells increase more rapidly and are the predominant plasma cell isotype in infant gut within 3 weeks of life [123]. Thus, plasma cell antibody responses in the gut seem to develop along with the establishment of the gut microbiota. Interestingly, it has been shown that Pakistani infants from poor urban areas have an accelerated maturation of the salivary secretory IgA system compared to age-matched Swedish infants [125]. At 2-3 weeks of age, the Pakistani infants had significantly higher levels of secretory IgA against *E. coli* compared to Swedish infants, and this difference increased during the first months of life [125]. Indeed, gut colonization by *E. coli* starts earlier in Pakistani compared to Swedish infants [126]. Similar associations were observed in another prospective study where salivary secretory IgA was compared between Swedish and Estonian children [127]. According to the authors, the accelerated maturation of the salivary IgA system in the Estonian children was most probably a consequence of higher microbial pressure in their environment [127]. It has further been shown that the number of *Bifidobacterium* species in the infantile gut flora of Swedish children correlate with salivary secretory IgA levels during the first year in life [128].

By multivariate factor analysis, we have previously shown that infantile gut colonization by *E. coli* and/or bifidobacteria (i.e. presence yes/no in faecal cultures) is associated with higher numbers of circulating $CD27^+$ memory B cells in children up to up to 18 months of age in our prospective birth-cohort study [80]. Early gut colonization by *E. coli* and bifidobacteria also seems to be associated with an enhanced PHA-induced capacity of circulating PBMCs to produce cytokines, such as TNF, IL-6, IL-5 and IL-13, at 3 years of age (Rabe et al, *in manuscript*; Appendix A). In our cohort, PHA-induced production of IFN-γ was unrelated to the early gut colonization pattern. Others have reported that PHA-induced IFN-γ production by PBMCs at 1 year of age is unrelated to infantile colonization by *Bacteroides fragilis* [128].

Although the infantile gut colonization pattern has changed over the last decades in Western infants, it is still not known whether these alterations

influence child health. Nevertheless, alterations in gut colonization patterns have been implicated in the predisposition of several different diseases, such as for example allergy (see section 9.5), and the link between acquisition of the gut flora and development of allergy is indeed an area of intense research.

6.4 Delivery mode

The rate of children born via cesarean section has increased rapidly both in the US and Europe over the last decades [129, 130]. It is known that the intestinal microbial colonization pattern differs between infants delivered by cesarean section or vaginally. For example, sectio-delivered infants display a delayed intestinal colonization by *E. coli* and *Bacteroides* and they also have a lower intestinal microbial diversity [120, 131]. Surprisingly little is however known regarding long-term differences in adaptive immune maturation between children born vaginally or by cesarean section.

When we prospectively investigated associations between delivery mode and adaptive immune maturation in childhood, we found that children born by cesarean section had significantly higher proportions of circulating Tregs at 18 months of age, compared with vaginally born children [132]. Multivariate analyses further showed that delivery by sectio was associated with higher proportions of naïve $CD5^+$ B cells in infancy as well as with higher total numbers of T and B cells at 18 months of age. In contrast, vaginal birth was associated with higher proportions of $CD45RO^+$ memory T cells and $CD27^+$ memory B cells [132]. Even though only 9 out of 55 children were born via cesarean section in our birth cohort, these results indicate that delivery by sectio is associated with delayed adaptive immunity in early childhood. However, no associations between delivery mode and adaptive immune maturation were observed when the children had reached school age (unpublished results). Our results from early childhood are in accordance with a recent prospective study demonstrating that 2-10 year old sectio-delivered children present with lower proportions of memory T cells compared to vaginally born children [133]. Further, during the first two years in life, $CD4^+$ T cells from children delivered by cesarean section show a decreased proliferative capacity after *in vitro* stimulation [133]. In contrast to our results, Puff et al found no difference in proportions of Tregs between sectio-delivered and vaginally born children through childhood [133]. Thus, further studies are needed to validate the observations above, and to establish how delivery by cesarean section influences adaptive immunity in children.

6.5 Sex-related differences

In the majority of developing countries, the mortality rate is significantly higher among boys than girls during the first 5 years in life, although they have equal access to food and medical care [134]. This is suggested to be a consequence of a higher vulnerability to infections among boys. Indeed, sex-related differences in immune responses have been demonstrated for several cell subsets of both the innate and adaptive arm of the immune system in adults, and women generally mount stronger and more effective immune responses than men to viral and bacterial infections [135]. However, few studies have investigated sex-related differences in immune maturation during childhood.

By using multivariate analyses, we have shown that adaptive immunity differ considerably between girls and boys during the first three years in life [132]. We show that being a girl is associated with higher proportions of $CD45RO^+$ memory T cells at birth and in infancy, and also with mononuclear cells with a higher capacity to produce IL-13 and TNF at 4 months of age [132]. Additionally, girls have significantly lower proportions of Tregs than boys at birth and in infancy as well as lower proportions of naïve $CD5^+$ B cells during the first 3 years in life [111, 132]. Girls also present with higher BAFF levels at birth compared to boys [111]. In all, our results indicate that girls have a more activated adaptive immunity in infancy and early childhood in comparison to boys. However, other studies show that boys mount higher PHA-induced IFN-γ responses at 1 year of age and higher IL-5 and IL-13 responses at 3 years of age compared to girls [136], and that Treg frequencies do not differ between girls and boys in the first year of life [137].

Regarding lymphocyte counts, girls appear to have significantly higher numbers of $CD4^+$ T cells in cord blood and also in the first year of life, while B cell numbers do not seem to differ between girls and boys at this age [132, 138]. In line with others, we show that girls also mount greater antibody responses to certain vaccines [132, 139], which will be further discussed in section 8.3. In all, biological sex may be an important source for variations in early life immune maturation. However, further studies are needed in order to establish the observed associations between sex and early life immune maturation, and also to investigate the cause for these associations.

7 VACCINATION

Successful vaccination relies on the induction of an immunological memory that mediates protection from infection upon re-exposure to the pathogen. The concept of vaccination is believed to originate several hundred years ago. However, the major breakthrough in vaccination came in 1796 when Edward Jenner used cowpox as a vaccine against smallpox. This act was based on the observations that dairy maids who had suffered from cowpox were naturally protected from smallpox. Smallpox is the first infection that has been fought by vaccination on a global scale, and in 1980, almost 200 years after Jenner's initial experiments, smallpox was officially declared eradicated.

