UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Heinrich-Pette-Institut, Leibniz-Institut für Experimentelle Virologie

Wissenschaftlicher Direktor: Prof. Dr. Thomas Dobner

Abundant specialized T follicular helper cells in the lamina propria of human infant intestines

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
an der Medizinischen Fakultät der Universität Hamburg.

vorgelegt von:

Fenja Linnea Steinert
aus Hamburg

Hamburg 2020

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 19.08.2021

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, der Vorsitzende: Prof. Dr. Hans-Willi Mittrücker

Prüfungsausschuss, zweiter Gutachter: Prof. Dr. Marcus Altfeld

Inhaltsverzeichnis

1 Problem definition

The number of deaths among under-five year old infants worldwide remains at six million in 2015 (United Nations 2015). The main reasons for the high mortality in this age group are infectious diseases (Mathers et al. 2008). Neonates and infants are more susceptible to numerous infectious diseases while having a reduced protective immune response to vaccinations compared to adults. It is assumed that this is due to the immature immune system, which is characterized by tolerogenic and suppressive immune responses (Zhang et al. 2017). Neonatal vaccines would provide a potent strategy to protect neonates and infants from infectious diseases. Developing the existing knowledge regarding immune responses upon vaccination especially in infants will help to develop vaccines and effective adjuvants tailored to neonates and infants.

Although it was assumed that infant immune cells in blood and lymphoid tissues are rather immature in infants, our group could previously show that in the intestine, effector memory T cells are present prior to birth (Bunders et al. 2012). This indicates that memory can be induced especially in intestines. Therefore oral vaccines could be a key strategy for efficient infant vaccinations (Lycke 2012).

One of the critical factors towards the protective immune response upon vaccinations are T follicular helper (T_{FH}) cells, which support B cells in producing high-affinity antibodies (Crotty 2011). The hypothesis underlying this study is that mature and functional T_{FH} cells in intestinal mucosal tissues are induced early in life. In order to test this hypothesis, intestinal tissues from infants and adults were collected during surgery, single cells were isolated, and T_{FH} cells analysed using multiparameter flow cytometry.

2 Introduction

2.1 Paediatric infectious diseases

In 2000, the hitherto biggest summit conference of the United Nations took place in New York. Heads of state and government from 189 countries around the world adopted a United Nations Millennium Declaration, from which the eight Millennium Development Goals were developed (Bundesministerium für wirtschaftliche Zusammenarbeit und Entwicklung 2018). They all concern the most fundamental needs. Target number four is to "reduce by two thirds, between 1990 and 2015, the under-five mortality rate" (United Nations 2015).

In 1990, out of 1000 live births, 90 under-five year old infants died. Even though the global rate of under-five year old infants deaths could be decreased to 43 of 1000 live births by 2015, the fourth Millennium Development Goal was not achieved (United Nations 2015).

The main causes of infant mortality worldwide are infectious diseases. Taken together, diarrheal diseases, acute respiratory infections, neonatal infections, and malaria account for 50% of all deaths in the under-five years age group. Other frequent causes for infant mortality, especially for neonates, are prematurity and low birth weight as well as birth asphyxia and trauma (Mathers et al. 2008). Furthermore, Black et al. (2008) demonstrated that in more than a third of all deaths of under-five year old infants, maternal and child undernutrition is an underlying factor, increasing the children's vulnerability to severe infectious diseases.

Vaccines are an efficient approach to prevent infants from communicable infectious diseases. By promotion of the measles vaccination, almost 15.6 million deaths could be prevented between 2000 and 2013 (United Nations 2015). Next to measles, there are vaccines against major diseases including poliomyelitis, diphtheria, tetanus, and pertussis as well as pneumonia caused by *Haemophilus influenzae* type b (Hib) and diarrhoea caused by rotavirus (WHO 2017).

Subsequent to the Millennium Goals, 17 Sustainable Development Goals were set by the United Nations in 2015. Until 2030 the countries ambition, amongst others, to "end preventable deaths of neonates and children under five years of age". Mortality of neonates must be reduced "to at least as low as 12 per 1,000 live births and under-five mortality to at least as low as 25 per 1,000 live births" in every country (United Nations 2018). Due to the fact that infectious diseases are the main reason for the under-five years old infants mortality

(Mathers et al. 2008), improving vaccinations for this group of age, could make a big contribution to achieve the Sustainable Development Goal 3: “Ensure healthy lives and promote well-being for all at all ages” (United Nations 2018).

2.2 The adaptive immune system

2.2.1 T-cell activation

The human immune system subdivides into the innate and the adaptive immune system. The innate immune system prevents pathogens to enter the body and is the first to be activated after a pathogen overcomes a barrier. Humoral and cellular elements act together as the innate immune system, which is unspecific and has no immunological memory (Murphy and Weaver 2017). The adaptive immune system, on the contrary, is responsible for the specific immune reaction and later immunological memory. The most important actors are T and B cells, as well as antibodies produced by B cells (Adkins et al. 2004).

The adaptive immune response is initiated by antigen-presenting cells which take up fluids in the peripheral tissues continuously. Monocytes and dendritic cells (DC) are activated after recognition of pathogen-associated molecular patterns (PAMP) by toll-like receptors (TLR) or other pattern recognition receptors (PRR) (Medzhitov 2001). Upon activation, the cells produce proinflammatory cytokines and chemokines that cause extravasation and attraction of more immune cells, including monocytes, granulocytes, and natural killer cells (Iwasaki and Medzhitov 2004, Pashine et al. 2005). The activation of DCs modifies the expression of lymph node (LN) homing receptors at the cell surface, allowing migration of the DCs to the LNs. The antigen is presented on major histocompatibility complex class II (MHC-II) molecules and the costimulatory molecules CD80 and CD86 are upregulated (Siegrist 2018).

In the T-cell zone of the lymph nodes, DCs can present the antigen to antigen-specific naïve $CD4^+$ T cells. Further costimulatory signals and signalling through cytokines are mandatory for activation of naïve $CD4^+$ T cells, proliferation and differentiation into memory cells. Depending on the cytokines produced by the antigen-presenting cells (APC) upon bacterial, virus or fungal particles a different T helper subtype differentiation is initiated (see Figure 1). T helper (T_H) cells can differentiate into T_H1, T_H2, T_H17 or T_{FH} cells and perform their different effector functions (Murphy and Weaver 2017). For the differentiation into T_{FH} cells (see chapter 2.4) further interactions with B cells, additional to DC signals, are required (Crotty 2014).

In case further danger signals are missing, DCs would stay immature so that after interaction with naïve T cells, the latter do not differentiate into effector but into regulatory T (T_{reg}) cells. T_{reg} cells consequently sustain tolerance of the immune system (Bacchetta et al. 2005).

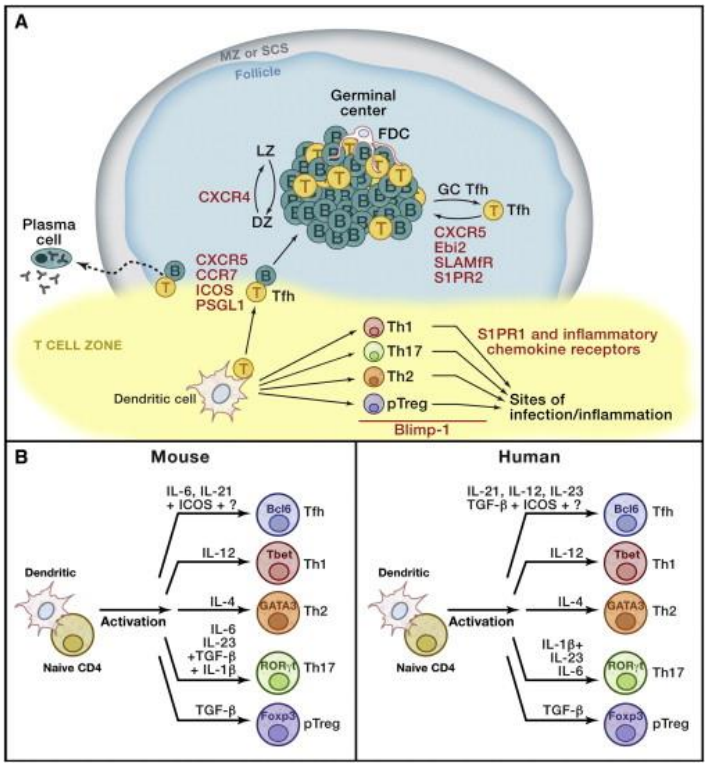

Figure 1. Germinal centre reaction (A) and T-cell activation and differentiation of naïve T cells into T-cell subtypes (A/B). In the T-cell zone of a lymph follicle, T-cell activation takes place. Activated T_{FH} cells are capable of activating B cells, initiate a germinal centre formation and the differentiation of B cells into antibody-producing plasma cells (A). Upon activation of naïve $CD4^+$ T cells by DCs, different T-cell subtypes evolve depending on the cytokines produced by the DCs. These cytokines can differ between mice and humans (B). Certain master transcription factors, specific for the lineages, increase the activity (Crotty 2014).

For an effective immune response upon a pathogen, adaptive immune responses need to be induced. To ensure long-lasting protection, the persistence of antibodies and the generation of memory T and B cells is indispensable. The latter can be quickly reactivated during an infection upon repeated exposure to the same antigen (Siegrist 2018).

2.2.2 Immune cascade to antibody production

Vaccination is aimed to initiate an immune cascade that leads, mainly through efficient antibody production, to protection against a specific pathogen. The injected vaccine antigens attract patrolling immature APCs, including dendritic cells and monocytes. When detecting a foreign antigen, DCs migrate to the lymphatic vessels and to the lymph nodes, where they

present the antigen to B cells. Antigen-specific B cells can recognize this antigen and are activated. B-cell activation can follow mainly two routes, the extrafollicular reaction and the germinal centre reaction.

The extrafollicular reaction is initiated by B cells that bind with the specific surface immunoglobulin (Ig) M receptor to an antigen upon contact. Consequently, activation leads to the upregulation of the lymph node homing C-C chemokine receptor type 7 (CCR7), whereby the antigen-specific B cells migrate to the T-cell zone. Encounters with recently activated DCs and $CD4^+$ T cells provide further activating signals. The extrafollicular reaction is characterized by rapid T-cell help, which induces B-cell differentiation into plasma cells. The latter generates low levels of low-affinity antibodies. This extrafollicular reaction is generally short-lived and plays a minor role in inducing efficient vaccination responses (MacLennan et al. 2003, Siegrist 2018).

The germinal centre reaction (see Figure 1A) is characterized by the induction of proliferating antigen-specific B cells that differentiate into plasma cells within the germinal centres (GC). GCs develop within lymph node follicles in secondary lymphoid tissues and there antigen-specific monoclonal B cells proliferate with the help of follicular dendritic cells (FDC) and T_{FH} cells (Qi 2016). FDCs present in the GCs are capable of capturing and presenting antigens for longer periods to antigen-specific B and T cells. B cells receiving activation and survival signals from FDCs and T follicular helper (T_{FH}) cells undergo extensive clonal proliferation and class-switching from IgM to other types of immunoglobulins. Consequently, plasma cells that are induced in germinal centres are able to produce large amounts of high-affinity antibodies in comparison to the extrafollicular reaction. After induction, small numbers of plasma cells exit the lymph nodes, migrate and persist in the bone marrow (Vinuesa et al. 2005, Siegrist 2018).

These large numbers of high-affinity antibodies are able to bind toxins or pathogens, ensure neutralization, and fast elimination (Cooper and Nemerow 1984). Consequently, most vaccines are aimed to induce large amounts of high-affinity antibodies and therefore initiate a germinal centre reaction (Siegrist 2018).

2.3 Vaccination

2.3.1 Vaccine qualities

Vaccines are antigens that induce an adaptive immune response with antibody and memory cell production. There are different types of antigens. A distinction is drawn between live-attenuated vaccines and non-live vaccines. Live-attenuated vaccines, including the mixed vaccine against measles, mumps, and rubella, are closely resembling a natural infection. Consequently, vaccination with live-attenuated vaccines can result in a better and longer-lasting immune response, so that boosters are less often required. Non-live vaccines, on the contrary, are used, amongst others, for vaccinations against hepatitis B and tetanus (Ständige Impfkommission 2019). Traditionally they consist of inactivated microorganisms or toxoids and newly comprise purified subunits including proteins and polysaccharides. Non-live vaccines are safer for immune-compromised people, however especially vaccines composed of subunits, induce a weaker immune response. Therefore, they must be given with adjuvants, and multiple booster injections might be required (Siegrist 2018).

In many cases, vaccinations were developed empirically. In order to develop new vaccines and to improve existing vaccines for children, knowledge regarding the infant immune system is essential.

2.3.2 Vaccination of neonates and infants

2.3.2.1 Immune system at the beginning of life

Pathogens including group B streptococcus (GBS) and respiratory syncytial virus (RSV) cause life-threatening infections in neonates and infants whereas infections with these pathogens in adults do not result in severe diseases. Although the exact reasons are unknown, it is thought to be due to memory, which is primarily established after the exposure to antigens at the time of birth (Levy 2007). It is assumed that an overall tolerogenic immune system, which is required for feto-maternal-tolerance before birth, results in a higher susceptibility to infections and reduced vaccine responses (Zhang et al. 2017).

Children are called “neonates” or “newborns” in their first four weeks of life (Oxford University Press 2018). The term “infant” is not exactly defined (Oxford University Press 2018). For convenience, children from birth up to five years of age are called “infants” in this study.

For protection against infections, it is assumed, that neonates mainly depend on the innate immune system due to an impaired adaptive immune system (Levy 2007). Comparing umbilical cord blood to adult's peripheral blood mononuclear cells (PBMC), qualitative and quantitative differences in the immune response are detected (Chen et al. 2006). Numerous cell types that are involved in the induction of an immune response upon infection or vaccination, have deficient responses in neonates and infants. First, antigen-presenting cells are lower in numbers and additionally have low levels of costimulatory molecules, MHC-II expression, and cytokine production upon toll-like-receptor stimulation (Krumbiegel et al. 2007, Basha et al. 2014). Second, T cells have a predominantly naïve phenotype and the infant immune system is mainly tolerogenic with abundant T_{reg}- and T_H2-cell responses (Levy 2007). T_{reg} cells are increased in neonates compared to adults. They can abrogate effector T-cell responses and provide support to tolerogenic APCs (Michaëlsson et al. 2006, Burt 2013). Third, T_{FH}-cell frequencies are reduced in neonatal mice lymph nodes upon immunization compared to adult lymph nodes (Debock et al. 2013, Basha et al. 2014). The limited T-cell help in neonates likely contributes to reduced B-cell activation resulting in smaller numbers of plasma cells which produce high titres of high-affinity antibodies in neonates (Siegrist and Aspinall 2009, Wood and Siegrist 2011, Alexander-Miller 2014).

However, upon antigen exposure in the first three months of life the adaptive immune system alters enormously (Siegrist 2007, Hodgins and Shewen 2012), nonetheless differences between infant and adult blood immune cells are retained at least until 18 months of life (Vosters et al. 2010). In conclusion, the neonatal immune system still reflects the focus of the foetal immune system to prevent uncontrollable proinflammatory processes in the first place rather than allowing responses that control infections (Kollmann et al. 2017). This leads to several challenges for neonatal and infant vaccine development.

2.3.2.2 Challenges for neonatal and infant vaccination

Even though great advancements have been made vaccinations against dangerous pathogens, such as human immunodeficiency virus (HIV), are missing. Paediatric HIV infection has a huge mortality rate, and maternal combined antiretroviral therapy (cART) can indeed avoid transmission but has several further disadvantages (Altfeld and Bunders 2016). However, due to the fact that life-threatening infections are mainly ascribable to a small number of pathogens, the development of only few more vaccines could have a substantial beneficial effect (Siegrist 2018).

A second crucial problem is that our existing vaccines are less effective for infants than they are in adults and most vaccines cannot be administered at birth, leaving a vulnerable phase (Zhang et al. 2017). For several vaccinations, which can be given a few weeks and months after birth, all children need to receive multiple priming and booster doses up to two years of age to induce sufficient long-lasting protection (Pichichero 2014). Thereby a serious problem worldwide is that a great number of infants do not receive any or less than the necessary number of vaccine doses (WHO and Unicef 2018). In many instances, neonates are only in contact with the health-care system once, at birth, in regions with poor infrastructure (Morris and Surendran 2016).

During pregnancy and breastfeeding infants acquire protection against foreign antigens through the transfer of maternal antibodies (Simister 2003, Adkins et al. 2004). However, in addition to their essential role of protection, maternal antibodies are also suspected to impact vaccine responses. The most likely mechanisms for the influence of maternal antibodies on the infant vaccine responses are the masking of antigens from infant B cells as well as the uptake of immune complexes consisting of maternal antibodies and vaccine antigens by APCs (Siegrist 2003, Hodgins and Shewen 2012).

Currently, few efficient vaccines are being administered at birth, while providing enough safety (Wood and Siegrist 2011). Vaccines against hepatitis B virus (HBV) and Bacille Calmette-Guérin (BCG), as well as the oral polio vaccine (OPV) are prescribed. These vaccines are currently given immediately after birth in numerous countries worldwide and BCG vaccination does provide acceptable safety and efficacy (Wood and Siegrist 2011, Prelog 2012). These observations confirm that early vaccination given in a single dose at birth can induce lasting, lifelong protection when appropriate immune responses are induced (Basha et al. 2014, Morris and Surendran 2016).