In 1974, the World Health Organization founded the Expanded Program on Immunization to provide all children in all countries with protection against 6 different infectious diseases, i.e. tuberculosis, diphtheria, tetanus, pertussis (whopping cough), measles and poliomyelitis, through routine vaccinations. Today, the global childhood immunization program recommends vaccination against 12 different pathogens [140], and the program is nationally designed to provide optimal protection in each country. A high vaccination coverage in a population is central for preserving the herd immunity, which offers protection also to unvaccinated individuals. In Sweden, 98-99% of all two-year-old children are immunized against diphtheria, tetanus and pertussis (the DTP-vaccine), and 95% have received vaccine against measles, mumps and rubella (the MMR-vaccine) [141]. During 2015, approximately 86% (116 million) of all infants in the world received complete DTP-vaccination. However, it is estimated that more than 19 million infants throughout the world do not receive the recommended doses of basic vaccines [142].

As described in chapter 5, babies and young children are particularly vulnerable to infections, and protection against infections through efficient vaccinations is one of the most effective methods to reduce morbidity and mortality among children across the world. However, as a consequence of the neonatal immune immaturity, vaccine-induced immune responses in early life

are generally low and the efficacy hence limited. Additionally, as described in section 5.2, maternal IgG antibodies are passively transferred to the child during pregnancy in order to protect the child and attenuate infections during initial maturation of the immune system. Although maternal antibodies are very efficient in protecting the infant during the first months of life from infections already experienced by the mother; these antibodies may unfortunately also hamper vaccine-induced immune responses, which may further complicates neonatal vaccine strategies [143].

7.1 Different types of vaccines

Vaccines can be classified into three major groups; live attenuated vaccines, inactivated vaccines, and subunit/acellular vaccines. **Live attenuated vaccines** comprise weakened versions of the pathogens that are still able to replicate and interact with the immune system in the host. This group includes vaccines against diseases caused by viruses, e.g. measles, mumps, rubella, and yellow fever, or by bacteria, e.g. tuberculosis. These vaccines are safe to use in healthy individuals but since these vaccine pathogens can replicate, they may cause severe or fatal reactions in individuals with immunodeficiencies, e.g. patients with common variable immune deficiency, leukemia, or HIV-infection.

The second major group of vaccines, **inactivated vaccines,** can be composed of either killed whole viruses or bacteria, while the third major group, **acellular** or **subunit vaccines**, consist of purified subunits of the pathogens that in turn can be either protein- or polysaccharide-based. Further, there are also vaccines based on detoxified bacterial toxins (toxoid vaccines), which are used against bacterial diseases in which the toxins are the cause of illness, such as diphtheria or tetanus. All these vaccines lack the ability to replicate and are thus safe to administer also to individuals with immune deficiencies and will collectively be referred to as **non-live vaccines** in this thesis.

7.2 How do vaccines induce protective immunity?

As for any natural infection, the very first requirement for a vaccine to trigger an efficient immune response is to provide **sufficient danger signals** that trigger activation of the innate immune system. As live attenuated vaccines consist of whole viruses or bacteria, it is proposed that they activate innate immunity through **several different PRRs**, such as TLRs.

Non-live vaccines may still contain PRRs that trigger an innate immune response. However, owing to the lack of replication, these vaccines induce a more limited immune activation as compared to live vaccines. Therefore, most non-live vaccines require formulation with specific **adjuvants** to trigger sufficient innate immune activation, and the choice of adjuvant will skew the immune response. Several compounds with adjuvant properties exist, and these compounds may be divided into two main categories based on their mechanisms of action: immune stimulatory adjuvants and delivery systems. **Immune stimulatory adjuvants**, e.g. agonists for various TLRs, trigger activation of APCs through different PRRs, which ultimately leads to adaptive immune responses. **Delivery system adjuvants**, such as the aluminum salt-based adjuvant alum and emulsion adjuvants, can function as carriers to which the vaccine antigens can be associated, and these types of adjuvants may also prolong the antigen deposit at site of injection and create local inflammatory responses. The adjuvant effect of **alum** was discovered almost a century ago, and alum is still by far the most widely used adjuvant. Previously, alum was thought to act only through depot formation at the injection site; today it is known that alum-based adjuvants have several effects that account for their adjuvant properties, which also includes direct immune stimulation. However, alum does not act via TLRs to activate innate immunity [144]. Instead, it is suggested that alum activates the NLRP3 inflammasome, which results in release of the proinflammatory cytokine IL-1 [145, 146]. Alum-based adjuvants preferentially induce T_H2 cells that ultimately results in a robust antibody response [147]; yet, it remains unclear by which mechanisms alum induces T_H2-responses.

If the vaccine antigen with or without the addition of adjuvants exhibit sufficient danger signals, it will trigger activation of dendritic cells that migrate to the draining lymph nodes. The immune responses to live attenuated vaccines are virtually identical to that produced by a natural infection and include activation of both $CD4^+$ and $CD8^+$ T cells. However, since non-live vaccines cannot replicate, vaccine antigens are not presented

on MHC-I molecules; $CD8^+$ T cells are thus generally neither generated nor needed. Yet, some dendritic cells have the ability to present ingested antigens on MHC-I to $CD8^+$ T cells in the process of cross-presentation. As described in detail in section 4.2, activated $CD4^+$ T cells provide crucial help to B cells that recognize the cognate antigen. This interaction results in generation of both long-lived plasma cells that produce high-affinity vaccine-specific antibodies as well as long-lived affinity-matured memory B cells, which is the ultimate goal of most vaccinations.

Since T cells only recognize protein antigens, vaccines purely based on polysaccharide antigens fail to activate T cells. B cell responses to polysaccharide antigens do not include formation of germinal centers and are accordingly characterized by the induction of moderate levels of low-affinity IgM antibodies that wane after a few months and a lack of B cell memory. In the absence of immunologic memory, subsequent re-exposure to the same polysaccharide antigen elicit the same immune response in vaccinated as in non-vaccinated individuals [148] However, if polysaccharides are conjugated to a protein antigen, antigen-specific T cells become activated and provide help to the B cells. This will result in germinal center formation, high-affinity antibody producing plasma cells, and memory B cells.

Almost all current vaccines work through induction of vaccine-specific antibodies, and antibodies in sufficient quantities are the predominant correlate of protection for most vaccines. For some vaccines, e.g. against diphtheria, tetanus, measles and rubella, a certain antibody level almost ensure protection. For other vaccines, however, infections may occur despite antibody titers above nominally protective levels [149].

8 DOES BASELINE IMMUNE MATURATION AFFECT VACCINE RESPONSES?

Although vaccine formulations and immunization schedules are similar in most countries, immune responses to certain vaccines differ significantly between populations across the globe [150]. The reasons underlying these variations are likely complex and may involve differences in practical handling of vaccines. For instance, vaccines generally need to be kept in a narrow temperature range from the point of manufacture to their use, and difficulties in keeping the cold-chain infrastructure in some geographical regions may contribute to population-based variations in vaccine responses. In addition, the impact of environmental exposures that may shape baseline immune maturation and activation prior to vaccination may also impact the magnitude and profile of vaccine-induced immune responses.

8.1 ...to live attenuated vaccines?

Although several studies show variations in vaccine-induced immune responses, few longitudinal studies have investigated whether baseline immune maturation and activation prior to vaccination is related to vaccine responses. It has been shown that young healthy adults in Uganda present with significantly lower vaccine-specific antibody titers in the circulation after vaccination with the live attenuated viral vaccine against yellow fever (YF-17D), compared with age-matched vaccinees in Switzerland [151]. These differences were attributed to variations in immune maturation and activation status between the populations before the vaccination; the Ugandan population having a heightened activation of innate immunity, as well as increased proportions of memory T and B cells prior to vaccination [151]. These results indicate that a heightened baseline immunity before vaccination negatively affects vaccine responses to the live attenuated vaccine against yellow fever. In line with this, we have shown that higher proportions of circulating memory T cells and higher cytokine producing capacity of PBMCs prior to vaccination in early childhood is associated with lower

vaccine-specific antibody titers after vaccination with the live attenuated vaccine against measles, mumps, and rubella (MMR) in our prospective birth cohort study [132]. Also, higher proportions of Tregs, which has previously been linked to delayed adaptive immunity [64], was associated with increased vaccine-induced antibody responses [132]. Interestingly, 7 days after vaccination against yellow fever, the YF-17D viral load in plasma was strikingly lower in the Ugandan compared to in the Swiss population [151]. The authors thus speculate that the heightened baseline immunity prior to vaccination negatively affects vaccine responses, at least partly, by limiting viral replication in these individuals.

In contrast to most vaccines, the live bacterial BCG-vaccine against tuberculosis does not mediate protective immunity by induction of vaccine-specific antibodies; instead, BCG-induced T cells are the main effectors. The BCG-vaccine is administered at birth or soon after in many high-risk countries and, as with many other vaccines, the efficacy of the vaccination show large geographical variations. Interestingly, after BCG- vaccination, infants in Malawi develop T cells with a cytokine profile that is strikingly different from that of infants in the UK [152]. Upon *in vitro* stimulation of whole blood with purified vaccine-antigen 3 months after vaccination, cells from UK infants produced higher levels of IFN-γ and IL-2, whereas the cells from Malawi infants generally produced higher levels of T_H2 related cytokines as well as higher levels of the regulatory cytokine IL-10 [152]. Indeed, T_H1-related cytokines, such as IFN-γ, have been shown to be advantageous for immunity to tuberculosis, whereas overproduction of T_H2 and regulatory cytokines may impede immunity [153]. The authors speculate that the difference in cytokine response between UK and Malawi infants are most probably due to disparities in baseline immune maturation and activation prior to vaccination; and since the BCG-vaccine is administered to neonates or early in infancy, differences in pre- or perinatal environmental factors may be pivotal [152]. Indeed, as described in section 6.1, children in regions with a more traditional lifestyle show a higher degree of adaptive immune maturation already at birth as compared to children born in more industrialized regions of the world [91]. In all, these studies point to that live vaccines induce more efficient immune responses in hosts with a lower degree of immune maturation and activation prior to vaccination.

8.2 ...to non-live vaccines?

In contrast to live attenuated vaccines, a heightened baseline immune activation before vaccination seems to favor immune responses to the non-live DTP-vaccine. In a randomized study, Turkish infants responded with markedly higher antibody production against all antigens in the DTP-vaccine, as compared to Belgian infants [154]. Further, Senegalese infants who received the same batch of DTP-vaccine at corresponding ages, presented with vaccine-specific antibody titers in the same range as the Turkish infants [155]. Although factors associated with differences in vaccine-induced antibody responses observed between populations were not investigated, the authors stress that there is a trend for higher immune response in less industrialized countries, i.e. Turkey and Senegal. The authors also here hypothesize that possible explanations may include differences in immune maturation and activation prior to vaccination. We obtained similar results in our Swedish prospective cohort, as we found that higher DTP-induced antibody levels at 18 months of age were associated with lower absolute numbers of circulating T and B cells in infancy, which according to our study reflect a more mature adaptive immune system during childhood [89]. Also, a higher proportion of memory B cells prior to vaccination has been identified as a significant predictor of higher antibody responses to the non-live hepatitis B vaccine in adults [156]. However, if these associations are valid also for other non-live vaccines remains to be determined.

Thus, in view of the studies discussed above, it appears as a heightened baseline immune maturation and activation prior to vaccination augments protective immune responses conferred by the non-live DTP-vaccine, but impede the efficacy of several live vaccines. The reasons for these differences are poorly understood, but it is likely that intrinsic differences in vaccine formulations between live and non-live vaccines and their respective way to induce immune responses are of importance. A heightened baseline immune maturation and activation, which for example is the concept of 'trained immunity' described in section 2.2, is characterized by improved responsiveness to antigen-stimulation with increased production of inflammatory mediators and an enhanced capacity to eliminate pathogens. Thus, similarly to natural infections, a more efficient host immunity could possibly combat the pathogens of live viral vaccines before an efficient immune response has been elicited. However, since the immunogenicity of the non-live DTP-vaccine rather relies on alum as an adjuvant, it is plausible that a heightened baseline immune maturation and activation do not

interfere with its mechanisms of action, but rather enhances it. Although other factors than baseline immune maturation may influence vaccination outcomes, the results discussed above point to that further studies of associations between baseline immune maturation and responsiveness to different vaccines may be of great importance for the development of new vaccination strategies.

8.3 Genetics and sex as determinants of vaccine responses

In addition to baseline immune activation, other factors may also influence vaccine-induced immune responses. It has been proposed that **genetic polymorphisms** play a substantial role in the variation of vaccine-induced antibody levels [157, 158]. Genetic variations can occur via polymorphisms of genes involved directly or indirectly in the generation of immune responses, e.g. genes encoding various pathogen receptors, cytokines, cytokine receptors, MHC-II molecules, and many other structures.