Two approaches are currently pursued to achieve high levels of protection. On the one hand, maternal vaccination, which can take place before, during, or after pregnancy, is a promising strategy. Through this, the maternal IgG antibodies produced upon the vaccination are transferred through the placenta to the foetus or IgG, IgA and IgM antibodies are transferred through the breastmilk to the neonate. This results in the infant receiving protection until sufficient vaccinations of the same are possible (Faucette et al. 2015). On the other hand, there is a strategy to optimize vaccines that are specific to the administration early in life.

Therefore, an appropriate combination of vaccine antigens with safe and effective adjuvants is required to specifically enhance the immune activity early in life (Kollmann et al. 2017).

A better understanding of early immune responses in children could help identify targets to improve and design early childhood vaccination. A pivotal role in the production of high-affinity antibodies in the germinal centre reaction upon pathogen exposure or vaccination is assumed by T_{FH} cells.

2.4 Role of T_{FH} cells in antibody production following vaccination

2.4.1 Function and nomenclature of T_{FH} cells

Follicular T helper (T_{FH}) cells are fundamental for the formation of germinal centres (GC), crucial to provide specialized B-cell help and the consequent production of high-affinity antibodies (Crotty 2011). Within the GCs, B cells undergo affinity maturation and immunoglobulin class switch as well as differentiation into plasma or memory B cells (Cooper 2015).

T_{FH} cells were recently identified as $CD4^+$ T cells expressing the C-X-C chemokine receptor type 5 (CXCR5) (Breitfeld et al. 2000, Schaerli et al. 2000). The CXCR5 receptor binds the chemokine C-X-C motif ligand 13 (CXCL13), which is primarily produced by FDCs in lymph nodes. CXCL13 enables the relocation of CXCR5 expressing T_{FH} and B cells to lymphoid follicles (Förster et al. 1994, 1996), where these cells can interact effectively (Ansel et al. 1999, Kim et al. 2001).

Initially, all $CD4^+$ T cells expressing CXCR5 were defined to be T_{FH} cells. Meanwhile, the term "T_{FH} cell" is more specific. Nevertheless, numerous different nomenclatures are used by different authors (Crotty 2011). The difficulty with the identification of T_{FH} cells is caused by the fact that the markers by which T_{FH} cells are usually identified, can also be expressed in general by activated $CD4^+$ T cells. Consequently, T_{FH} cells are currently characterised by expressing the highest amount of markers, including programmed cell death protein 1 (PD-1), inducible T-cell costimulatory (ICOS) and CXCR5 (Deenick and Ma 2011).

The co-inhibitory receptor PD-1 is a regulatory molecule, expressed by activated T cells. PD-1 is produced in germinal centre-associated T cells assumably upon constant stimulation of GC B cells (Qi 2016). It is lower expressed in other T-cell zones and T cells derived from T_H1 and T_H2 cells, which confirms that PD-1 closely correlates with germinal centre-

associated T_{FH} cells (Dorfman et al. 2006). Upon T-cell priming resulting in T_{FH} cells, CXCR5 is upregulated first, followed by B-cell lymphoma 6 (Bcl-6) and PD-1 (Qi 2016).

For several years, it was controversial whether the T_{FH} cells are a distinct cell type next to T_H1, T_H2, and T_H17 cells, since distinct characteristics were not identified at that time. Meanwhile, they are accepted as a distinct lineage, due to the identification of Bcl-6 as their master transcription factor and interleukin (IL) 21 as their effector cytokine (Nurieva et al. 2008, 2009, Yu et al. 2009).

2.4.2 Regulators of T_{FH}-cell differentiation

In general, differentiation of naïve $CD4^+$ T cells into the different subsets is determined by several different transcription factors. Specific cytokines induce the differentiation through various signal transducers and activators of transcription (STAT). These factors upregulate the expression of lineage-specific transcription factors. Afterwards, the specific transcription factors promote the lineage differentiation and inhibit the differentiation pathways of other T-cell lineages (Liu et al. 2013).

Human T_{FH}-cell development mainly depends on IL-12, IL-6, and IL-21, which can activate STAT3, in turn increases the expression of master transcription factor Bcl-6 in T cells (Liu et al. 2013). Bcl-6 supports the characteristics of T_{FH} cells by suppressing transcription factors specific for other T-cell lineages, as well as suppressing B lymphocyte-induced maturation protein 1 (Blimp-1), which inhibits, as antagonist of Bcl-6, the T_{FH}-cell differentiation (Johnston et al. 2009, Ma et al. 2012b).

The identification of Bcl-6 as the master transcription factor is based on the failure of T_{FH}-cell differentiation in absence of Bcl-6 in vivo, whereas other cell types stay unaffected (Nurieva et al. 2009, Yu et al. 2009). However, there are some contradictory results regarding Bcl-6. The transcription factor Bcl-6 is not able to induce essential expression of CXCR5 or IL-21, which are the main characteristics of T_{FH} cells (Ma et al. 2012b). Moreover, Kerfoot et al. (2011) and Kitano et al. (2011) could identify T_{FH}-like cells that did not express Bcl-6 and cells that expressed Bcl-6, but no other T_{FH}-cell markers. Consequently, Ma et al. (2012b) reason that additional transcription factors are most likely part of the T_{FH}-cell differentiation and they even suggest that “Bcl-6 may not be the “master regulator” of the Tfh lineage” (Ma et al. 2012b).

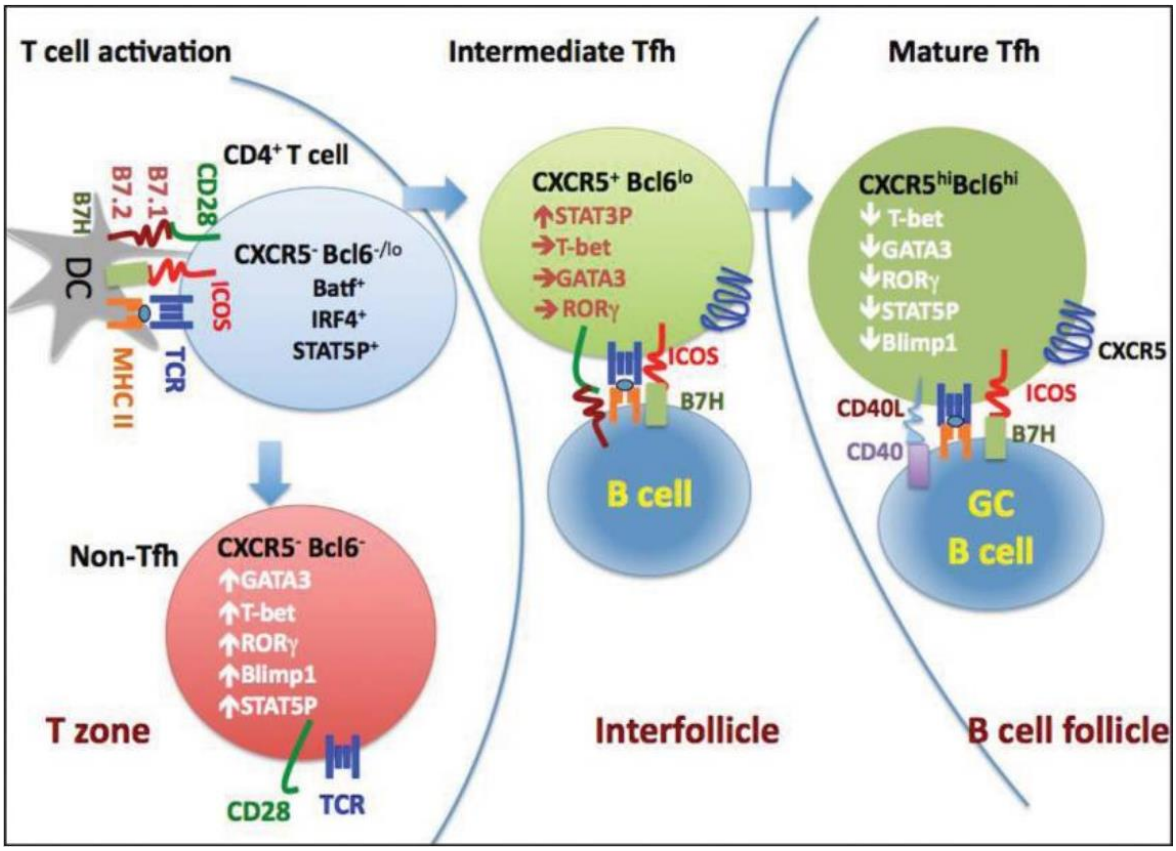

Figure 2. Activation of transcription factors in the differentiation of naïve T cells into mature T_{FH} cells. Upon activation of naïve $CD4^+$ T cells by DCs, $CXCR5^+$ $Bcl\text{-}6^{-/lo}$ pre-T_{FH}-like cells develop. The transcription factors Batf and IRF4 increase their activity. The CXCR5 expression induces the cells to migrate towards the intrafollicular zone where T-B-cell interactions lead to upregulation of STAT3 and sustain the expression of Bcl-6. In the B-cell follicle, the interaction between T and B cells completes the T_{FH}-cell development and simultaneously gives help to B cells. The mature T_{FH} cell expresses $Bcl\text{-}6^{hi}$ and downregulates the transcription factors T-bat, GATA3, and RORγ for other T_H-cell lineages, as well as Blimp-1 (Liu et al. 2013).

To determine further transcription factors involved in the generation of T_{FH} cells, $CD4^+$ T cells with deficiencies in different transcription factors were examined by several authors. Upon a mutation in STAT3, naïve $CD4^+$ T cells still acquired characteristics of T_{FH} cells such as increased expression of ICOS, CXCR5, and Bcl-6. However, T_{FH} cells developed a defect in IL-21 production leading to diminished T_{FH}-cell differentiation and reduced support of B cells (Ma et al. 2012a). Consequently, STAT3 can be seen as an additional critical factor for human T_{FH}-cell differentiation as shown in Figure 2 (Liu et al. 2013).

In addition, several other transcription factors are involved in the T_{FH}-cell differentiation as well. Together with STAT3, cytokine signals induce basic leucin zipper transcription factor activation transcription factor-like (BATF) and interferon regulatory factor 4 (IRF4). BATF can directly regulate Bcl-6 and musculoaponeurotic fibrosarcoma oncogene homolog

(c-Maf), with the latter transcription factor being important for IL-21 production. The role of IRF4 in T_{FH}-cell differentiation needs to be determined, although in murine $CD4^+$ T cells IRF4 is involved in the IL-21 expression. Studies in mice showed that the lack of one of these transcription factors, c-Maf, BATF, and IRF4 results in diminished numbers of T_{FH} cells (Ma et al. 2012b). In the end, most of the transcription factors influence the interaction between Bcl-6 and the counterpart Blimp1 (Qi 2016).

2.4.3 B-cell activation by T_{FH} cells

Important actors in the induction of T_{FH}-cell-dependent B-cell activation and differentiation, but also for bidirectional signalling, are costimulatory molecules including CD40 Ligand (CD40L) and ICOS (Ma et al. 2012a). ICOS belongs to the essential actors for T_{FH}-cell generation. There are two ways ICOS promotes T_{FH}-cell differentiation. On the one hand, ICOS signals given by DCs at the T-cell zone induce Bcl-6 expression in T cells (Choi et al. 2011). On the other hand, ICOS signals provided by follicular B cells at the T-B-cell border promote the migration of T_{FH} precursors into the germinal centre (Xu et al. 2013). Additionally, ICOS can potentiate, as co-stimulatory molecule, the IL-21 production by T_{FH} cells (Bauquet et al. 2009). CD40L on the surface of T_{FH} cells induces B-cell differentiation and class-switching through CD40 on B cells (Banchereau et al. 1994). Furthermore, the CD40-CD40L interaction ensures GC B-cell maintenance, preventing apoptosis (Crotty 2011).

T_{FH} cells express IL-21 as their most important effector cytokine. IL-21 has several roles in B-cell help, such as establishing GCs (Tangye et al. 2013) and effectively promoting plasma cell differentiation and B-cell proliferation in GCs. IL-21 production by T_{FH} cells is STAT3 dependent (Crotty 2011). Furthermore, depending on environmental factors, T_{FH} cells can produce cytokines associated with other T-cell lineages. For instance, the T_H2 cytokine IL-4 is often produced by T_{FH} cells as well. It promotes B-cell survival and differentiation, as well as class switching (Crotty 2011). To sum up, a combination of costimulatory molecules and cytokines are mandatory for the B-cell activation.

2.4.4 T follicular regulatory cells

Due to the strong potential of T_{FH} cells to activate B cells, mechanisms regulating the GC response and prevent the production of autoantibodies are highly mandatory. They are necessary to avoid the emergence of systemic autoimmune diseases, chronic inflammation, allergic responses, and B-cell malignancies (Küppers 2005, Vinuesa et al. 2005, Townsend

et al. 2010). Main mediators of regulation are, in addition to negative feedback mechanisms and inhibitory receptors, T follicular regulatory (T_{FR}) cells. T_{FR} cells act as a negative regulator in the germinal centre response by migrating to the GCs and suppressing T_{FH}-cell-mediated B-cell activation and antibody production. T_{FR} cells, similar to T_{FH} cells, highly express CXCR5, PD-1, and Bcl-6, although to a lesser extent than T_{FH} cells. Whereas T_{FH} cells derive from naïve $CD4^+$ cells, T_{FR} cells are thought to arise from natural T_{reg} precursors (Sage and Sharpe 2016). Therefore, T_{FR} cells express FoxP3 comparable to T_{reg} cells, which T_{FH} cells do not (Sage and Sharpe 2016). T_{FH} cells and their antagonistic T_{FR} cells regulate the function of GC and hence are important actors in successful vaccine responses and might present suitable targets for vaccine design (Linterman and Hill 2016).

2.4.5 T_{FH} cells in human organs

Data regarding bona fide T_{FH} cells in humans is rare due to their residence in tissues, which is difficult to obtain. Recently Roider et al. (2018) determined the T_{FH}-cell frequency in blood and lymphoid tissues of HIV infected children and adults. They revealed a higher frequency of T_{FH} cells in children compared to adults (Roider et al. 2018).

Extensive research has been done on blood circulating $CXCR5^+$ $CD4^+$ T cells, which represent circulating memory cells of the T_{FH}-cell lineage and are much easier to acquire. Even though blood memory T_{FH} cells express CXCR5, they are different to tissue-resident T_{FH} cells when looking at other markers including CD69 and ICOS (Schmitt et al. 2014). In addition, the majority of blood memory T_{FH} cells do express CCR7, which is required to be downregulated in bona fide T_{FH} cells to enable migration from the T-cell zone to the B-cell follicle (Haynes et al. 2007).

Murine models have allowed more extensive studies of T_{FH} cells. Allam et al. (2015) revealed T_{FH} frequencies in different tissues of humanized mice. $CXCR5^+$ $PD\text{-}1^{++}$ $CD4^+$ T_{FH} cells were largely present in the mesenteric lymph nodes (MLN), in Peyer's patches (PP) and, at lower frequencies, in intraepithelial (IEL) and lamina propria derived lymphocyte (LPL) populations (Allam et al. 2015). A Study by Debock et al. (2013) demonstrated that the proportion of $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ T_{FH} cells were significantly reduced and showed a reduced expression of Bcl-6 compared to adult mice T_{FH} cells. Nevertheless, studies on neonatal tissues are mostly restricted to neonatal mice lymph nodes and there is a lack of data regarding T_{FH} cells in human tissues.

2.5 Intestinal mucosal barrier

2.5.1 Intestinal anatomy and associated lymphoid tissue

The effector site of the intestinal immune system is the epithelial and lamina propria layers, which separately build distinct immunological compartments. Histological the gastrointestinal tract consists of the four layers of mucosa, submucosa, muscular layer, and adventitia. The mucosa is the luminal layer and can be further subdivided into the epithelium, the lamina propria, and the muscularis mucosae (Schünke et al. 2012). The lamina propria layer contains different cell types such as B cells, T cells, and several cells from the innate immune system. Meanwhile, the overlying epithelium primarily contains T cells (Mowat and Agace 2014).

Whereas effector immune cells are distributed throughout the mucosa, additional organized structures of the gut-associated lymphoid tissue (GALT) are localized in the mucosa and submucosa. Due to the enormous intestinal surface, the GALT has a special role for the immune system and contains most of the body's T cells, plasma cells, and macrophages (Mowat and Agace 2014). The GALT comprises isolated and aggregated lymphoid follicles, as well as the mesenteric lymph nodes (Neutra et al. 2001). Peyer's patches are aggregated lymphoid follicles within the jejunum and ileum that consist of B-cell lymphoid follicles, surrounded by T-cell areas. Their counterparts in the colon are colonic patches (Mowat and Agace 2014).

The PPs are surrounded by follicle associated epithelium (FAE). This FAE contains cluster of microfold cells (M-cells), which are specialized epithelial cells that give antigens or bacteria via transcytosis from the lumen to APCs that stay in pockets underneath (Corr et al. 2008). Activated dendritic cells then migrate towards the T-cell area and activate naïve T cells, which in turn migrate to GCs and promote the IgA class-switch in B cells. After transcytosis of specific secretory IgA back into the intestinal lumen, it is able to impede pathogens from adhering to the mucosa, infecting the epithelial cells, and breaching the mucosal barrier (Pabst 2012, Brown et al. 2013, Kim and Jang 2017). In addition, specific effector T cells traveling to the mucosal sites support the barrier function (Lycke 2012).

2.5.2 Influence of the microbiota on the intestinal immune system

The intestinal mucosa is responsible for the uptake of nutrients and therefore must provide a certain permeability that also enables pathogens to use the intestinal mucosa as an entry

point to the human body (Cebra 1999, Brown et al. 2013, Mowat and Agace 2014). A barrier consisting of physical, biochemical, and immunological elements ensures protection (Sánchez de Medina et al. 2014). However, in neonates the intestinal barrier is very vulnerable mainly due to decreased acid secretion, mucous, and motility (Basu 2015).