Further, there are clear **sex-related differences** in vaccine-induced immune responses among both children and adults. In general, females mount stronger antibody responses than males in response to both live attenuated and non-live vaccines [139, 159]. Specifically, higher vaccine-induced mumps and rubella antibody titers have been reported in blood from adolescent girls compared to boys in the same age [160, 161]. This observation was also confirmed among children in our prospective cohort, since girls presented with significantly higher vaccine-induced mumps and rubella antibody titers at 36 months of age [132]. Whether sex-related differences in antibody responses to vaccines are caused by genetic, hormonal or environmental factors, or a combination, remains to be established.

9 SENSITIZATION AND ALLERGIC DISEASE

A top priority for the immune system is to mount an effective defense against pathogenic microorganisms. In parallel, it needs to avoid aggressive immune responses to self-antigens; this is primarily managed in thymus by the mechanism of central tolerance, as described in section 3.1. In addition, the immune system should also tolerate encounters with the infinite number of harmless antigens that are present in food and in the surrounding environment, here referred to as allergens. This is accomplished by the mechanism of **peripheral tolerance**. Upon antigen/allergen-recognition without adequate co-stimulation from APCs, the T cell does not become activated. Instead, the T cell receptor may lose its ability to transmit activating signals and the cell becomes **anergic**; a second fate includes death by **apoptosis**. However, some T cells may also develop into **Tregs** that suppress proliferative and cytokine responses against the allergen.

These tolerance mechanisms do however fail in some individuals, which results in activated T cells that trigger B cells to produce **allergen-specific IgE antibodies** to these harmless allergens, which is referred to as **sensitization** [162]. In some sensitized individuals, these antibodies can further trigger inflammation upon renewed exposure to the allergen, giving rise to immune-mediated hypersensitivity, i.e. **allergy**. It is still not known why some individuals mount allergen-specific IgE-antibodies or why re-exposure to allergens cause allergic reactions in some but not all sensitized individuals.

Although sensitization can occur without symptoms of allergic disease, there is a strong association between these two conditions. Children who are sensitized to any allergen at 18 months of age but without any allergic symptoms, have a higher risk than non-sensitized children to develop symptoms of wheezing, asthma and rhinitis at 5 years of age [163]. The prevalence of sensitization increases with age; 13% at 1.5 years, 50% at 5 years, and 61% at 26 years of age, according to a prospective cohort published in 2013, where Danish subjects were followed from birth to early adulthood [164]. In this cohort, the children were primarily sensitized to food

allergens during the first 5 years of life, whereas sensitization to house dust mites and pollen dominated in school age and adulthood [164].

9.1 Sensitization upon first encounter with the allergen

The sequence of events in the allergic reaction begins with the capture of allergens by APCs, primarily dendritic cells, at mucosal surfaces. Allergens have the capacity to prime dendritic cells for **T_H2-induction** by engaging an array of surface receptors, for example TLRs [165, 166]. The primed dendritic cells migrate to the nearest lymphoid tissue where they present processed peptides from the allergen to naïve T cells (see section 3.2 for a more detailed description of the T activation process). If the naïve T cell recognizes the peptide and is exposed to IL-4 it will be stimulated to differentiate into a T_H2 cell.

In parallel, intact allergens will be transported by the lymphatics to the lymphoid tissue where it can be recognized by B cells with the right receptor specificity. As for any protein-antigen, primed B cells are activated through interactions with cognate T_H cells. Secretion of IL-4 and IL-13 by T_H2 cells or T_{FH} cells activated by the same allergen, stimulates **allergen-specific B cells** to **class switch to IgE production**. In addition, it was recently shown that allergen-specific B cells are essential for optimal expansion and activation of the allergen-specific T_H2 cells [167].

The **allergen-specific IgE antibodies** bind to high-affinity Fcε-receptors expressed on **mast cells**, which reside in connective tissue, especially below epithelia and next to blood vessels. IgE antibodies also bind to circulating **basophils**. Thus, in sensitized individuals, mast cells and basophils are coated with IgE-antibodies specific for the allergen to which the person is sensitized. Importantly, as also mentioned above, the presence of allergen-specific IgE antibodies in blood does not mean that the person is allergic; in many cases, sensitized individuals do not have any clinical symptoms.

Tests for IgE sensitization

IgE sensitization can be diagnosed by sensitive detection of allergen-specific IgE antibodies in blood, by skin prick test, and by basophil activation test. A skin prick test is usually performed by placing a drop of the allergen extract solution on the skin, usually on the forearm. The skin beneath the drop is then pricked with a thin needle. If the subject has allergen-specific IgE

antibodies bound to the surface of mast cells in the skin, this will lead to activation of the mast cells and immediate release of inflammatory substances, such as histamine, as further described in section 9.2 below. This elicits a small local allergic response with swelling and redness in the skin at the site of testing. As mentioned above, similar to mast cells, basophils express surface receptors for IgE. The basophil activation test is a flow cytometry-based assay where expression of activation markers on basophils is measured after ***in vitro*** stimulation with allergen.

9.2 The allergic reaction

In some of the sensitized individuals, an allergic reaction is triggered by the **activation of mast cells** upon re-encounter with the specific allergen to which the individual is sensitized. Activation occurs when two or more IgE antibodies on the cell surface of mast cells are crossed-linked by the specific allergen. This activation triggers rapid release of granules containing histamine and proteases, which gives rise to an '**immediate allergic reaction**'. **Histamine** activates endothelial cells, which leads to increased vascular permeability and dilatation of small blood vessels, whereas **proteases** may cause local tissue damage. Histamine also stimulates transient contraction of smooth muscles.

The 'immediate allergic reaction' is followed by a '**late phase reaction**', which can develop and last for hours after allergen encounter. This reaction is mediated and maintained by synthesis and secretion of **prostaglandins** and **leukotriens**, and also by synthesis and secretion of various **cytokines** and **chemokines**. Similarly to histamine, prostaglandins and leukotriens increase vascular permeability and stimulate smooth muscle contraction, but the effects are **more potent** and **last longer**. In addition, these mediators also stimulate mucus secretion and promote influx and activation of leukocytes in the tissue.

A hallmark of the IgE-mediated inflammatory response is the **recruitment of leukocytes from the circulation**, e.g. eosinophils, neutrophils and T_H2 cells, into the tissue. This recruitment is mediated by IL-4, IL-5 and TNF produced by T_H2 cells and mast cells and induce endothelial cells to upregulate the expression of adhesion molecules, which in turn promote extravasation of circulating leukocytes. Recruitment of circulating immune cells is also mediated by chemokines produced by tissue resident macrophages

and by T cells that has infiltrated the inflamed tissue, and also by endothelial cells.

Eosinophils contain several different types of granule proteins that exhibit both tissue-damaging/remodelling as well as immune-modulatory activities. Despite that the allergic inflammation is characterized by tissue infiltration by eosinophils and eosinophilia in the blood, the full role of these cells in allergic inflammations is not yet understood.

The physiological effects of the allergic reaction differ considerably depending on which tissue is affected. For example, hay fever and asthma are symptoms from the upper and lower respiratory tract, respectively, and are caused by inhalant allergens, such as those derived from pollen and animal dander. Food allergy can be manifested by allergic reactions in the gut with increased fluid secretion and peristalsis followed by vomiting and diarrhea, but may also manifest as urticaria in the skin.

9.3 The atopic march

The term 'atopic march' describes the natural history of sensitization to allergens and symptoms of allergic disease that may develop during a certain period in childhood; some symptoms may persist over several years, whereas other show a more rapid spontaneous remission with age [168]. Atopy is defined as a personal or familial predisposition to become sensitized, and thus to produce IgE antibodies in response to harmless ingested or inhaled allergens. As described in the beginning of this chapter, some sensitized individuals also develop symptoms of allergic disease, such as eczema and asthma; in sensitized individuals, these symptoms may also be referred to as *atopic* asthma, *atopic* eczema and so forth. Important to note in this section is that the prevalence rates of allergic diseases may differ considerably between studies, which can be explained by differences in definitions and criteria used for diagnoses as well as differences between the populations studied.

9.4 Diagnoses of allergic disease in children

Eczema

Eczema is generally a very early clinical manifestation of atopy in a child and the debut is before 6 months of age in half of all affected children [164, 169]. The prevalence of eczema in children peaks at around 15% during the first years of life, and thereafter decreases with age [164, 170]. The criteria for diagnosis of atopic eczema includes itchy skin conditions in the past 12 months, generally dry skin in the past year, visible flexural dermatitis, and onset of symptoms before 2 years of age [171]. The risk of other allergic diseases, primarily asthma and hay fever is markedly increased in children with eczema [172], and it has been suggested that a defect in epithelial barrier integrity caused by eczema may increase the exposure to allergens, which may contribute to an increased risk of allergy development.

Food allergy

Around one third of all children with atopic eczema also report to have skin symptoms provoked by food allergy [173]. Food allergy occurs very early in childhood or infancy, and symptoms can range in severity from mild to life threatening. Exposure to very small amounts of food allergens can trigger clinical symptoms such as urticaria, eczema and bouts of vomiting and diarrhea, as well as airway inflammation. More than 170 food items have been reported to cause IgE-mediated allergic reactions, but the allergens most commonly involved are proteins from cow's milk, hen's egg, tree nuts, peanuts, fish and shellfish.

As mentioned in the beginning of this section, the estimated prevalence of allergic disease in children differs considerably between studies, and this applies particularly well to the reported prevalence of food allergies. In addition to differences in criteria and study populations, self-reported prevalence of food allergy is known to be vastly overestimated. However, it has been suggested that the prevalence of food allergy diagnosed by health care peaks at around 5-7% within the first 2 years in life and then drop with age [164, 174]. Around half of all children with IgE-mediated allergy to milk, egg, soy or wheat outgrow their allergy during childhood, whereas allergies to other foods, e.g. peanuts and tree nuts are more likely to persist for life [175].

Asthma

The airways are an important route for allergen entry, and IgE-mediated allergic asthma, simply referred to as asthma in this thesis, is an allergic reaction in the lower airways. In asthmatic individuals, allergen exposure to the airways can lead to bronchial constriction within seconds, as well as an increased secretion of fluid and mucus that makes breathing even more difficult by trapping inhaled air in the lungs. The incidence rate of diagnosed asthma peaks at 1-2 years of age [174, 176], and symptoms start before 8 years of age for 90% of all asthmatics. The prevalence of asthma in children is highly dependent on age and geographic location, but is estimated to approximately 10-20% [164, 172, 174].

Allergic rhinoconjunctivitis

Allergic rhinoconjunctivitis is a reaction to airborne allergens deposited on the mucosal membrane inside the nose in rhinitis, and eyes in conjunctivitis, and is generally considered as the last step of the atopic march. Symptoms include stuffy and runny nose, sneezing, redness and swelling of the eyelids and increased production of tears. Allergic rhinoconjunctivitis typically develop later in childhood and the prevalence is estimated to 15% in adolescents and 30% in young adults [164].

9.5 Allergy – genetics or environment?

Allergic diseases have a complex etiology, and appear to be caused by an interplay between genetic factors and environmental exposures. It has been shown that parental allergic disease is the strongest risk factor for allergy development in the offspring. Thus, **the genetic component** is of major importance, and the heritability has been estimated to vary between 71-84% for eczema, 33-91% for allergic rhinitis, and between 35-91% for asthma [177]. Variations within numerous different genes appear to contribute to this strong genetic component, but the mechanisms by which these genes influence the development of allergies are still poorly understood [177].

The prevalence of allergic disease during early childhood is generally **higher among boys compared to girls** [81, 178, 179]. It has been suggested that the higher rate of asthma in boys could be a result of their smaller airways relative to lung size as compared to girls [180]. However, a more immature adaptive immunity among boys compared to girls in early childhood could also contribute to a higher prevalence of allergic disease among boys [111,

132, 135]. During adolescence, there is however a shift, with asthma becoming more common in girls [181].

In addition to genetic predisposition and sex, epidemiological studies have emphasized many associations between early life environmental exposures and subsequent **risk for allergy development**. For instance, an altered gut colonization pattern during infancy has been associated with subsequent development of various allergic diseases in several studies. Specifically, a low gut microbial diversity in infancy has been associated with eczema in children up to two years of age [182, 183], and with asthma in school-age children [184]. This association has been proposed to be a consequence of that a less complex gut flora fails to induce sufficient immune stimulation to support normal maturation of the immune system. In line with this, it has been shown that the prevalence of eczema and asthma is strongly reduced in children whose parents consistently clean their pacifier by sucking it [185]. The authors speculate that this observed protective effect of parental pacifier sucking is due to transfer of parental oral bacteria to their infant.