Physiologically the intestinal mucosa is in symbiosis with a beneficial diverse microbiome. Neonates are only largely colonized with microbes from the environment after birth, primarily from the birth canal, including the mother's vaginal and faecal microbiomes (Stark and Lee 1982). It is subject of recent studies, whether the foetus and placenta are already colonized with microbiota in utero. Results from Aagaard et al. (2014) suggest that the placenta accommodates commensal microbiota, whereas Theis et al. (2020) could not confirm the in-utero colonization hypothesis.

The microbiota is involved in several processes in the intestine including the development and homeostasis of the immune system (Brown et al. 2013). Several studies showed in experiments with germ-free mammals that microbial colonization induces maturation of the GALT and the intestinal immune system (Arrieta et al. 2014).

2.5.3 Characterization of T-cell populations in infant intestinal tissue

2.5.3.1 Memory T cells in neonatal and infant intestinal mucosal tissues

It was assumed, that infants in their first years of life are dependent on their innate immune system because adaptive immune cells in blood and lymphoid tissues are reduced (Levy 2007). However, these assumptions mainly rely on experiments with human blood and are not transferable to all tissue sites. Bunders et al. (2012) showed through the context of HIV-1 mother-to-child transmission (MTCT) that infant blood and intestinal immune cells differ. It could be shown that memory $CD45RO^+$ $CD4^+$ T cells were largely present in the intestinal mucosa and submucosa of foetuses and young neonates, compared to lower frequencies in the spleen. In the lymph nodes (LN) and blood, memory T cells were scarce. In addition, T-cell clones in the intestine were unique and showed little overlap with those in spleen or blood, suggesting that T cells in the intestine are developing as a unique T-cell compartment (Bunders et al. 2012). This is most likely due to early exposure of the intestinal mucosa to antigens of the environment, with subsequent activation of the mucosal immune system.

Thome et al. (2016) also showed frequencies of more than 20% of memory T cells in the jejunum and ileum of infants up to two years of age and significantly less in the blood,

spleen, lymph nodes, and colon. This compartmentalization of memory T cells early in life leads to a concept of an early local immune response to ingested antigens (Thome et al. 2016). In T-cell compartmentalization tissue-resident memory T (T_{RM}) cells are important effector cells in tissue compartments.

2.5.3.2 Tissue-resident T cells

T_{RM} cells are anchored in tissues and provide protection specific to their tissue site. In addition, T_{RM} cells have an emerging role in tissue generation and regeneration by the production of tumour necrosis factor alpha (TNFα) as a pro-inflammatory cytokine (Schreurs et al. 2019). Mucosal T_{RM} cells have a distinct phenotype and functional profile. Because T_{RM} cells do not recirculate they cannot be measured by studying peripheral blood. The early activation marker CD69 and the epithelial cell-binding integrin αEβ7 (also CD103) are a marker for tissue residency and can further characterize memory T cells in tissues (Farber et al. 2014).

In different tissues, CD69 is expressed by memory T cells and distinguishes these cells from circulating T cells, which are mainly negative for CD69 (Sathaliyawala et al. 2013). The integrin CD103 is in turn associated with intestinal immune cells, especially memory $CD8^+$ T cells (Schön et al. 1999, Sheridan and Lefrancois 2011).

2.5.4 Oral vaccination

Several pathogens and most toxins enter and infect humans via the different mucosal routes of respiratory, genitourinary, and particularly the gastrointestinal tract (GIT). Nevertheless, vaccines administered by injection generally induce low protection at mucosal sites (Lavelle 2005). Mucosal vaccinations are able to induce immune protection in mucosa-associated lymphoid tissues (MALT) so that pathogens could be captured before they enter and establish an infection (Holmgren and Czerkinsky 2005, Neutra and Kozlowski 2006, Lycke 2012). Furthermore, mucosal vaccines can induce a systemic, long-lasting antigen-specific humoral and cellular immune response (Sallusto et al. 2010, Lycke 2012).

In addition to the immunological advantages of mucosal vaccines over parenteral vaccinations, there are clear advantages for the implementation of oral vaccines. Oral vaccines do not require extensive purification as are needed for parenteral use, therefore reducing the costs of production. Additionally, they are favourable for mass vaccination as they can be administered by persons without special training, improving compliance in less

developed regions. Moreover, the risk for blood-borne infections, due to contaminated needles, is decreased (Levine and Dougan 1998 Yuki and Kiyono 2009, Lycke 2012).

Oral vaccination is until now restricted to a limited number of live vaccines (WHO 2018). Other than for live vaccines the numerous barriers in the intestines pose a challenge for the development of a new vaccine (Holmgren and Czerkinsky 2005, Lavelle 2005). First, mucosal sites strictly regulate the influx of antigens from luminal and have additional barriers, such as a chemical or enzymatic environment that degrades vaccines. Second, the vaccines need to be delivered to the inductive site of the mucosa. A third big hurdle for oral vaccines is the risk of inducing oral tolerance instead of protective immunity. Taken together these hurdles for oral vaccines contribute to a decreased immunogenicity. Live-attenuated vaccines are capable of handling these hurdles better than non-living whole cell or subunit vaccines, due to their resemblance to natural pathogens (Lycke 2012, Kim and Jang 2017).

In order to reach sufficient immunogenicity through vaccines, strong and safe adjuvants are required to support the vaccine antigen. Since most adjuvants were developed for parenteral application (Kim and Jang 2017), and limited numbers are approved for infant vaccination (Morris and Surendran 2016), further understanding will improve their development.

Currently, the live attenuated vaccines against rotavirus (RV), poliovirus, *Salmonella Typhi*, *Vibrio cholerae*, and a non-living vaccine against cholera, are the only approved oral vaccines (Lycke 2012). Among these, the rotavirus vaccine is a representative example of successful oral vaccine development. The rotavirus is responsible for severe diarrhoea in under-five year old infants and the RV gastroenteritis causes approximately five percent of all child deaths worldwide. The rotavirus is highly infectious via the faecal-oral route and specific therapy is not available (WHO 2013). Since 2006 the live oral rotavirus vaccines RV1 and RV5 were approved and since 2009 the World Health Organization (WHO) recommended to include the vaccination in the national immunization schedules (Kollaritsch et al. 2015). The vaccination implementation demonstrably decreased the numbers of infant deaths and hospitalisations. In some countries deaths due to all causes of diarrhoea decreased by up to 50% (Tate and Parashar 2014). Most likely due to herd protection, in Austria, the number of cases was also decreased in infants younger than 90 days, which were too young to be vaccinated (Paulke-Korinek et al. 2011).

The development of an oral vaccine against the dangerous rotavirus was successful. However, it was not successful in mimicking the formulations and protocols for vaccines against other pathogens in the past so that further understanding is necessary (Lycke 2012).

2.6 Objectives of the study

At present, the knowledge of the local immune responses in the infant's intestines is not well understood. The infant immune system, in general, is assumed to be rather immature. However, Bunders et al. (2012) discovered activated memory T cells in the intestinal mucosa even before birth. Therefore, there might be a large potential of inducing a stronger and longer-lasting vaccine response through oral vaccines. T_{FH} cells are the limiting factor to the GC response and consequently to the production of high-affinity antibodies by B cells (Linterman and Hill 2016). So far it is unknown whether there are also T_{FH} cells with a memory phenotype present in the intestinal mucosa early in life. A better understanding would improve the research design for new and better vaccine strategies and enable the reduction of infant mortality due to infectious diseases.

Other studies on T_{FH} cells so far have methodical limitations regarding the study cohort or type of examined material. On the one hand, there are a couple of studies on mice tissues (Mastelic et al. 2012, Debock et al. 2013). In general mice studies are not completely applicable to humans, because the immune system of neonatal mice is less developed than the one of human neonates (Hodgins and Shewen 2012). Mice are usually in pathogen-free conditions often exposed to one type of pathogen in experiments, whereas humans are exposed to thousands of pathogens (Farber et al. 2014). The additional problem is that mice neonates develop quickly so that there is not much time for experiments, leaving the question if experiments were still performed on neonatal mice (Morris and Surendran 2016). On the other hand, studies were made with umbilical cord blood (Yu et al. 2015) that neither reflects the immune function after birth, which might be impacted by perinatal stress (Kovarik and Siegrist 1998), nor does it represent the conditions in tissues. The majority of the body's memory T cells are present in tissues and might be poorly represented by studies on peripheral blood (Farber et al. 2014). In the intestinal tissue, the close contact to antigens from food and the microflora, for instance, has an enormous influence on the development of the local immune system (Basu 2015).

This study hypothesises that mature and functional T_{FH} cells in intestinal mucosa are induced soon after birth. Therefore, it had been the aim to assess the occurrence and ontogeny of

T_{FH} cells. It was aimed to determine to what extent the T_{FH} cells in infant intestinal tissues differ the T_{FH} cells in adult intestinal tissues. In this study, T_{FH} cells were determined upon several characteristics, including their memory and tissue-resident phenotype, cytokine production, and their precondition to interact with DC and B cells. In order to test the hypothesis infant and adult intestinal tissue samples were collected from surgeries and mononuclear cells were isolated. T_{FH} cells were quantitatively and qualitatively determined using multiparameter flow cytometry.

3 Material and methods

3.1 Material

3.1.1 Disposable materials

Table 1. Disposable materials that were used and the company by which they were provided.

Disposable material	Provided by
Serological pipette (5 mL, 10 mL, 25 mL)	Sarstedt AG & Co. KG, Nümbrecht, Germany
Biosphere Filter Tips (100–1000 µL, 2–100 µL, 0.5–20 µL)	Sarstedt AG & Co. KG, Nümbrecht, Germany
TC Plate, Standard F (6/12/24/48 Well)	Sarstedt AG & Co. KG, Nümbrecht, Germany
Microtest plate, 96-well, polystyrene, conical bottom	Sarstedt AG & Co. KG, Nümbrecht, Germany
Cellstar Centrifuge Tubes (50 mL, 15 mL)	Greiner Bio-One GmbH, Kremsmünster, Austria
Transfer pipettes	Sarstedt AG & Co. KG, Nümbrecht, Germany
EASYstrainer 70 µm, for 50 mL tubes	Greiner Bio-One GmbH, Kremsmünster, Austria
Puradisc Sterile Syringe Filter with Cellulose Acetate Membrane, Diameter: 30 mm, Pore Size: 0.2 µm	Whatman/GE Healthcare, Little Chalfont, United Kingdom
Falcon 5 mL Polystyrene Round-Bottom Tube	Corning, Inc., Corning, USA
Single-use syringe 5 mL	B. Braun Melsungen AG, Melsungen, Germany

3.1.2 Reagents

Table 2. Reagents that were used and the company by which they were provided.

Reagent	Provided by
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Iscove's Modified Dulbecco's Medium (IMDM)	Life Technologies, Carlsbad, USA
Standardized Foetal Bovine Serum (FBS)	Biochrom GmbH, Berlin, Germany
1,4-Dithiothreit (DTT)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ethylenediaminetetraacetic acid solution (EDTA)	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Collagenase D	Roche Diagnostics GmbH, Rotkreuz, Schweiz
DNase I Solution (1 mg/mL)	STEMCELL Technologies Inc., Vancouver, Canada

Continuation of Table 2.

Reagent	Provided by
Hanks' Balanced Salt Solution	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Biocoll Separating Solution	Biochrom GmbH, Berlin, Germany
Percoll Density Gradient Media	GE Healthcare, Chicago, USA
Trypan Blue solution, 0.4%	Sigma-Aldrich Chemie GmbH, St. Louis, USA
BD CellFix	BD Bioscience, Franklin Lakes, USA
eBioscience IC Fixation Buffer	Thermo Fisher Scientific, Waltham, USA
eBioscience Permeabilization Buffer (10X)	Thermo Fisher Scientific, Waltham, USA
eBioscience Fixation/Permeabilization Diluent	Thermo Fisher Scientific, Waltham, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Ionomycin calcium salt	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Brefeldin A	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Penicillin-Streptomycin (Pen-Strep)	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Zombie Aqua Fixable Viability Kit	BioLegend, San Diego, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, St. Louis, USA
Anti-mouse Ig, Negative Control Compensation Particles Set	BD Bioscience, Franklin Lakes, USA
10x PBS	Sigma-Aldrich Chemie GmbH, St. Louis, USA

3.1.3 Solutions

Table 3. Solutions that were made and their respective components.

Solution	Composition
EDTA/DTT-Mix	95% IMDM, 5% FBS, 5 mM EDTA, 2 mM DTT
Collagenase-Mix	99% IMDM, 1% FBS, Dnase I (1:100), 1 mg/mL Collagenase D (sterilized)
60% Standard Isotonic Percoll solution (SIP)	9:1 Percoll and 10x PBS, afterwards diluted with 60% 1x PBS
Culture medium	94% IMDM, 5% FBS, 1% Pen-Strep

3.1.4 Equipment

Table 4. Equipment and the company by which it was manufactured.

Equipment	Manufacturer
Herasafe KS (NSF) Class II, Type A2 Biological Safety Cabinets (KS12)	Thermo Electron Corporation, Waltham, USA
Orbital Shaker 3005	GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany
Centrifuge 5810 R	Eppendorf AG, Hamburg, Germany
PG403-S DeltaRange	Mettler-Toledo, Columbus, USA
Counting chamber Neubauer improved bright-line	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Inverted Microscope Leica DM IL	Leica Microsystems GmbH, Wetzlar, Germany
Allegra X-22R Centrifuge	Beckman Coulter, Brea, USA
LSRFortessa	BD Bioscience, Franklin Lakes, USA
Hybridisation oven, HB-1000 Hybridizer	UVP, LLC, Upland, USA
Pipetboy acu/ acu 2	INTEGRA Biosciences AG, Zizers, Switzerland
Eppendorf Research plus (0.5–10 µL, 10–100 µL, 100–1000 µL)	Eppendorf AG, Hamburg, Germany
PCV-2400 Combined Centrifuge/Vortex Mixer (Combi Spin)	Grant Instruments, Shepreth, United Kingdom
Vortex mixer Certomat MV	B. Braun Biotech International, Melsungen, Germany
Heracell 240 L CO_2 Incubator	Thermo Electron Corporation, Waltham, USA

3.1.5 Antibodies

Table 5. Anti-human antibodies used for extracellular staining with information regarding the dye, the clone and the company by which the antibody was provided.

Anti-human antibody for extracellular staining	Clone	Provided by
CD25, BUV395	2A3	BD Bioscience, Franklin Lakes, USA
CD4, BUV737	SK3	BD Bioscience, Franklin Lakes, USA
CCR7 (CD197), PE-Cy7	G043H7	BioLegend, San Diego, USA
CD3, PE-CF594	SP34-2	BD Bioscience, Franklin Lakes, USA
CD45, PE	HI30	Thermo Fisher Scientific, Waltham, USA
PD-1 (CD279), BV785	EH12.2H7	BioLegend, San Diego, USA
CXCR5 (CD185), BV711	J252D4	BioLegend, San Diego, USA
CD45RA, BV650	HI100	BD Bioscience, Franklin Lakes, USA
CD27, APC-eFluor 780	323	Thermo Fisher Scientific, Waltham, USA
CD69, PerCP/Cy5.5	FN50	BioLegend, San Diego, USA
CD127, BB515	HIL-7R-M21	BD Bioscience, Franklin Lakes, USA
CD103, BUV395	Ber-ACT8	BD Bioscience, Franklin Lakes, USA
ICOS (CD278), APC	C398.4A	BioLegend, San Diego, USA

Table 6. Anti-human antibodies used for intracellular staining with information regarding the dye, the clone and the company by which the antibody was provided.

Anti-human antibody for intracellular staining	Clone	Provided by
Bcl-6, BV421	K112-91	BD Bioscience, Franklin Lakes, USA
FoxP3, APC	PCH101	Thermo Fisher Scientific, Waltham, USA
IL-21, APC	3A3-N2	BioLegend, San Diego, USA
TNFα, BUV395	Mab11	BD Bioscience, Franklin Lakes, USA
IL-4, PE-Cy7	8D4-8	Thermo Fisher Scientific, Waltham, USA
CD40L (CD154), BV421	24-31	BioLegend, San Diego, USA

3.1.6 Software

Table 7. Software that were used during the process of data acquisition and analysis, and the company by which the software was provided.

Software	Provided by
FlowJo v10	FlowJo LLC, Ashland, USA
ImageJ 1.50e	National Institutes of Health, USA
GraphPad Prism 7.04	GraphPad Software Inc., San Diego, USA
Microsoft Office 2010	Microsoft Corporation, Redmond, USA
BD FACSDiva Software v.7.0	BD Bioscience, Franklin Lakes, USA

3.2 Patient cohort

To determine T_{FH}-cell frequencies in infant and adult intestinal mucosae, immune cells from tissue samples were investigated. The tissue samples were obtained from residual tissues resected at surgeries performed at the University Medical Centre Hamburg-Eppendorf (UKE) and Altona Children's Hospital (AKK). The ethics committee of the medical association Hamburg (Ärztekammer Hamburg, PV5251) approved the study according to the Declaration of Helsinki. Informed consent was obtained from patients or guardians before surgery. Only tissues of patients without inflammatory diseases were included (Table 8).

Table 8. Information regarding foetal, infant and adult donors. The information comprises age and gender. The description of the age varies between gestational age in weeks (w) for foetal, in months (m) for infant and years (y) for adult patients. Furthermore, the main diagnosis that indicated the surgery the tissues had been acquired in and the part of the intestine that was examined are listed. Donors were numbered randomly.

No.	Group	Age	Gender	Main diagnosis	Surgery	Tissue
1F	**Foetuses**	18 w	unknown	-	abortion	whole intestine
2F		15-20 w				
3F		15-20 w				

Continuation of Table 8.