Identified **allergy-protective environmental factors** include large family size and having elder siblings [186, 187] (further described in section 10.1), increased diversity of food introduced in the first year of life [188] and pet ownership [187, 189]. Moreover, growing up in a traditional farming milieu confers a strong protection from development of allergic diseases. Indeed, the farming milieu is the most potent allergy-protective environmental factor known in affluent industrialized countries.

9.6 The allergy protective effect of farming environments

One of the first studies that tested the hypothesis of an allergy-protective farm effect was a cross-sectional study published in 1999, which demonstrated that farming as a parental occupation was associated with lower prevalence of sneezing attacks during pollen season as well as with lower prevalence of IgE-sensitization to outdoor as well as indoor allergens [190]. Interestingly, this study further showed a gradient in sensitization from non-farming to full-time farming as parental occupation, suggesting a dose-response effect [190]. Shortly after this, these associations were confirmed by other cross-sectional studies, and evidence of a protective effect of farming environments also on asthma and wheeze was added [191, 192]. These studies further showed an

association between regular contact with livestock and reduced risk of sensitization and allergic disease among the children [191, 192]. Since then, numerous large cross-sectional studies around the world have examined and confirmed the protective effect of a farming environment on development of sensitization and allergic diseases in childhood [193].

Although these epidemiological studies provide overwhelming evidence for this protective farm effect, the characterization of the protective components as well as the optimal timing for exposure are far from being understood, because most of these studies were of a cross-sectional design and included school-aged children. However, some of the **identified farm-related exposures** that independently contribute to the reduced risk of developing allergic disease among farmers' children are regular contact with livestock and animal feed as well as consumption of unpasteurized cow's milk [104, 105, 194]. Of note, the **timing of exposure** was crucial, since the strongest protective effects were found when exposed prenatally or during the first year of life [104, 194].

As described in section 6.2, environmental exposure to microbial products, as measured by endotoxin levels, are significantly higher in homes of farming- compared to non-farming families [99, 100]. Increased endotoxin exposure is associated with a significantly lower risk of asthma, hay fever, and IgE-sensitization in childhood [100]. As mentioned in section 6.2, the endotoxin levels in airborne dust differ significantly between homes of the two farming populations Amish and Hutterite, who practice a more traditional compared to a more industrialized farming, respectively [101]. Indeed, the prevalence of asthma also differed significantly between these two groups of children; none of the 30 Amish children in the study reported symptoms of asthma, whereas 6 (20%) of the Hutterite children did [101]. This is in line with previous studies that show associations between high exposure to endotoxins and low prevalence of asthma. As described above, a traditional farming environment is associated with an environment with a high microbial antigen diversity. Thus, increased exposure to microbes and microbial products, which may be reflected by higher endotoxin levels, may stimulate enhanced immune maturation that could lead to a reduced risk of allergy among farmer´s children.

10 IMMUNE MATURATION IN RELATION TO SENSITIZATION AND ALLERGIC DISEASE

10.1 The hygiene hypothesis

A striking increase in the prevalence of allergic conditions has occurred during the second half of the twentieth century [195-198]. Since this rapid increase cannot be explained by genetic alterations, and many of the children who become allergic do not have allergic parents, other factors must account for this increase. Today, allergy is the most common chronic disease in children and adolescents in westernized affluent countries. At the age of 5, approximately one third of all children in Sweden and Denmark are or have been affected with one or more allergic manifestations [176]. This dramatic increase, in combination with large variations in the worldwide prevalence of allergic diseases, suggest a strong impact of environmental factors on allergy development.

The publication of David Strachan in 1989 is regarded as the starting point for the research regarding the environmental influence on allergy development in children. In a prospective birth cohort, including almost 17,500 British children, Strachan found that the prevalence of self-reported hay fever during childhood was inversely related to the number of older siblings [186]. Strachan hypothesized that infections, prenatally or in early childhood, 'transmitted by unhygienic contact with older siblings', might prevent the development of allergic diseases in childhood; this was the origin of the 'hygiene hypothesis'. Over the last decades, new angles and aspects of environmental effects on development of allergic diseases in children have been proposed with the hygiene hypothesis as basis, and the role of viral or bacterial infections as well as the impact of exposure to non-viable microbial products on the innate and adaptive immune system have been explored [199]. Although a wide range of possible allergy-protective exposures, the core of the hygiene hypothesis remains, namely that reduced exposure to microbes or microbial products early in life may lead to delayed adaptive

immune maturation and as a consequence development of allergic disease. However, the relationship between postnatal adaptive immune maturation and allergic disease has not yet been established.

10.2 $CD4^+$ T cells

As described previously, ongoing allergic inflammation is generally characterized by excessive T_H2 immune responses with increased production of T_H2 related cytokines, such as IL-4, IL-5 and IL-13. This was also observed in our prospective study, since current allergic disease at 3 years of age was associated with higher mitogen-induced production of IL-5 and IL-13 by PBMCs [76].

However, the relation between early life cytokine producing capacity in general prior to development of allergic diseases is not established. Regarding **T_H2-related cytokines**, some studies show a strong association between increased proportions of IL-4^+CD4^+ cells upon polyclonal *in vitro* stimulation of cord blood and development of allergic disease in early childhood [200, 201], while others do not [202]. Furthermore, a higher production of IL-13 upon polyclonal stimulation of PBMCs at 3 months of age, but not at birth, was strongly associated with subsequent development of both atopic and non-atopic asthma between 1 and 5 years of age in a birth cohort study comprising 450 US children [68].

Regarding **T_H1-related cytokines**, it has been shown that children with lower proportions of IFN-γ producing $CD4^+$ T cells after polyclonal stimulation of cord blood, had a fivefold higher risk to develop allergic disease during the first 2 years in life [201]. This result was obtained after adjustment for potential confounders, such as parental history of atopy, sex, pet ownership and maternal smoking during pregnancy [201]. Thus, this indicates that hampered IFN-γ production at birth is associated with subsequent development of allergic disease, which could be due to both genetic and prenatal environmental factors. Several other studies also demonstrate an association between impeded IFN-γ production at birth and subsequent allergic disease [202-204]. In some reports, however, these two factors were unrelated [68, 200, 205]. Nevertheless, an attenuated *in vitro* induced IFN-γ production in the first year of life is strongly associated with subsequent development of sensitization and/or allergic disease later in childhood and in early adolescence [68, 206, 207]. Although several studies

suggest that a T_H2-skewed immune response in infancy precede allergic disease in children, the biological mechanisms behind these associations are probably very complex. Not only an excessive T_H2 or impaired T_H1 response, but rather an imbalance between these two immune profiles early in life may influence the risk for later development of allergic disease.