No.	Group	Age	Gender	Main diagnosis	Surgery	Tissue
4I	**Infants**	3 m	female	anorectal malformation	reconstruction	ileum
5I		4 m	male	unclear defect in intestinal transport	ileostomy reversal	ileum
6I		4 m	male	gastroschisis	ileostomy reversal	ileum
7I		4 m	female	Hirschsprung's disease	ileostomy reversal	ileum
8I		5 m	male	anal atresia	colostomy reversal	colon
9I		6 m	female	Hirschsprung's disease	ileostomy reversal	ileum
10I		6 m	male	Hirschsprung's disease	ileostomy reversal	ileum
11I		6 m	male	anal atresia	colostomy reversal	colon
12I		7 m	female	Hirschsprung's disease	ileostomy reversal	ileum
13I		8 m	unknown	anorectal malformation	reconstruction	colon
14I		8 m	male	Hirschsprung's disease	ileostomy reversal	ileum
15I		9 m	unknown	anorectal malformation	reconstruction	colon
16I		10 m	male	Hirschsprung's disease	ileostomy reversal	ileum
17I		11 m	female	intestinal duplication	resection of duplicated portion	colon
18I		24 m	male	neuroblastoma	nephrectomy and resection colon	colon
19I		30 m	female	gastroschisis	jejunostomy reversal	jejunum
20A	**Adults**	34 y	male	colorectal cancer	ileostomy reversal	ileum
21A		50 y	male	state after rectum resection	ileostomy reversal	ileum
22A		55 y	male	neuroendocrine tumour terminal ileum	hemicolectomy with terminal ileum	ileum
23A		60 y	female	neuroendocrine tumour terminal ileum	hemicolectomy with terminal ileum	ileum
24A		61 y	male	colon cancer	ileostomy reversal	ileum
25A		62 y	female	rectal cancer	ileostomy reversal	ileum
26A		64 y	female	rectal cancer	ileostomy reversal	ileum
27A		65 y	male	gastric carcinoma	gastrectomy	duodenum
28A		67 y	female	neuroendocrine tumour coecum	hemicolectomy with terminal ileum	ileum
29A		68 y	male	peridiverticulitis	ileostomy reversal	ileum
30A		69 y	male	hernia	ileostomy reversal	ileum
31A		74 y	female	pancreatic cancer	Whipple procedure	jejunum
32A		81 y	male	rectal cancer	ileostomy reversal	ileum
33A		> 18 y	female	familial adenomatous polyposis	unknown	unknown

Within the infant cohort (median age: 7.0 months, interquartile range (IQR) 4.9–9.9 months, n = 16) samples were frequently obtained from patients with Hirschsprung's disease and anorectal malformation. The Hirschsprung's disease is a disease where the distal section of the intestine is not innervated and thus must be removed. Because of these diseases being of structural origin, the distribution of immune cells is assumed to be unaffected. The occurrence of diseases in the adult cohort (median age: 64.1 years, IQR 58.0–68.8 years,

n = 14) was different. Most of the adult samples were received from patients with cancer at different anatomical locations, such as rectal, colonic, or pancreatic. Often hemicolectomies were required for the treatment of patients, including residual tissues next to the cancers. For infants as well as adults the stoma reversal surgery is a routine surgery in which tissues remain frequently. Because a stoma is localized in an unaffected part of the intestine, it is presumed to be comparatively healthy.

The foetal data (n = 3) was acquired from elective abortions at the Stichting Bloemenhove clinic in Heemstede, the Netherlands, after obtaining informed consent. At the time of abortion, all the pregnancies were in the second trimester between 15 to 20 weeks of gestational age. Donors 1F, 2F, 3F, 4I, 13I, 15I, and 33A were processed and measured at the Academic Medical Centre (AMC) in Amsterdam, the Netherlands.

3.3 Isolation of mononuclear leukocytes from intestinal mucosae

3.3.1 Collection of mucosal samples

The tissue processing was initiated within an hour after removal. The tissue samples were provided by the surgeons in the native condition, occasionally in saline solution to prevent the tissues from drying out. Tissues from the AKK were transported on ice in IMDM with 5% FBS (see Table 2) to preserve cell viability.

In the laboratory, the tissues were placed in a petri dish and the mucosae were removed from the underlying muscular layer (see Figure 3). In vivo, the intestinal mucosa is natively folded with valves of Kerckring to enlarge the surface area. The valves emerge from the mucosa that is kept together by fibres of the submucosa (Schünke et al. 2012). These valves of Kerckring were removed after severing the submucosal fibres leading to the bona fide size of the mucosa. The mucosae were completely spread out and pictures were taken with a scale for later determination of cell yield per area. The size of the mucosae was estimated to determine the volume of required reagents for the following steps.

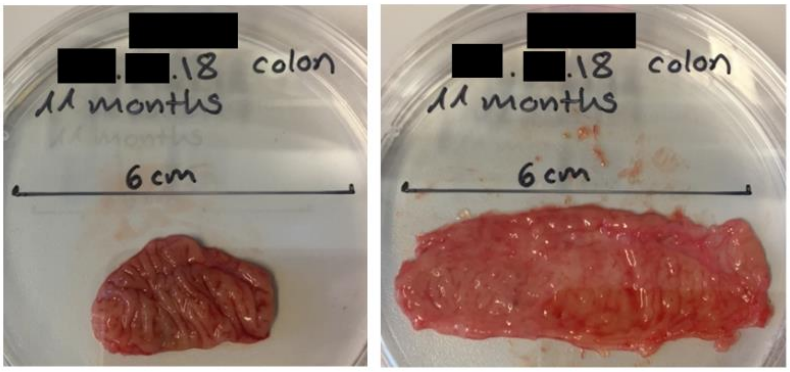

Figure 3. Identical piece of infant colon at the beginning of processing in a petri dish, before (left) and after removal of the submucosal fibres (right). The right picture shows mucosa only, with the underlying layers removed. A scale serves as reference for determination of the area. A donor number, the date of surgery, the patient's age, and the origin in the intestine were labelled. Infant donor no. 17I.

Afterwards, the mucosa was cut into equal pieces of approximately 0.5 cm^2. All the pieces were placed in a tube with IMDM and 0.8 mM dithiothreitol (DTT). The tube was shaken to remove mucus and stool if present (Schreurs et al. 2017). When necessary this step was repeated several times.

3.3.2 Isolation of mononuclear cells from epithelial layers

For isolation of mononuclear cells from epithelial layers, the tissue pieces were placed in 6-well-plates (see Table 1). The 0.5 cm^2 pieces of approximately 5 cm^2 tissue were collected in one well. Per well 6 mL of EDTA/DTT-Mix (see Table 3), consisting of IMDM with DTT and ethylenediaminetetraacetic acid (EDTA), was added to remove the mucus and segregate the epithelial cells. The plates were placed at 4 °C on an orbital shaker (see Table 4) for one hour. Every 20 to 30 minutes the mixes were pipetted up and down up to twenty times with a 25 mL pipette to further disrupt the epithelial cells. At the end of the incubation, the tissue pieces were removed and stored to isolate immune cells from the lamina propria layer. The supernatants, containing the detached epithelial cells, were placed on a 70 µm single-cell strainer. The wells and the strainers were washed with IMDM and the tubes were centrifuged at 500 x g (multiples of gravitational acceleration force) at 4 °C for 10 minutes. The intraepithelial single-cell suspensions were washed a second time and placed at 4 °C until the lamina propria cells were obtained (Schreurs et al. 2017).

3.3.3 Isolation of mononuclear cells from lamina propria layers

After intraepithelial cell isolations, the tissue pieces were minced with scissors creating small pieces of approximately 2 to 3 mm^2 and transferred in 10 mL of Collagenase-Mix (see

Table 3), to a 50 mL tube. For mucosa samples bigger than 10 cm^2 the amount of Collagenase-Mix was doubled. Collagenase degrades collagen and is therefore used to disaggregate tissues and to prepare single cell suspensions (Sigma-Aldrich 2018b). The tubes were placed in a rotating incubator at 37 °C. After 30 minutes the tubes were vortexed at the highest speed of 3200 rpm for at least 10 seconds to loosen the cells mechanically as well. After letting the tissue pieces settle, the supernatants were strained through 70 µm single-cell strainers to obtain single-cell suspensions. Afterwards, 10 mL, or 20 mL for larger tissue samples, of fresh Collagenase-Mix were added to the remaining tissue pieces and the incubation step, along with vortexing and straining the suspensions, was repeated. The tubes and strainers were rinsed with IMDM and the cells from the two subsequent Collagenase incubations were merged. The single-cell suspensions from the lamina propria layers were washed with IMDM twice, each centrifuged at 500 x g at 4 °C for 10 minutes, and stored at 4 °C until density gradient centrifugation (Schreurs et al. 2017).

3.3.4 Purification of cells by gradient centrifugation

Using the gradient centrifugation, the two cell suspensions from epithelial and lamina propria layers were purified respectively to remove erythrocytes, epithelial cells, and detritus. The gradient centrifugation enables the separation of cells and other complexes based on their molecular masses (Fuss et al. 2009). The isolated cells were resuspended in 10 mL Hanks' Balanced Salt Solution. The IEL suspension was carefully layered on top of 4 mL Biocoll Separating Solution, whereas the LPL suspension was layered on top of 4 mL 60% SIP (see Table 3). Tubes were centrifuged at room temperature with 1000 x g (acceleration 1, deceleration 0) for 22 minutes without a break. During centrifugation, the mononuclear cells accumulate on top of the Biocoll Separating Solution, or SIP respectively, while the detritus and erythrocytes accumulate at the bottom of the tube. This enables the careful collection of cells between the two layers with a transfer pipette. Cells were washed twice with IMDM and centrifuged at 500 x g for 10 minutes at 4 °C during each washing step and stored at 4 °C.

Afterwards, 10 µL of the single-cell suspensions were mixed with an equal amount of trypan blue solution and counted with a Neubauer counting chamber under the microscope. Only leukocytes that were alive were counted while excluding erythrocytes and residual epithelial cells.

3.4 T-cell stimulations

To determine the cytokine production of T cells, cells were stimulated. The mononuclear cell fractions were resuspended in culture medium (see Table 3), resulting in an end concentration of $2x10^6$ cells/mL and were distributed in a 24-well-plate with 1 mL in each well. For stimulation, 1 mL of culture medium containing phorbol 12-myristate 13-acetate (PMA) and ionomycin calcium salt was added to the wells. The control wells received only the culture medium. The end concentrations were 40 ng/mL PMA and 1 µg/mL ionomycin. PMA, as an activator of the protein kinase C, and ionomycin, as a calcium ionophore, omit the T-cell receptor activation and mediate T-cell activation as well as production of a variety of cytokines (Ai et al. 2013). The plates were stored in an incubator at 37 °C with 5% carbon dioxide (CO_2) throughout the whole stimulation time.

After one hour incubation, brefeldin A was added to stimulated and unstimulated cells to prevent the cytokines from leaving the cells. Brefeldin A disrupts the structure and the function of the Golgi complex and therefore inhibits the transport from the endoplasmic reticulum to the Golgi complex (Sigma-Aldrich 2018a). After 12 to 14 hours total incubation time, cells were washed and intracellularly stained for the cytokines IL-21, IL-4, and TNFα.

3.5 Flow cytometry

3.5.1 Principles of flow cytometry

Flow cytometry allows multiparametric analysis of physical and chemical characteristics for thousands of cells per second. It relies on a single cell suspension that allows single cells to pass several lasers at a high speed. Different detectors can detect a fluorescent signal or scattered light. Whereas the side-scattered (SSC) light is proportional to the intracellular granularity of cells, the forward-scattered (FSC) light correlates to the volume of the cells. Furthermore, cell surface structures and intracellular proteins, which were previously labelled by different fluorescent antibodies, can be determined as well. The intensity of their emitted fluorescent light is thereby directly proportional to the expression level of the protein. This technique enables the collection of information regarding cell size, granularity, and cell surface as well as intracellular proteins for every single cell (Ibrahim and van den Engh 2007).

3.5.2 Antibody staining

To detect specific surface and intracellular molecules, cells were stained with antibodies that bind to specific molecules on human cells (see Table 5 and 6). The antibodies used to identify and specifically characterize the T_{FH} cells were combined in three different panels. Occasionally, not all panels could be stained for every tissue sample due to low number of cells. Usually, $2x10^6$ cells were stained per condition and per panel in a well of a 96-V-bottom-plate. After transferring the cells to the plates, they were washed with phosphate buffered saline (PBS), before adding 30 µL of antibody mix to each well. The antibody mixes contained all the different required antibodies and the Zombie Aqua Fixable Viability Kit in PBS, which is only adherent to cells with compromised membranes. This is used for discrimination of live and dead cells (BioLegend 2018). The optimal concentration for an antibody was determined beforehand by titration of each antibody respectively. Cells were incubated on an orbital shaker at 4 °C in the dark for 30 minutes. Afterwards, 100 µL PBS was added to each well and the plates were centrifuged at 4 °C, 500 x g for 5 minutes and the supernatants were removed. To fixate the cells for flow cytometry analyses, they were resuspended in 100 µL BD CellFix.

Usually additional intracellular staining, for detection of transcription factors or cytokines after stimulation, were performed. For intracellular staining, 150 µL of 1:4 eBioscience IC Fixation Buffer and eBioscience Fixation/Permeabilization Diluent was used for fixation. After incubation in the dark, at 4 °C on the orbital shaker for 15 minutes, cells were washed with PBS. Cells were permeabilized with 30 µL 1x eBioscience Permeabilization Buffer containing the intracellular antibodies for 30 minutes at 4 °C on the orbital shaker. For the following washing step, 1x eBioscience Permeabilization Buffer was used again. Cells were resuspended in 100 µl PBS for flow cytometric analyses. Cells from extracellular, as well intracellular staining were stored up to maximal 24 hours in the dark at 4 °C before flow cytometric analyses.

3.5.3 Data acquisition

Except for the tissues of donors processed and analysed in Amsterdam, all cells were measured at the same flow cytometer BD LSRFortessa with support of BD FACSDiva Software (see Table 7). Most of the samples were measured with the same application settings, which were generated after compensation of the different fluorescent lights emitted by the different antibodies from one panel. The application settings were always adapted to

the respective BD Cytometer Setup and Tracking (CS&T) protocol, which is a performance quality control run daily. The use of application settings ensures consistency of results throughout different experiments measured on different days (Meinelt et al. 2012). During measurement, the flow rate was always chosen to be similar and kept the abort rate below 5%. The acquisitions were terminated when the single-cell suspensions were exhausted and the approximately two million cells were acquired. The BD FACSDiva Software saved the data in a flow cytometry standard (fcs) 3.1 file format, which could be analysed in FlowJo. For each cell and panel of antibodies, next to SSC and FSC, additional 12 to 14 parameters were collected.

3.6 Analysis

3.6.1 Gating strategy

The data measured by flow cytometry were analysed with FlowJo (see Figure 4). Total cells were first gated on single cells. In case of clumps and clotted cells, the width of side-scattered light (SSC-W) and forward-scattered light (FSC-W) becomes larger and could be excluded by gating. Afterwards, $CD45^+$ alive cells were selected roughly to remove the dead cells. CD45 is the leukocyte antigen. By plotting the area of FSC (FSC-A) and SSC (SSC-A) against each other, lymphocytes were identified by cell size and granularity.

After the gating of the lymphocytes, a more precise gate identifies the $CD45^+$ alive leukocytes again. The $CD3^+$ $CD4^+$ lymphocytes were selected from the total leukocyte populations. T_{FH} cells were identified as $CD4^+$ T cells expressing CXCR5, and high levels of PD-1. Qi (2016) described this as the most common method to identify T_{FH} cells. The T_{FH} cells were examined for different cell markers, including CD45RA and CD69, to further characterize these cells. Thereby the T_{FH} cells were usually compared to the $CXCR5^+$ $PD\text{-}1^-$ pre-T_{FH} population, which does express $CXCR5^+$ but lacks PD-1 expression (see Figure 5). In other analyses, the T_{FH} and the pre-T_{FH} cells were compared to $CXCR5^-$ $PD\text{-}1^-$ non-T_{FH} population and the intermediate $CXCR5^+$ $PD\text{-}1^+$ population for further sub-division. Occasionally a comparison between the total $CXCR5^+$ population and the $CXCR5^-$ $CD4^+$ T-cell population was performed, due to low frequencies of T_{FH} cells, to increase validity. Gates were positioned equally for all samples.

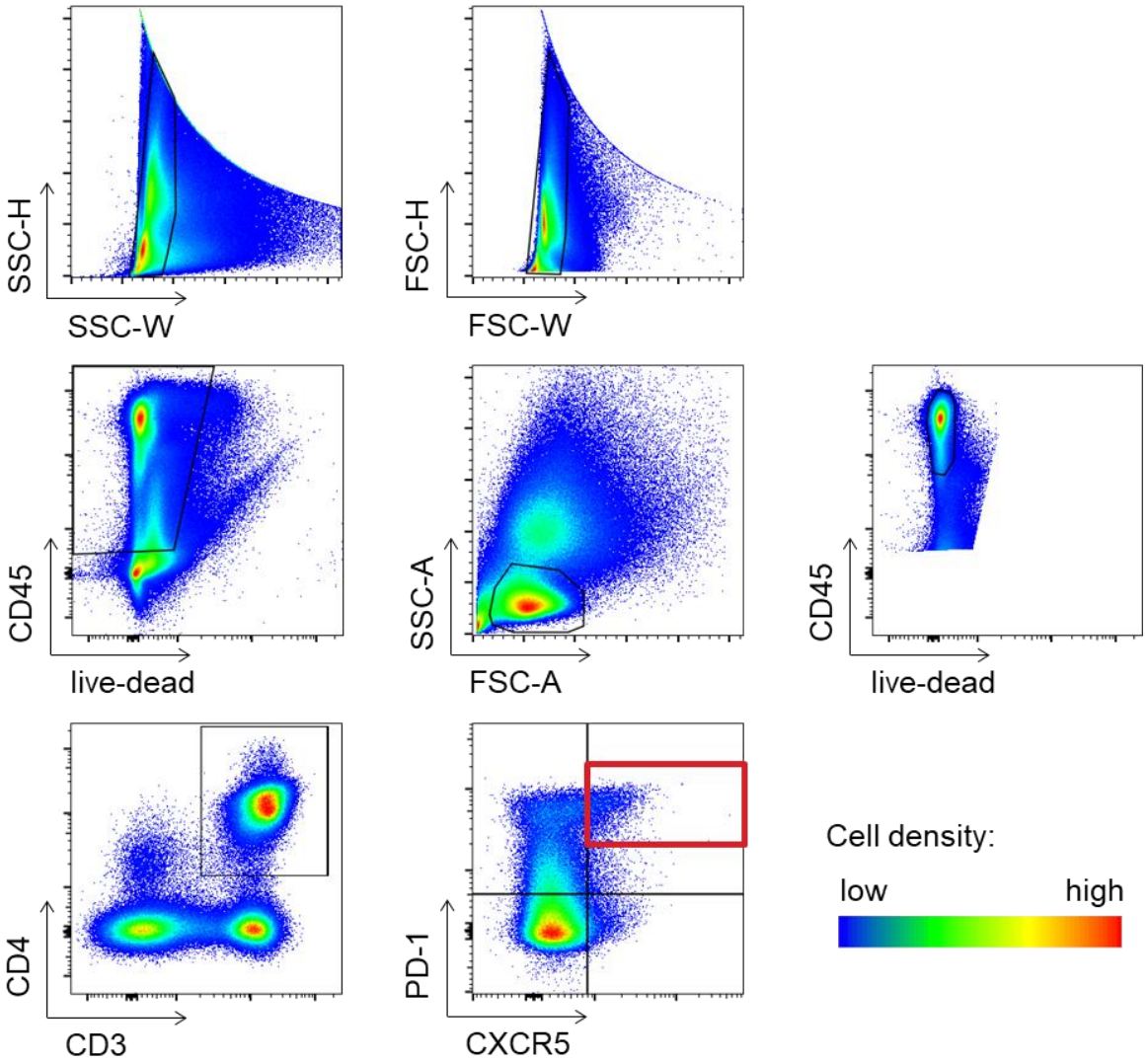

Figure 4. Gating strategy of flow cytometry data from a representative infant intestinal tissue sample. The first two pseudocolor dot plots show the gating towards single cells. The following gating on $CD45^+$ alive leukocytes done twice is displayed in the second row, with lymphocyte gating in between. From the lymphocyte population going to the last row, only $CD3^+$ $CD4^+$ cells were selected. Among the $CD4^+$ T cells, T_{FH} cells are $CXCR5^+$ and $PD\text{-}1^{++}$ (red square). Every plot shows the cells that were selected by the gate in the prior plot. The axes are in general logarithmic, but linear for SSC and FSC. The colors demonstrate the cell density going form low cell density in blue to high cell density in red. Infant donor no. 11I.

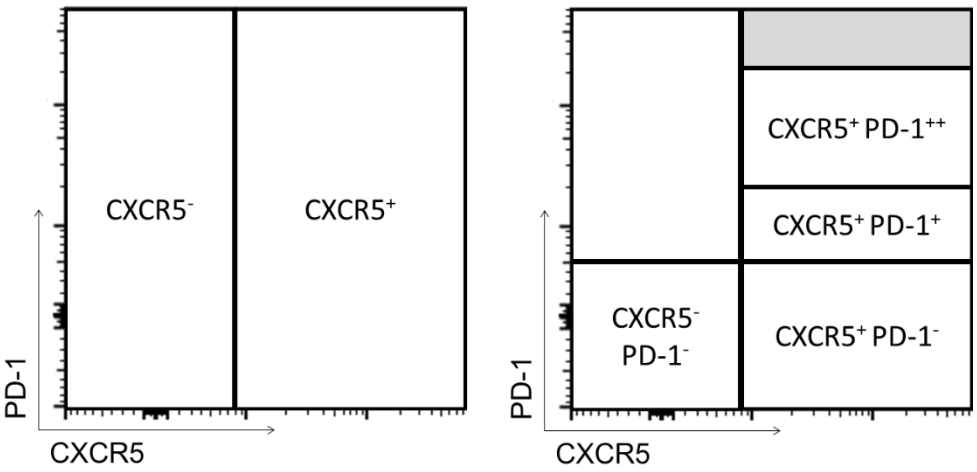

Figure 5. Gating strategy on the different populations used in the analysis to identify the specific populations. Starting with $CD4^+$ T cells two populations divided by the expression of CXCR5 were compared. Furthermore, the $CXCR5^+$ $PD\text{-}1^{++}$ T_{FH} population, an intermediate $CXCR5^+$ $PD\text{-}1^+$ population, and the $CXCR5^+$ $PD\text{-}1^-$ pre-T_{FH} population were sub-divided and compared to the $CXCR5^-$ $PD\text{-}1^-$ non-T_{FH} population. The gray space was free of any cells.

3.6.2 Evaluation of selected data and creation of figures

The figures and statistics were created with GraphPad Prism. Populations smaller than ten cells were excluded for further analysis due to their low validity. For the statistics, usually a non-parametric Mann-Whitney U test was used. Differences were identified as significant with a p-value below 0.05. A trivariate linear regression model was used in two cases with the support of Laura Richert. Medians and interquartile ranges (IQR) are shown in figures.

The area of the mucosa after preparation (see Figure 3, right) was exactly determined using the image processing program ImageJ and further calculations were made with Microsoft Excel. Determinations of cells per area were only feasible when the total cell suspensions had been measured by flow cytometry.

4 Results

4.1 CXCR5^{+} PD-1^{++} T_{FH} cells are present in infant and adult intestinal mucosal tissues

In order to understand and improve vaccine responses in neonates and infants, this study aimed to identify and characterize T_{FH} cells in infant intestinal mucosae in comparison to T_{FH} cells in adults. Therefore, mononuclear cells isolated from intestinal mucosae were analysed using flow cytometry. Presumed healthy intestinal tissues from infants ranging from 4 to 30 months of age, were compared to intestinal tissues from adults. T_{FH} cells were identified in this study as CD4^{+} T cells expressing CXCR5 and high amounts of PD-1.

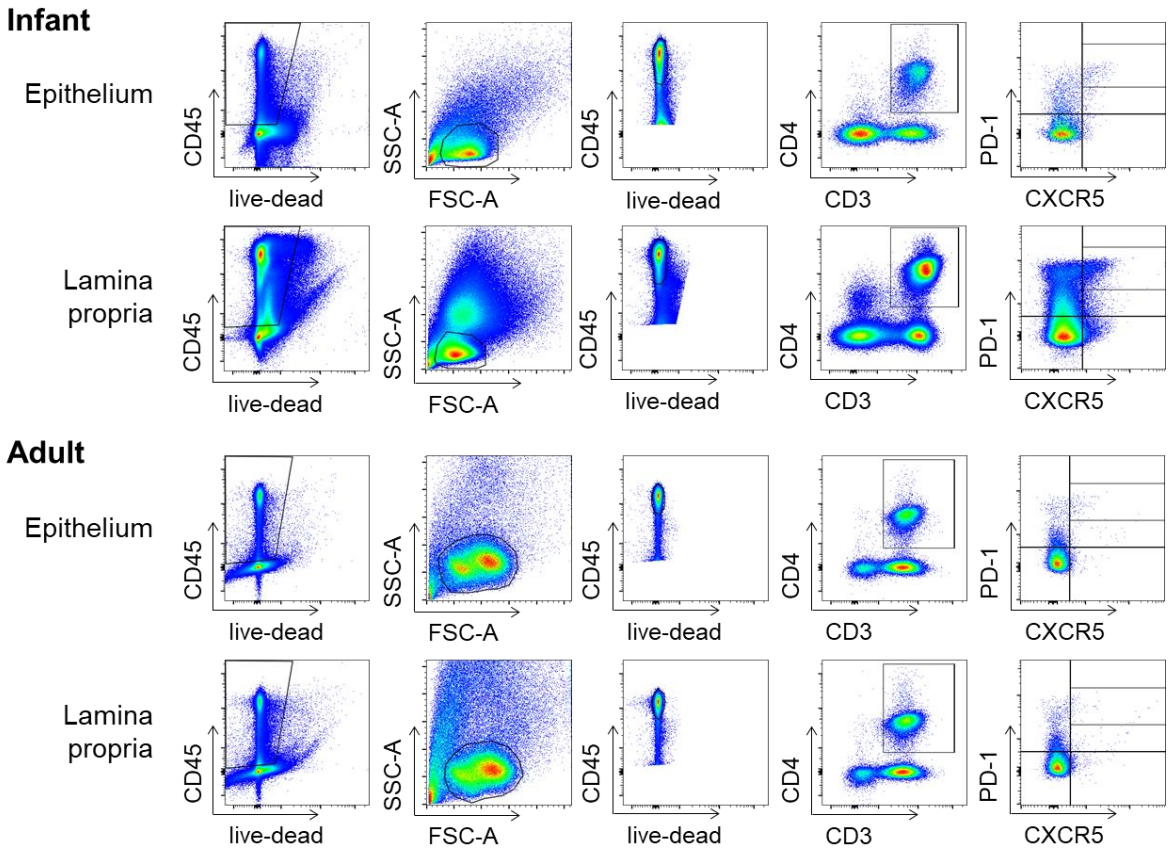

Figure 6. Representative dot plots from flow cytometric analysis of epithelial and lamina propria mononuclear cells. The single-cell gating is shown above (see Figure 4). T_{FH} cells are within the CD45^{+} live CD3^{+} CD4^{+} lymphocytes group. Among the cells expressing the lymph node homing receptor CXCR5^{+}, there are specialized PD-1^{++} T_{FH} cells. Infant donor no. 11I and adult donor no. 29A are shown.

CXCR5^{+} PD-1^{++} T_{FH} cells were detected in infants as well as in adults throughout every age. They were further identified in the epithelial, as well as in the lamina propria layers, although at different frequencies (see Figure 6).

4.2 T_{FH} cells are abundant in infant lamina propria intestinal mucosae

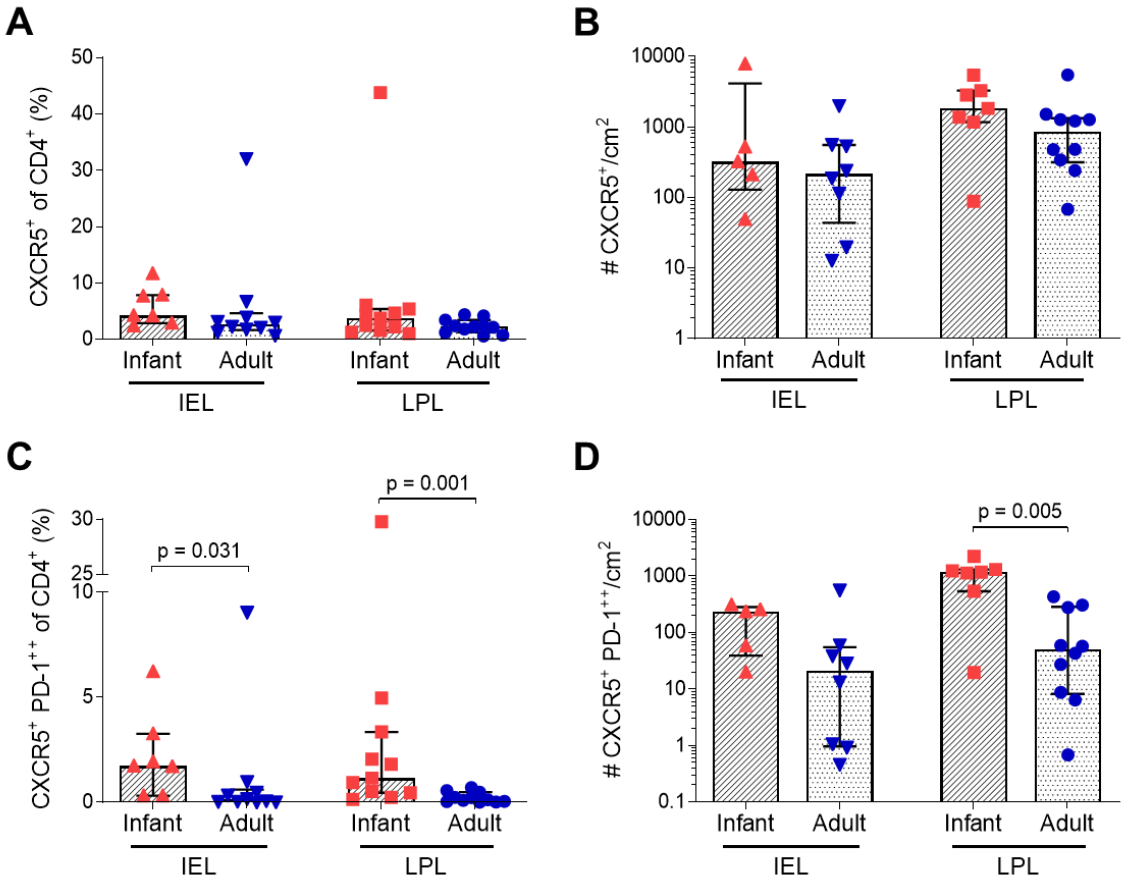

Figure 7. Frequencies of CXCR5^{+} (A) and CXCR5^{+} PD-1^{++} (C) expression by CD4^{+} cells and absolute numbers of CXCR5^{+} (B) and CXCR5^{+} PD-1^{++} cells (D) per area of mucosa. CXCR5^{+} CD4^{+} T cells were detected frequently in epithelial and lamina propria layers of infants as well as adults (A/B). CXCR5^{+} PD-1^{++} T_{FH} cells were more frequent in the infant intestines compared to the adult intestines (C/D). In the epithelial layer the frequencies of T_{FH} cells in infants were significantly higher than in adults ($p = 0.031$). In the lamina propria layers the difference between infants and adults was significant in frequencies ($p = 0.001$) and absolute numbers ($p = 0.005$). Median frequencies (± IQR) are given. Linear (A/C) and logarithmic (B/D) scales were used. The "#" stands for the quantity. Mann-Whitney U tests were used for statistical analysis. Intestinal tissue samples: Infants IEL n = 7, infants LPL n = 11, adults IEL n = 10, adults LPL n = 11 (A/C). Infants IEL n = 5, infants LPL n = 7, adults IEL n = 8, adults LPL n = 10 (B/D).

Within the CD4^{+} T-cell populations, CXCR5^{+} cells were present in infant and adult epithelial as well as lamina propria layers. A proportion of 4.26% (median, IQR 2.89–7.86%, n = 7) of infant CD4^{+} T cells in intraepithelial lymphocyte (IEL) populations expressed

CXCR5, whereas 3.79% (median, IQR 1.58–5.41%, n = 11) in infant lamina propria lymphocyte (LPL) populations did. Compared to infants, adults had less $CXCR5^+$ $CD4^+$ T cells in IEL with 2.70% (median, IQR 1.69–4.64%, n = 10) and 2.28% (median, IQR 1.26–3.45%, n = 11) in LPL layers (see Figure 7A).

This trend was reflected by the absolute numbers of cells, which were calculated in proportion to the area of the mucosae. 1830 cells/cm^2 (median, IQR 1167–3275 cells/cm^2, n = 7) in infant and 851 cells/cm^2 (median, IQR 316–1333 cells/cm^2, n = 10) in adult intestinal mucosae were observed in the lamina propria layers. Similarly, infants (median 321 cells/cm^2, IQR 129–4139 cells/cm^2, n = 5) had more cells per square centimetre than adults (median 216 cells/cm^2, IQR 44–557 cells/cm^2, n = 8) in epithelial layers (see Figure 7B).

As observed for the total $CXCR5^+$ $CD4^+$ T cells, the trend towards increased frequencies in infants also holds true for specialized T_{FH} cells. Frequencies of infant T_{FH} cells were significantly higher for IEL (median 1.73%, IQR 0.31–3.26%, n = 7) as well as LPL (median 1.15%, IQR 0.45–3.35%, n = 11) compared to adult intestines (IEL: p = 0.031, LPL: p = 0.001), with adult intestines having comparatively lower frequencies of T_{FH} cells in epithelial (median 0.12%, IQR 0.03–0.61%, n = 10) and lamina propria layers (median 0.12%, IQR 0.03–0.48%, n = 11) as shown in Figure 7C.

The absolute numbers of cells were significantly higher (p = 0.005) in infants with 1177 T_{FH} cells/cm^2 (median, IQR 539–1312, n = 7) than in adults with 50 T_{FH} cells/cm^2 (median, IQR 8–287 cells/cm^2, n = 10) in LPL. The same trend was also apparent in IEL, where infants had 236 T_{FH} cells/cm^2 (median, IQR 39–283 cells/cm^2, n = 5), while adults had a median of 21 T_{FH} cells/cm^2 (IQR 1–55 cells/cm^2, n = 8) as shown in Figure 7D.

In summary, T_{FH} cells were abundant in infant intestinal mucosae, whereas in adulthood, T_{FH} cells were comparatively decreased. This age-dependent difference was furthermore distinct for LPL. As total numbers of lymphocytes in lamina propria were higher compared to epithelial lymphocytes, following experiments were performed investigating LPL.