An impaired responsiveness to polyclonal stimulation early in childhood has been suggested to indicate an immaturity in the T cell compartment. In addition to attenuated IFN-γ responses, the immaturity in neonatal T cell function in children who go on to develop allergic disease may also include **hampered proliferative capacity**. For instance, the lymphoproliferative responses upon polyclonal stimulation of cord blood cells are significantly hampered in neonates who subsequently develop allergic disease [208], as well as in neonates with a high risk of developing allergic disease, i.e. both parents atopic, compared to low-risk neonates, i.e. both parents non-atopic [209]. Interestingly, parental atopy was not predictive for proliferative responses at 1 year of age, since there were no difference between the two groups of children at this age [209]. According to the authors, this indicates that genetic factors are of less relevance for the T cell responses at 1 year than at birth and that these responses instead may correlate with environmental exposures. In line with the hypothesis that a more immature T cell compartment in infancy may predispose for allergic disease, our prospective birth cohort shows that higher proportions of naïve $CD45RA^+$ cells among $CD4^+$ T cells in infancy is associated with allergic disease at 8 years of age (Strömbeck et al *in manuscript*: paper IV in this thesis).

10.2.1 Regulatory T cells

Since Tregs have suppressive effects on both B cells [210, 211] and T cells [16, 27, 77], these regulatory cells play a master role in controlling adaptive immune responses. One could thus hypothesize that Tregs are central for preventing development of sensitization and allergic disease in children. Significant efforts have been made to identify the role played by Tregs in the development of allergic sensitization and disease; however the vast majority of these studies have focused on adults or on children with established allergy. Since allergic symptoms often occur in the first years of life, studies of neonatal and infantile Tregs and subsequent development of sensitization and allergic disease are of significant interest. However, studies with this prospective design are few and results are inconsistent. This inconsistency may, as described in section 6.2.2, in part confer to different phenotypic

characterization of Tregs in these studies, as well as differences in experimental settings when assessing the suppressive function of these cells.

A longitudinal study showed that the suppressive capacity of cord blood Tregs on lymphoproliferative responses did not differ between children who developed allergic sensitization or disease at 1 or at 2 years of age [212]. At one year of age, however, children who were sensitized or became sensitized within one year had Tregs with a reduced suppressive capacity, a difference that was not detectable at two years of age [212]. It has further been shown that neonates who developed allergy at one year of age had cord blood Tregs with reduced capacity to suppress IFN-γ production compared to neonates who did not develop allergy [213]. This effect was specific only for IFN-γ with no effects on other cytokine responses [213]. Further, studies differ regarding suppressive function of Tregs in cord blood of children with a high risk of developing allergic disease, i.e. babies born by atopic mothers; one study showed that maternal atopy was associated with an impaired Tregs suppressive capacity in the baby [214], whereas another study found no such association [200]. A reduced suppressive capacity of neonatal Tregs could be a logical contributing factor to the failure of normal tolerance mechanisms. A reduced suppressive capacity of Tregs among children who later become sensitized or allergic could be a further indication of delayed adaptive immune maturation in children at high risk of developing allergic disease. However, since longitudinal studies are few and the outcomes vary, further research is required to establish the relationship between Treg function and subsequent development of sensitization and allergic disease.

Additionally, it has been shown that lower numbers of Tregs in cord blood is associated with a higher risk of developing eczema and sensitization at 1 year of age [215]. Other studies report that the proportions of Tregs in cord blood do not differ between children who develop allergic sensitization or disease later in childhood and children who do not [212, 213]. In a prospective cohort of Australian children, it was shown that children who developed sensitization and/or allergic disease at 2 years of age had significantly higher proportions of circulating Tregs compared with non-allergic children at 6 months of age [207]. This was observed for multiple atopic outcomes, i.e. sensitization to food and inhalant allergens as well as for eczema [207]. This association could be due to that proportions of Tregs may expand as a protective mechanism during an allergic inflammatory response, as observed in children with current sensitization or allergic disease, but also in other chronic inflammatory conditions [108, 212]. However, to rule out this as an

explanation for the observed associations between higher proportions of Tregs prior to development of sensitization and allergic disease, children who already had eczema at 6 months of age were excluded from the analysis. Exclusion of these children did not alter the outcome of the analysis, and additional regression analyses demonstrated strong associations between high proportions of Tregs and subsequent sensitization [207]. These associations were confirmed in our prospective birth-cohort study, since we found that children who became sensitized in the first three years of life had significantly higher proportions of circulating Tregs in the neonatal period compared to non-sensitized children [76]. In the follow-up study of the same cohort, we further found that allergic disease at 8 years of age is associated with higher proportions of Tregs from infancy throughout childhood (Strömbeck et al *in manuscript*: paper IV in this thesis). We also found that children with current disease at 8 years of age had significantly higher proportions or circulating Tregs, thus confirming results from previous studies mentioned above [108, 212]. We speculate that higher proportions of Tregs early in life may impede adaptive immune maturation, which in turn could favor development of allergic disease. Still, additional prospective studies in larger birth cohorts are required to confirm that higher proportions of Tregs precede development of sensitization and allergic disease in children. Due to the inherent obstacles to collect human tissue samples, all studies above have investigated total Treg numbers or frequencies in the circulation. This is important to consider when interpreting the results, since lower Treg numbers or proportions may be a consequence of migration of these cells into target tissues where they execute their suppressive functions.

10.3 B cells

Although B cells are the precursors of IgE-producing plasma cells and, hence, key players in allergic disease, the relationship between early life B-cell maturation and subsequent allergy development has just started to be explored. To the best of our knowledge, our prospective FARMFLORA cohort is the first study that demonstrates that higher proportions of immature/naïve $CD5^+$ B cells already at birth but also throughout childhood is associated with allergic disease in school-age children (Strömbeck et al *in manuscript*: paper IV in this thesis). We also demonstrate that a higher proportion of $CD5^+$ B cells at 1 month of age is, indeed, a risk factor for subsequent development of allergic disease [81] (and Strömbeck et al *in manuscript*: paper IV in this thesis). In addition, low BAFF levels at birth are associated with higher proportions of $CD5^+$ B cells and allergic disease in early childhood [81]. In this study, current allergic disease was associated with higher proportions of circulating immature/naïve and immature/transitional B cells (Strömbeck et al *in manuscript*: paper IV in this thesis); this is in accordance with others who have demonstrated that allergic adults have higher proportions of circulating immature/transitional B cells compared to non-allergic subjects [216, 217].