Recently we have shown that $CD4^+$ T cells are present in foetal intestines from 13 weeks of gestation (Schreurs et al. 2019), therefore we examined in these samples whether a population of T_{FH} cells was also already present prior to birth. This substudy was performed in Amsterdam. Foetal intestinal tissue samples from 15 to 20 weeks of pregnancy were investigated. Flow plots from foetal intestinal cells showed no expression of CXCR5 on

$CD4^+$ T cells (see Figure 8A). The frequencies of foetal T_{FH} cells from all $CD4^+$ cells as shown in Figure 8B with 0.44% (median, IQR 0.03–0.45%, n = 3) was lower than in infants (median 1.15%, IQR 0.45–3.35%, n = 11).

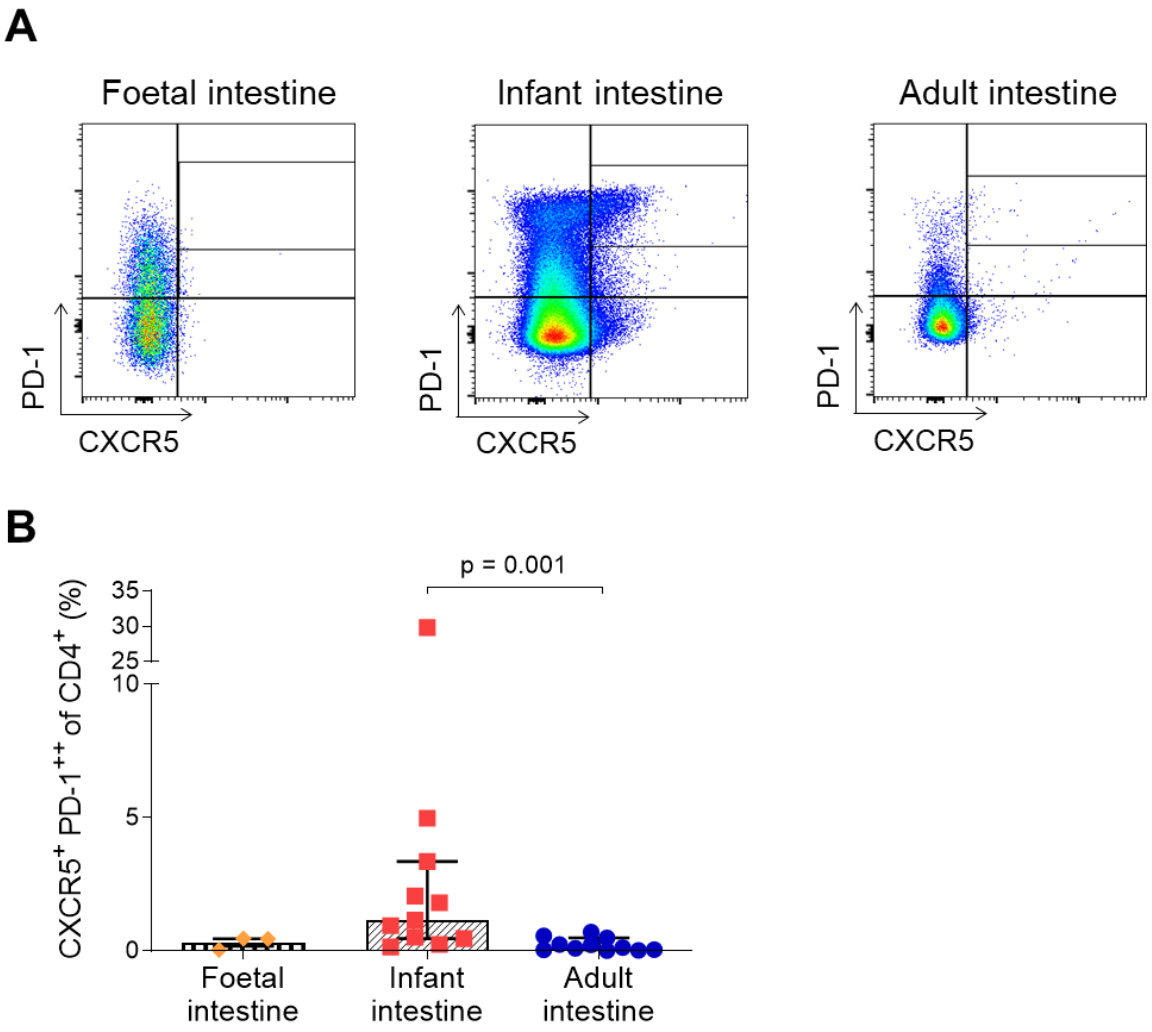

Figure 8. Representative flow cytometric plots of foetal, infant, and adult lamina propria $CD4^+$ cells for PD-1 and CXCR5 (A). Frequencies of $CXCR5^+$ $PD\text{-}1^{++}$ $CD4^+$ T cells were compared in statistical analyses (B). Foetal intestinal samples were obtained from abortions in the second trimester of pregnancy. The frequencies of $CXCR5^+$ $PD\text{-}1^{++}$ $CD4^+$ T cells in foetal intestinal mucosae was lower than in infants. Median frequencies (± IQR) are given. Mann-Whitney U tests were used for statistical analysis. Dot plots form donors no. 2F, 11I, and 29A are shown. Intestinal tissue samples: Foetus n = 3, infants n = 11, adults n = 11.

4.3 Intestinal T_{FH} cells are effector memory cells

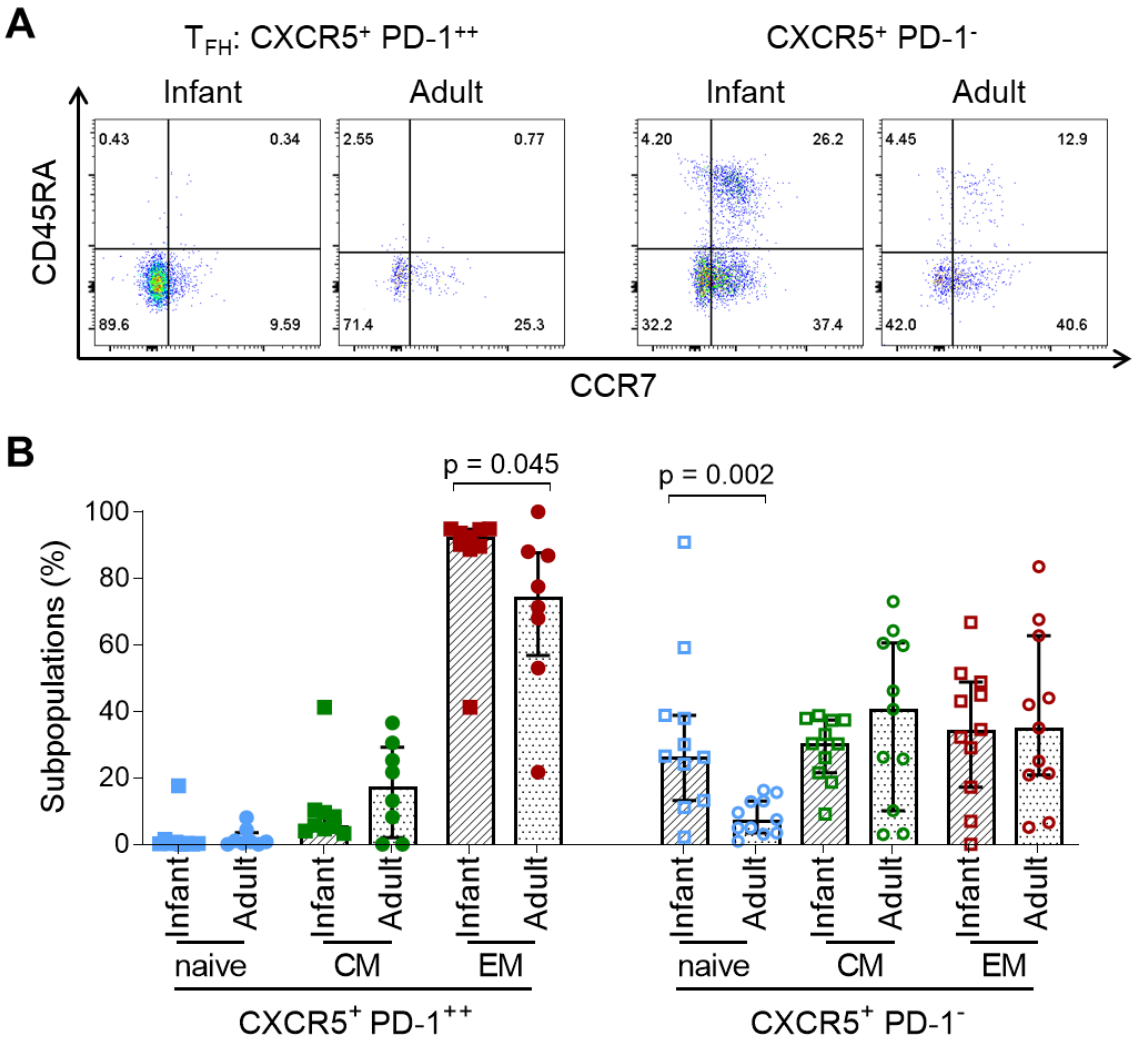

Figure 9. Representative flow plots of infant and adult CXCR5$^+$ PD-1^{++} and CXCR5$^+$ PD-1$^-$ CD4$^+$ T cells for CD45RA and CCR7 respectively (A). Frequencies of CD45RA$^+$ CCR7$^+$ naïve, CD45RA$^-$ CCR7$^+$ central memory (CM) and CD45RA$^-$ CCR7$^-$ effector memory (EM) cells within the CXCR5$^+$ PD-1^{++} and CXCR5$^+$ PD-1$^-$ CD4$^+$ T-cell populations (B). Within the CXCR5$^+$ PD-1^{++} T_{FH}-cell populations most of the cells were effector memory cells with significantly higher frequencies in infants than in adults ($p = 0.045$). Naïve CXCR5$^+$ PD-1$^-$ pre-T_{FH} cells were significantly more frequent in infants than in adults ($p = 0.002$). Median frequencies (± IQR) are given. Mann-Whitney U tests were used for statistical analysis. Flow plots from donors no. 11I and 22A are shown. Intestinal tissue samples: Infants n = 11, adults n = 11.

CD45RA is a marker for naïve T cells, and absence of CD45RA identifies memory T-cell subsets (Thome et al. 2016). The expression of CCR7 allows to identify effector memory (EM) and central memory (CM) T cells. T_{EM} cells migrate to multiple peripheral tissue sites and produce effector cytokines, whereas T_{CM} cells travel to lymphoid tissues due to their CCR7 expression which binds to its ligand expressed in lymphoid tissues (Farber et al.

2014). In sum, the characterization of CCR7 and CD45RA enables the identification of CD45RA$^+$ CCR7$^+$ naïve, CD45RA$^-$ CCR7$^-$ T_{EM} and CD45RA$^-$ CCR7$^+$ T_{CM} cells (see Figure 9A).

The majority of T_{FH} cells in infant and adult intestinal tissues consisted of T_{EM} cells and only small numbers of T_{CM} cells were observed. Naïve T_{FH} cells were scarce for both, infants (median 0.32%, IQR 0.16–1.09%, n = 9) and adults (median 0.80%, IQR 0.09–3.55%, n = 8) as shown in Figure 9B. The median frequency of EM T_{FH} cells in infant intestines was 92.4% (IQR 89.15–94.8%, n = 9) of all T_{FH} cells. Meanwhile, the frequency of adult EM T_{FH} cells was significantly lower ($p = 0.045$) with 74.45% (median, IQR 56.75–87.73%, n = 8) of all T_{FH} cells. Although not significant, there was a trend towards more CM T_{FH} cells in adults (median 17.4%, IQR 2.06–29.2%, n = 8; infants median 5.78%, IQR 4.31–10.0%, n = 9).

In the T_{FH}-cell development the follicular homing receptor CXCR5 is upregulated first, followed by PD-1 (Qi 2016). Consequently, CXCR5$^+$ PD-1$^-$ CD4$^+$ T cells were addressed as pre-T_{FH} cells. Among the naïve cells the frequencies of infant pre-T_{FH} cells (median 26.5%, IQR 13.2–38.8%, n = 11) was significantly higher ($p = 0.002$) than the frequencies of adult naïve pre-T_{FH} cells (median 7.48%, IQR 3.49–13.0%, n = 11) as shown in Figure 9B.

In summary, T_{FH} cells in the intestinal mucosae mainly presented an EM phenotype in infants, whereas in adults also CM cells were present. Naïve T_{FH} cells were absent. Whereas T_{FH} cells were mostly T_{EM} cells, CXCR5$^+$ PD-1$^-$ pre-T_{FH}-cell populations showed a more diverse pattern.

4.4 Intestinal T_{FH} cells express markers allowing tissue residency

Immune cells in the intestine can remain tissue-resident memory T (T_{RM}) cells to provide efficient protection at tissue sites. The expression of CD103 and the early activation molecule CD69 allow residency in these tissues (Thome and Farber 2015). To investigate whether infant T_{FH} cells would comprise a tissue-resident population the expression of CD69 and CD103 was determined on T_{FH} cells (see Figure 10A).

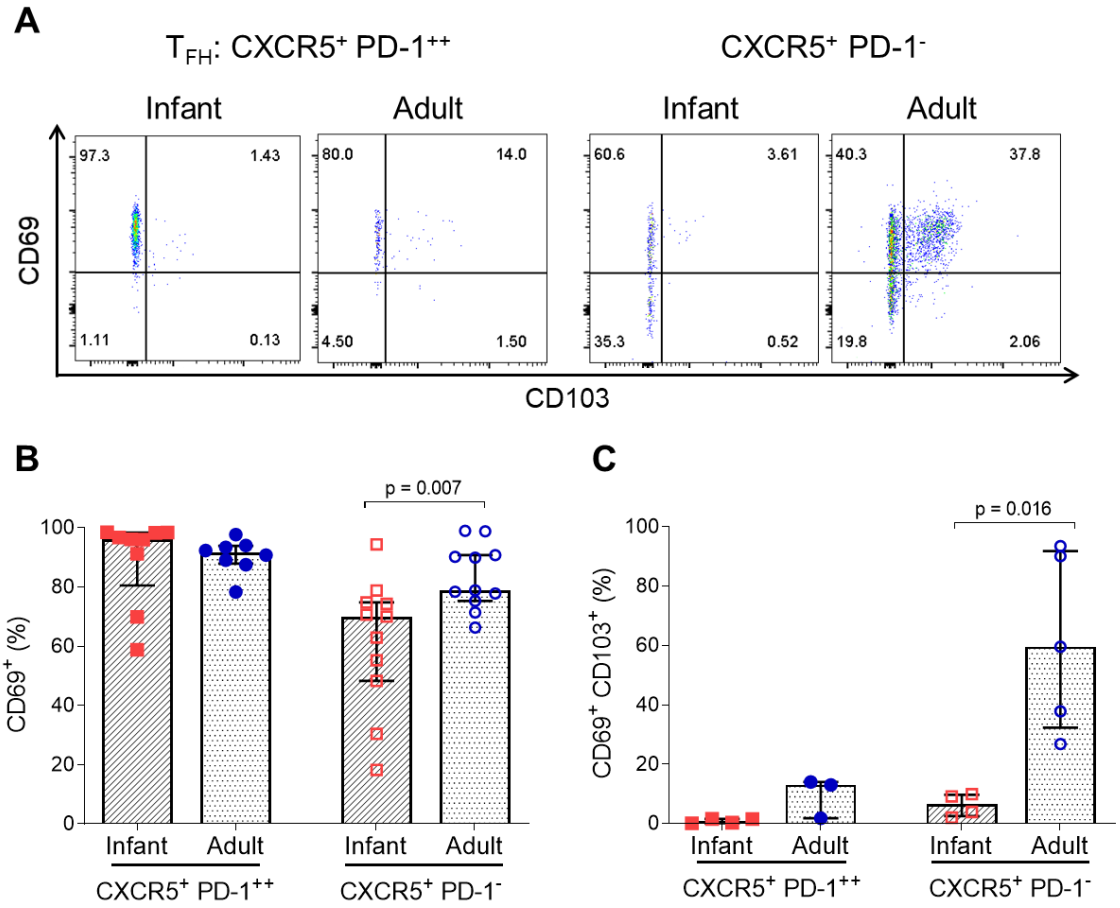

Figure 10. Representative flow cytometric plots of CXCR5$^+$ PD-1^{++} and CXCR5$^+$ PD-1$^-$ CD4$^+$ T cells for the tissue residency markers CD69 and CD103 (A). Frequencies of infant and adult CXCR5$^+$ PD-1^{++} and CXCR5$^+$ PD-1$^-$ CD4$^+$ T cells for CD69$^+$ (B) and CD69$^+$ CD103$^+$ (C) respectively. CXCR5$^+$ PD-1^{++} cells in infant and adult intestines were mainly CD69$^+$. The pre-T_{FH}-cell populations showed a lower CD69 expression, with a significantly lower ($p = 0.007$) expression in infants compared to adults. The co-expression of CD69 and CD103 was less in infant compared to adult intestines. In adults the frequencies of CD69$^+$ CD103$^+$ T_{FH} cells was non-significantly higher and significantly higher ($p = 0.016$) in the CXCR5$^+$ PD-1$^-$ pre-T_{FH} population than in infants respectively. Median frequencies (± IQR) are given. Mann-Whitney U tests were used for statistical analysis. Flow plots of infant donor no. 14I and adult donor no. 30A are given. Intestinal tissue samples: Infants n = 11, adults n = 11 (B). Infants n = 4, adults n = 5 (C).

The majority of T_{FH} cells were CD69$^+$. Almost all infant T_{FH} cells expressed CD69 (median 96.1%, IQR 80.55–98.35%, n = 9), whereas 91.55% (median, IQR 87.9–93.85%, n = 8) of adult T_{FH} cells did. Among the pre-T_{FH} cells the CD69 expression was more diverse. In infant pre-T_{FH}-cell populations 70.0% (median, IQR 48.3–74.8%, n = 11) expressed CD69, while the expression was significantly higher ($p = 0.007$) on adult pre-T_{FH} cells with 78.9% (median, IQR 75.3–90.8%, n = 11) expressing CD69 (see Figure 10B).

A proportion of 0.83% (median, IQR 0.06–1.52%, n = 4) of infant $CD69^+$ T_{FH} cells showed a co-expression of CD103, whereas 6.48% (median, IQR 2.42–9.67%, n = 4) of $CD69^+$ pre-T_{FH} cells did. On adult $CD69^+$ cells the expression of CD103 was higher on T_{FH} (median 13.0%, IQR 1.73–14.0%, n = 3) and significantly higher (p = 0.016) on pre-T_{FH} cells (median 59.6%, IQR 32.3–91.8%, n = 5) than on infant cells respectively. However, the adult cells showed a distributed pattern (see Figure 10C).

4.5 Expression of T_{FH}-cell master regulator Bcl-6

Transcription factors determine T_H-cell lineages by using T_H-cell polarization. The identification of these master regulators of polarization allows to further classify T_H-cell populations. The master transcription factor of T_{FH} cells is Bcl-6 (Crotty 2011).

The total $CXCR5^+$ $CD4^+$ T cells showed a higher expression of Bcl-6 versus $CXCR5^-$ $CD4^+$ T cells (see Figure 11A). The ratio, however, of the median fluorescence intensity (MFI) for Bcl-6 of $CXCR5^+$ to $CXCR5^-$ $CD4^+$ T cells was significantly lower in infants (median 1.32, IQR 1.11–1.50, n = 11) than in adults (median 1.84, IQR 1.31–2.78, n = 10) as shown in Figure 11B.

Bcl-6 expression by $CXCR5^+$ $PD\text{-}1^{++}$, $CXCR5^+$ $PD\text{-}1^+$ and $CXCR5^+$ $PD\text{-}1^-$ $CD4^+$ T-cell populations was significantly lower in infants than in adults. In infants 8.22% (median, IQR 6.05–9.15%, n = 8) of the T_{FH} cells expressed Bcl-6, whereas in adults 18.0% (median, IQR 10.0–38.5%, n = 7) T_{FH} cells expressed Bcl-6 (p = 0.021). The highest frequencies of Bcl-6 in infant intestines were observed in $CXCR5^+$ $PD\text{-}1^+$ $CD4^+$ T-cell populations with 15.45% (median, IQR 6.27–28.28%, n = 8) being positive for Bcl-6, compared to 40.8% (median, IQR 15.95–52.25%, n = 10) expressing Bcl-6 in adult $CXCR5^+$ $PD\text{-}1^+$ $CD4^+$ T-cell populations (p = 0.034). The frequencies of Bcl-6 expression on $CXCR5^+$ $PD\text{-}1^-$ $CD4^+$ T cells were significantly lower (p = 0.034) in infants (median 6.66%, IQR 3.69–27.88%, n = 10) than in adults (median 32.25%, IQR 20.03–44.03%, n =10). In the $CXCR5^-$ $PD\text{-}1^-$ non-T_{FH} population infants expressed Bcl-6 to 3.51% (median, IQR 2.24–5.57%, n = 10) compared to 13.25% (median, IQR 3.98–17.03%, n = 10) in adults (see Figure 11C).

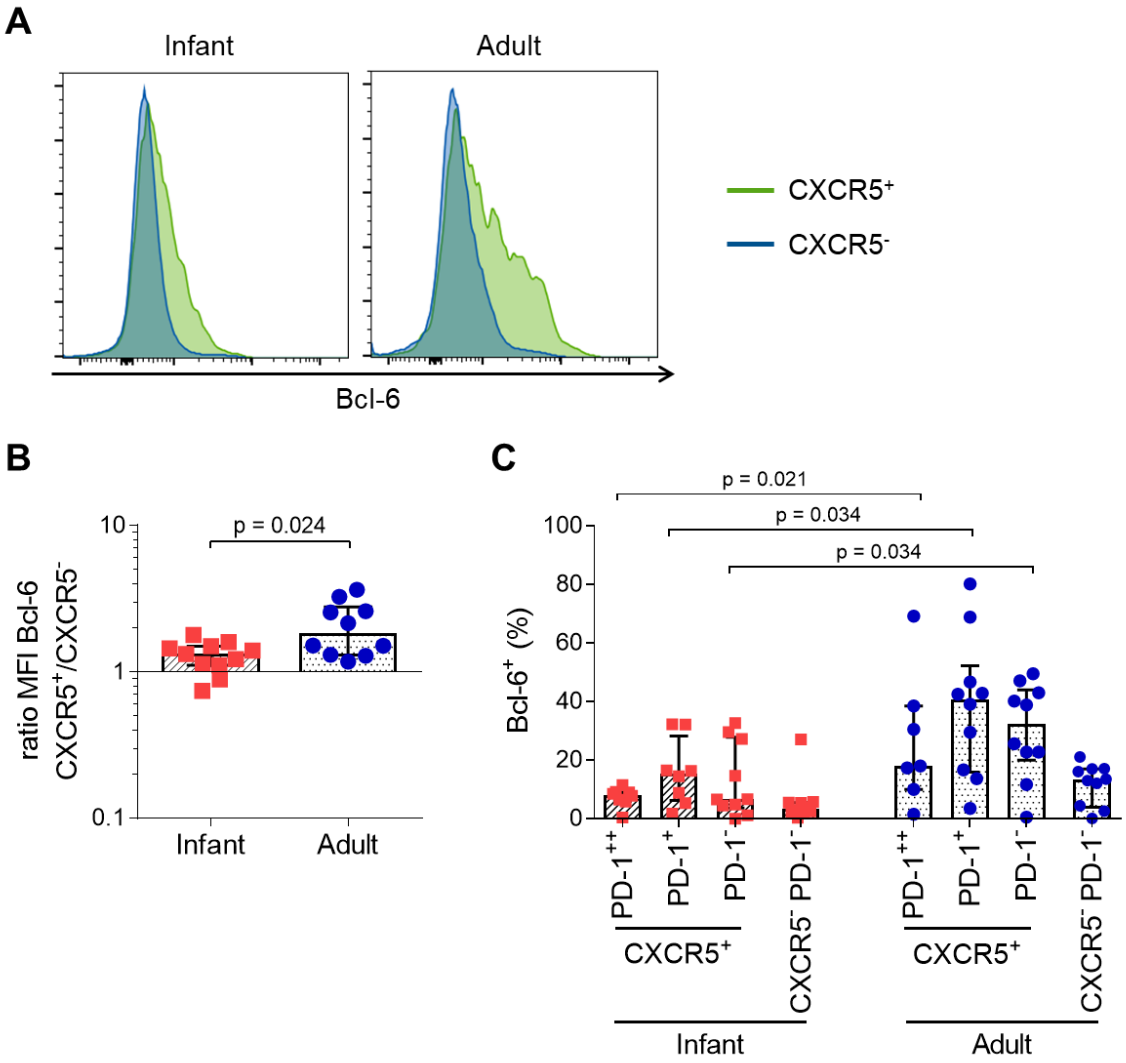

Figure 11. Representative histograms of Bcl-6 expression in infant and adult intestinal CXCR5$^+$ (green) and CXCR5$^-$ (blue) CD4$^+$ T cells (A). Ratio of CXCR5$^+$/CXCR5$^-$ CD4$^+$ T cells for mean fluorescence intensity (MFI) of Bcl-6 (B). Frequencies of Bcl-6$^+$ CXCR5$^+$ (PD-1^{++}, PD-1$^+$, PD-1$^-$) and CXCR5$^-$ PD-1$^-$ CD4$^+$ T cells in infant and adult intestines (C). Histograms show a higher expression of Bcl-6 in the CXCR5$^+$ population compared to CXCR5$^-$ CD4$^+$ T-cell populations (A). The ratio of the median fluorescence intensity (MFI) of CXCR5$^+$/CXCR5$^-$ CD4$^+$ T cells for Bcl-6 expression in infants is significantly lower ($p = 0.024$) than in adults. A lower expression of Bcl-6 in infants than in adults for all the CXCR5$^+$ CD4$^+$ T-cell subpopulations was observed. Median frequencies (± IQR) are given. Linear (C) and logarithmic (B) scales were used. Mann-Whitney U tests were used for statistical analysis. The histograms from infant donor no. 11I and adult donor no. 30A are given. Intestinal tissue samples: Infants n = 11, adults n = 10.

The results above were supported by a trivariate linear regression model calculated by Laura Richert: "The overall effect of adult compared to infant CXCR5$^+$ CD4$^+$ T cells was significant ($p < 0.001$). The overall mean difference of Bcl-6$^+$ cells in adults compared to children across the three CXCR5$^+$ [CD4$^+$ T-cell] subpopulations is +19%. The effect of age, infant or adult, did not change between subpopulations."

4.6 Frequencies of regulatory cells in T_{FH}- and non-T_{FH}-cell populations

T follicular regulatory (T_{FR}) cells inhibit T_{FH} and B cells in the germinal centre and therefore contribute to the inhibition of GC responses. The T_{FR} cells develop from natural T_{reg}-cell precursors, express FoxP3, and develop CXCR5 and PD-1 expression (Sage and Sharpe 2016).

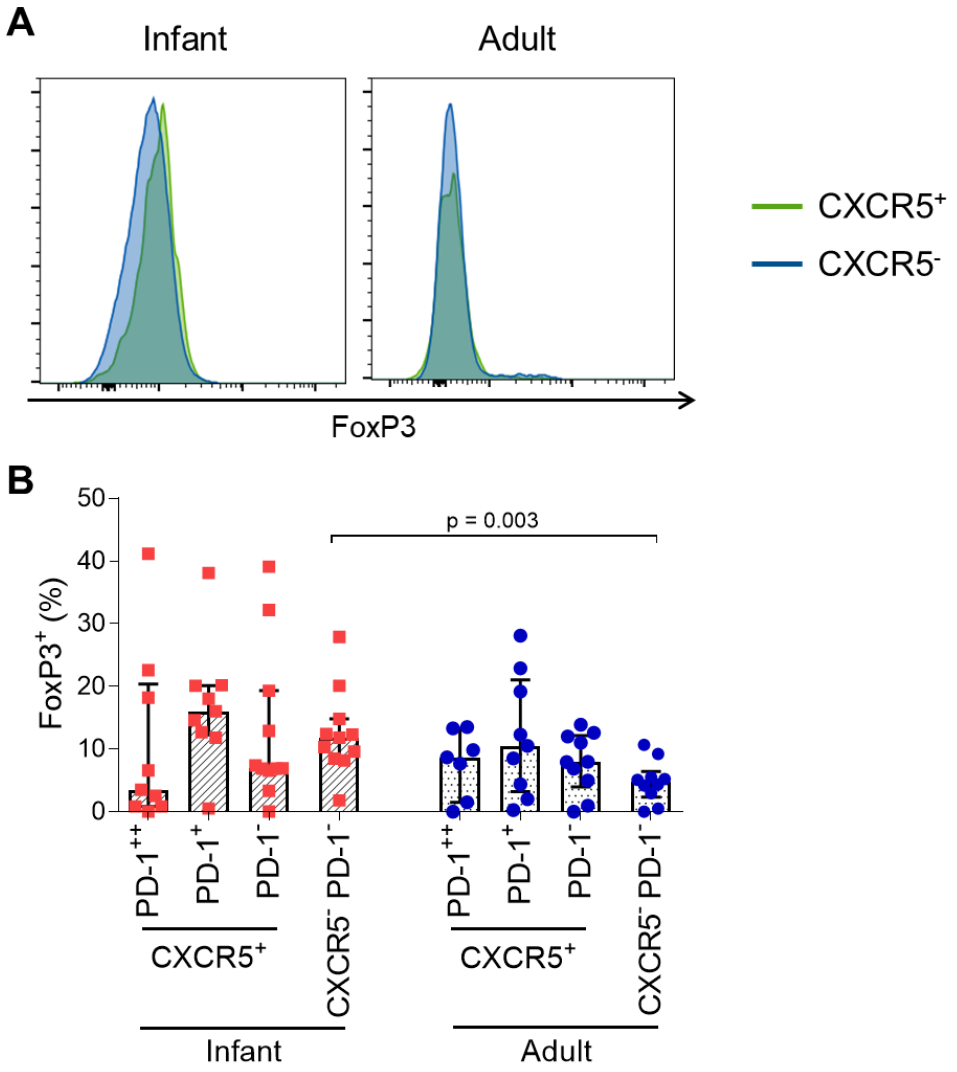

Figure 12. Representative histograms of CXCR5$^+$ (green) and CXCR5$^-$ (blue) CD4$^+$ T-cell populations expressing FoxP3 (A). Relative FoxP3 expression of three different CXCR5$^+$ CD4$^+$ T-cell populations (PD-1^{++}, PD-1$^+$, and PD-1$^-$) and the CXCR5$^-$ PD-1$^-$ CD4$^+$ T-cell population comparing infants and adults (B). No significant differences in the expression of FoxP3 between CXCR5$^+$ and CXCR5$^-$ CD4$^+$ T-cell populations for both, infants and adults was detected, however FoxP3 expression by CXCR5$^-$ PD-1$^-$ non-T_{FH} cells was significantly higher (p = 0.003) in infants than in adults. Median frequencies (± IQR) are given. Mann-Whitney U tests were used for statistical analysis. Histograms of donor no. 7I and 24A are given. Intestinal tissue samples: Infants n = 11, adults n = 10.

The transcription factor FoxP3 was equally expressed among the CXCR5$^+$ as well as the CXCR5$^-$ CD4$^+$ T cells (see Figure 12A). There was no difference of the FoxP3 expression between infants and adults for the CXCR5$^+$ CD4$^+$ T-cell populations. A proportion of 3.54% (median, IQR 0.87–20.4%, n = 9) of infant T_{FH} cells expressed FoxP3, whereas 8.70% (median, IQR 1.53–13.3%, n = 7) of adult T_{FH} cells did.

However, as shown in Figure 12B in the CXCR5$^-$ PD-1$^-$ non-T_{FH} population the expression of FoxP3 in infants (median 11.8%, IQR 8.46–14.8%, n = 11) was significantly higher (p = 0.003) compared to adults (median 4.72%, IQR 2.36–6.46%, n = 10). Results where similar when determining regulatory T cells based on CD25$^+$ and CD127$^-$ (data not shown)

4.7 Expression of ICOS and CD40L on intestinal T_{FH} cells

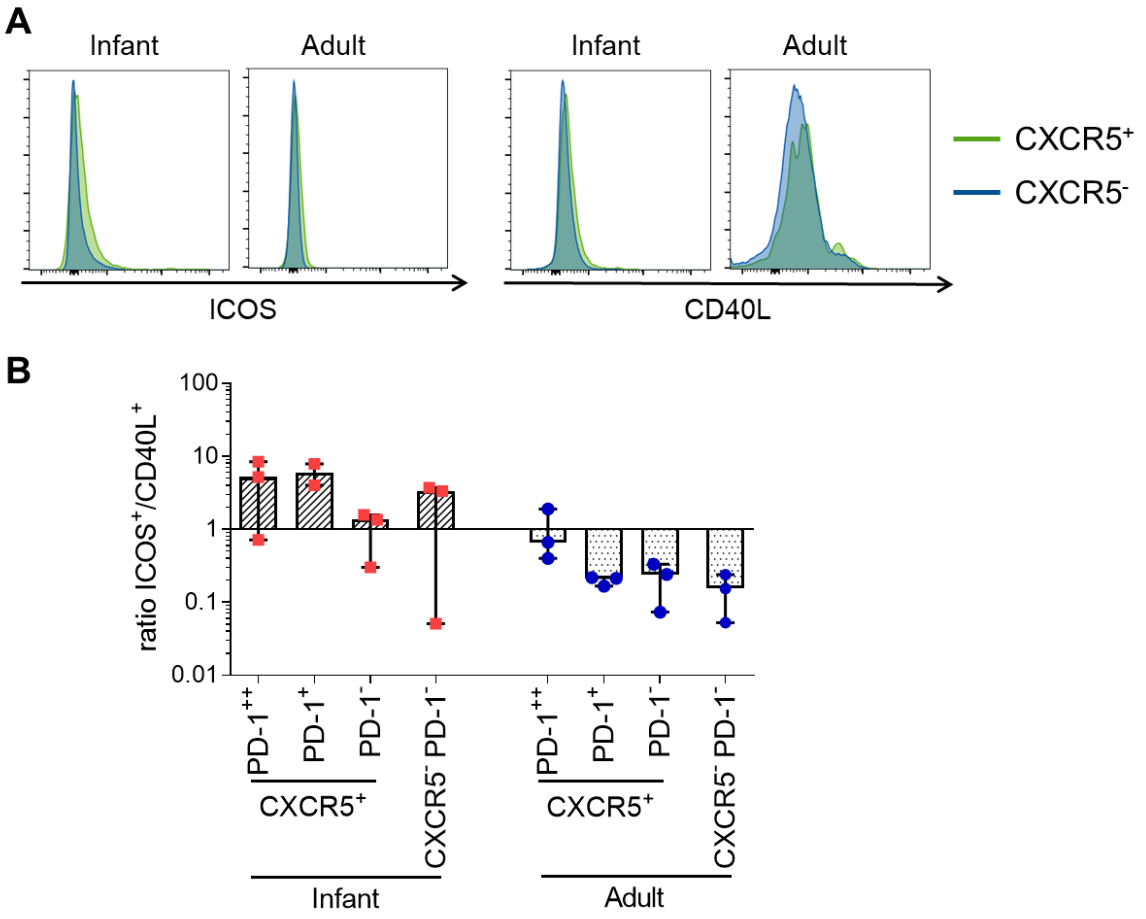

Figure 13. Representative histograms of the expression of ICOS and CD40L on CXCR5$^+$ (green) and CXCR5$^-$ (blue) CD4$^+$ T cells (A). Ratio of ICOS to CD40L expression, comparing three different CXCR5$^+$ CD4$^+$ T-cell populations (PD-1^{++}, PD-1$^+$, PD-1$^-$) and the CXCR5$^-$ PD-1$^-$ CD4$^+$ T-cell populations of infants and adults (B). Compared to CD40L there was a trend towards a higher expression of ICOS in infants, while, in comparison, adults expressed more CD40L. Median frequencies (± IQR) are given. A logarithmic scale was used (B). Histograms of donor no. 17I and 31A are shown. Intestinal tissue samples: Infants n = 3, adults n = 3.