The finding of a more immature B cell compartment in children with a higher risk of allergy is supported by a recent study of pregnant women with allergic asthma. In this study, there is a strong association between higher proportions of circulating transitional B cells during pregnancy and allergy development in the offspring at the age of 6 months. In addition, the proportion of transitional B cells in these women could be used as a predictive marker for allergy development in their offspring [217]. However, most allergic diseases arise after 6 months of age and these preliminary results need to be confirmed also at older ages. Yet, these results collectively provide further support to studies suggesting that delayed adaptive immunity may predispose for allergic diseases later in childhood.

CONCLUDING REMARKS

Adaptive immune maturation comes with age and is likely the result of a complex interplay between both intrinsic and numerous environmental factors, as pointed out through this thesis. However, surprisingly little is known regarding this adaptive immune maturation progress and its association with development of allergic disease and vaccine-responsiveness in children. In the FARMFLORA-study we have had the unique opportunity to prospectively follow children from birth throughout childhood, and we have performed detailed immunological analyses of blood samples obtained at several occasions. By the use of multivariate factor analyses we have, for the first time, been able to visualize longitudinal patterns of adaptive immune maturation in relation to clinical outcomes, i.e. sensitization and allergic disease, as well as in relation to certain environmental factors and immunologic effector responses.

The rapid increase in the prevalence of allergic diseases in affluent countries in the last half-century points to an essential role of environmental factors in the development of these conditions. Although the hypothesis that compromised immune maturation during early childhood could increase the risk of allergic disease was introduced almost 30 years ago, the relationship between postnatal adaptive immune maturation and allergic disease has not yet been established. When analyzing associations between T cell maturation and sensitization up to 3 years of age in **paper I**, we found that, of all immunological parameters analyzed, the strongest association was observed between higher proportions of neonatal Tregs and subsequent sensitization. Indeed, children who were sensitized at 18 and/or 36 months of age had significantly higher proportions of Tregs within the circulating $CD4^+$ T cell population at birth and early in infancy as compared to children who remained non-sensitized. In the 8 years follow-up study, presented as **paper IV**, we further show that higher proportions of Tregs during childhood was associated with allergic disease at 8 years of age. It is important to emphasize that our results are based on proportions of Tregs among $CD4^+$ T cells in the circulation and not in the tissues where they execute there suppressive functions. Higher proportion of Tregs in the circulation could be a

consequence of a lower degree of migration of these cells into target tissues, but could also reflect a higher proportion of these cells among $CD4^+$ T cells in general. Thus, it is somewhat difficult to interpret these results and to speculate on the immunological consequences of higher proportions of these cells in the circulation in early childhood. However, our results do not support the hypothesis that higher proportions of Tregs in childhood is protective against development of allergic disease. Instead, a high proportion of these cells early in life may hamper activation and maturation of the adaptive immune system, which in turn may predispose for development of allergic disease. **Paper IV** further shows that allergic disease at 8 years of age is associated with higher proportions of naïve $CD45RA^+$ T cells in infancy as well as with higher proportions of immature/naïve $CD5^+$ B cells from birth up to 8 years of age. Thus, according to our study, allergic disease in childhood is preceded by a heightened immaturity among circulating $CD4^+$ T cells and B cells.

Growing up in a traditional farming environment is the most potent allergy-protective environmental factor known in westernized countries. Indeed, in **paper IV** we show that the incidence of allergic disease was significantly lower amongst farmers' compared to non-farmers children during the first 8 years in life, even in our small cohort. Interestingly, in **paper I** we found that a farming environment was strongly associated with lower proportions of circulating Tregs up to 3 years of age. This association did, however, not appear as strong later in childhood. Instead, **paper IV** shows that of all parameters analyzed during the first 8 years of life, growing up in a farming environment was most strongly associated with higher proportions of memory B cells as well as with lower proportions of naïve B cells at 8 years of age. Thus, growing up in a farming environment seems to be associated with a higher degree of immune maturation, which also may explain the lower incidence of allergic disease among these children. However, these results need to be confirmed in larger birth-cohort studies before any vast conclusions can be drawn. The traditional farming environment provides an environment with a high microbial antigen diversity. It is likely that this diversity contributes to increased immune stimulation and, as a consequence, a higher degree of adaptive immune activation and maturation in these children. A deeper understanding of the relevant stimuli to provide an efficient immune maturation may ultimately pave the way for development of effective strategies for prevention of allergic disease in children.

The impact of intrinsic and environmental factors that may shape immune maturation may also be central for how children respond to vaccination. In **paper II** and **III**, we show that baseline immune maturation and activation prior to vaccination is associated with the magnitude of vaccine-induced immune responses in children. In **paper II**, we investigated associations between adaptive immune maturation and DTP vaccine-induced antibody levels at 18 months of age. The most striking association pattern displayed for all three vaccines included was that higher vaccine-specific antibody levels were associated with lower absolute numbers of B cells and $CD4^+$ T cells the first months in life. Further analyses showed that absolute numbers of $CD4^+$ T cells correlated with proportions of naïve $\alpha4\beta7^+$ T cells, but correlated inversely with proportions of $CD45RO^+$ memory T cells, at several occasions in childhood. A similar association pattern was observed for infant B cell counts. Thus, in this study, a higher lymphocyte count in infancy appears to reflect a more immature/naïve adaptive immunity in childhood. In all, this indicates that a heightened baseline immune maturation and activation prior to vaccination enhances protective antibody responses conferred by the non-live DTP-vaccine in Swedish children. An opposite trend was however observed for antibody responses against the live attenuated MMR-vaccine (**paper III**). Higher vaccine-induced antibody titers were generally associated with a lower degree of adaptive immune maturation prior to vaccination. The reasons behind these differences are not fully understood, but one possible explanation lies within the intrinsic differences in vaccine formulations of the DTP- and the MMR-vaccine, and hence their respective way to induce immune responses in the host. Although other factors than baseline immune maturation prior to vaccination might be central for vaccination outcomes, our results indicate that further studies in this area are needed. A greater understanding of the relation between immune maturation and vaccine responsiveness may be of great importance for developing better vaccination strategies.

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