ICOS and CD40L are two surface molecules, which are essential for bidirectional interaction to DCs and B cells (Crotty 2011). There was a trend towards a higher expression of ICOS and CD40L on $CXCR5^+$ compared to $CXCR5^-$ $CD4^+$ T cells (see Figure 13A). Infant $CXCR5^+$ $PD\text{-}1^{++}$ $CD4^+$ T cells had a five-fold higher expression of ICOS (median 5.18, IQR 0.71–8.39, n = 3) compared to CD40L. The ratio of ICOS/CD40L was lower (median 0.66, IQR 0.40-1.90, n = 3) in adults. The same trend was observable for $CXCR5^+$ $PD\text{-}1^+$ $CD4^+$ T cells with a ratio of 5.93 (median, IQR 4.02–7.83, n = 2) in infants and 0.21 (median, IQR 0.17–0.22, n = 3) in adults. As shown in Figure 13B the trend continued for all other subpopulations ($CXCR5^+$ $PD\text{-}1^-$ infants: median 1.35, IQR 0.30–1.58, n = 3; $CXCR5^+$ $PD\text{-}1^-$ adults: median 0.24, IQR 0.07–0.33, n = 3; $CXCR5^-$ $PD\text{-}1^-$ infants: median 3.34, IQR 0.05–3.73, n = 3; $CXCR5^-$ $PD\text{-}1^-$ adults: median 0.15, IQR 0.05–0.24, n = 3).

The trivariate linear regression model by Laura Richert "showed an overall significance (p = 0.044) of adults versus infants, with a mean difference of -12% of $ICOS^+$ cells in adults compared to infants across the three $CXCR5^+$ [$CD4^+$ T-cell] subpopulations. The subpopulation effect itself was not significant."

4.8 Cytokine production by intestinal T_{FH} cells

To provide B-cell help, T_{FH} cells can produce their main effector cytokine IL-21. However, T_{FH} cells are also able to produce cytokines, that are typically produced by other T-cell lineages, including IL-4, which has an antiapoptotic effect on B cells (Crotty 2011). TNFα furthermore is a pivotal cytokine for lymphoid tissue development and we have recently shown that early in life this cytokine can contribute to both intestinal tissue development and destruction (Schreurs et al. 2019).

After phenotypical characterisation of T_{FH} cells, cytokine staining was performed after 12 hours of stimulation with PMA and ionomycin, to functionally characterize the T_{FH} cells. First, cytokine productions on the overall $CD4^+$ populations were assessed (see Figure 14).

The TNFα production of $CD4^+$ T cells upon stimulation significantly increased (p = 0.002) from 0.97% (median, IQR 0.35–1.49%, n = 6) to 20.7% (median, IQR 13.45–41.03%, n = 6) in infants upon PMA stimulation. In adults the TNFα production also increased from 0.34% (median, IQR 0.27–1.83%, n = 3) in unstimulated condition to 24.3% (median, IQR 8.54–75.2%, n = 3) in stimulated cells (see Figure 15A).

The IL-4 production in infant CD4$^+$ T cells declined from 8.77% (median, IQR 3.59–18.78%, n = 6) to 4.03% (median, IQR 1.58–6.42%, n = 6) upon stimulation. In adults however a trend towards a higher production upon stimulation from 1.56% (median, IQR 0.73–4.15%, n = 3) to 2.88% (median, IQR 2.76–4.64%, n = 3) could be detected in intestinal CD4$^+$ T cells (see Figure 15B).

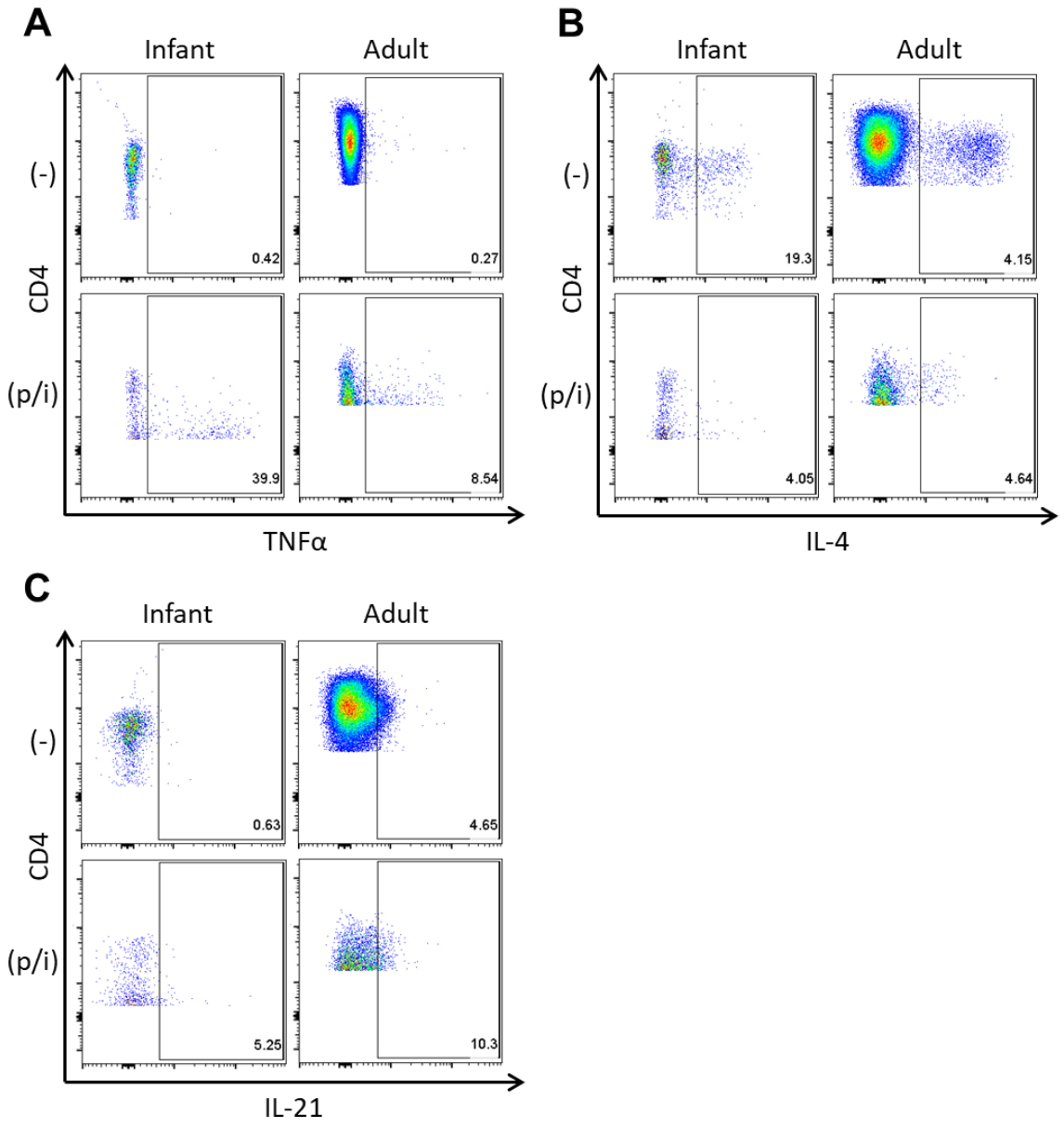

Figure 14. Representative flow cytometric plots of total CD4$^+$ T cells from infant and adult intestinal tissues. The production of TNFα (A), IL-4 (B) and IL-21 (C) is shown upon stimulation with PMA and ionomycin (p/i) for 12-14 hours and for unstimulated cells (-). Brefeldin A was added after one hour. Flow plots from donor no. 10I and 33A are shown.

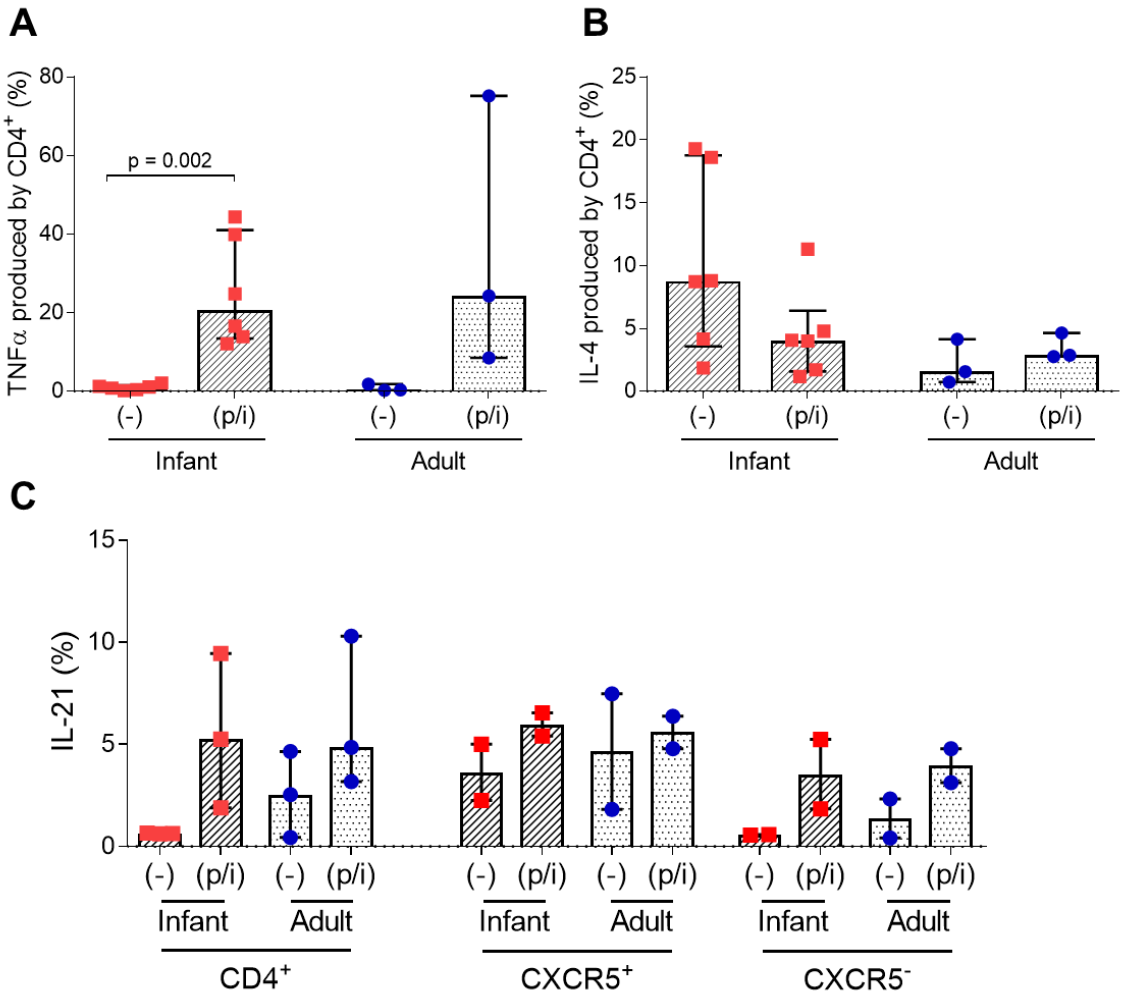

Figure 15. TNFα (A), IL-4 (B) and IL-21 (C) production by all CD4$^+$ cells and IL-21 production for CXCR5$^+$ and CXCR5$^-$ CD4$^+$ T cells (C). Cytokine production is shown for unstimulated cells (-) and upon stimulation with PMA and ionomycin (p/i). Median frequencies (± IQR) are given. Mann-Whitney U tests were used for statistical analysis. Intestinal tissue samples: Infant n = 6, adult n = 3 (A). Infant n = 6, adult n = 3 (B). Infant n = 3, adult n = 3 (C, CD4$^+$). Infant n = 2, adult n = 2 (C, CXCR5$^{+/-}$).

The production of IL-21 on CD4$^+$ T cells increased from 0.63% (median, IQR 0.62–0.65%, n = 3) to 5.25% (median, IQR 1.90–9.46%, n = 3) in infant and from 2.54% (median, IQR 0.44–4.65%, n = 3) to 4.86% (median, IQR 3.18–10.3%, n = 3) in adult intestines. The CD4$^+$ T cells were further differentiated in CXCR5$^+$ and CXCR5$^-$ cells. CXCR5$^+$ CD4$^+$ T cells showed a baseline production of IL-21 with 3.63% (median, IQR 2.25–5.00%, n = 2) in infants and 4.65% (median, IQR 1.82–7.48%, n = 2) in adults, whereas for unstimulated CXCR5$^-$ CD4$^+$ T cells the IL-21 was nearly absent (median 0.57%, IQR 0.55–0.58%, n = 2) in infant intestines and 1.38% (median, IQR 0.42–2.33%, n = 2) in adult intestines. Upon stimulation IL-21 production increased to 5.98% (median, IQR 5.41–6.54%, n = 2) for infant

and to 5.59% (median, IQR 4.80–6.38%, n = 2) for adult $CXCR5^+$ $CD4^+$ T cells. The IL-21 production upon stimulation in the $CXCR5^-$ $CD4^+$ T-cell population was lower with 3.54% (median, IQR 1.84–5.24%, n = 2) for infant cells and 3.97% (median, IQR 3.13–4.80%, n = 2) for adult cells. Statistical analysis was not performed (n = 2 in each group) for IL-21 (see Figure 15C).

5 Discussion

5.1 Summary of the results

This study demonstrates that, at four months of age, infants have an abundance of intraepithelial and lamina propria derived T_{FH} cells in their intestines. In adult intestines T_{FH} cells were reduced compared to infant intestines. Foetal intestinal tissues did not harbour any T_{FH} cells. Infant and adult T_{FH} cells had an effector memory phenotype. Furthermore, the majority of T_{FH} cells had a tissue-resident phenotype. In comparison to adult T_{FH} cells, infant T_{FH} cells showed a reduced expression of the master transcription factor Bcl-6. The ratio of costimulatory molecules ICOS/CD40L was higher on infant $CXCR5^+$ and $CXCR5^-$ $CD4^+$ T cells compared to adults. In total $CD4^+$ T cells, the production of TNFα increased upon stimulation in adult and infant populations. Infant $CD4^+$ T cells had a high baseline expression of IL-4, which was not detected in adult $CD4^+$ T cells. $CXCR5^+$ $CD4^+$ T cells showed a baseline expression of the main T_{FH}-cell effector cytokine IL-21, which could not be detected in $CXCR5^-$ $CD4^+$ T cells. Infant $CXCR5^+$ and $CXCR5^-$ $CD4^+$ T cells could produce IL-21 similar to adults. These primary results will be discussed in the context of the current state of knowledge below.

5.2 Interpretation

This study is the first to describe frequencies of T_{FH} cells in human infant intestinal tissues. In human adult intestinal tissues $CXCR5^+$ $CD4^+$ T cells were described (Carlsen et al. 2002), however not compared to infant intestines. On the one hand, Roider et al. (2018) revealed that human infants had increased frequencies of T_{FH} cells in tonsils than adults. On the other hand, there are studies on human blood $CXCR5^+$ $CD4^+$ T cells showing increasing levels with age (Schatorjé et al. 2012). Recently, Allam et al. (2015) revealed T_{FH}-cell frequencies in different tissues of humanized DRAG mice, which are mice infused with human stem cells to produce human immune cells. Even though it is a humanized tissue model in mice, it is worth a comparison with the human tissue for the present study. It is well comparable because T_{FH} cells are chosen based on the same criteria as $CXCR5^+$ $PD\text{-}1^{++}$ $CD4^+$ T cells. T_{FH} cells are largely present in the mesenteric lymph nodes (MLN), in Peyer's patches (PPs), and at lower frequencies in IEL and LPL (Allam et al. 2015). The T_{FH}-cell frequencies Allam et al. (2015) determined for murine IEL and LPL, resemble the frequencies determined for humans in the present study. Other authors reported lower frequencies in neonatal murine

lymphoid tissues and murine blood, compared to adults upon immunization (Mastelic et al. 2012, Debock et al. 2013). In contrast, this study of human intestinal mucosa could reveal that T_{FH} cells in infants were significantly more frequent than in adults.

In the structural development of intestinal lymphoid tissues at 14 to 16 weeks of gestation, small groups of T and B cells arise. The first recognizable Peyer's patches (PP) with follicular dendritic cells (FDC) form in week 19. At week 24, PPs are microscopically visible, but germinal centres (GC) do not exist. The GCs develop and the number of PPs increase, when extensively exposed to commensal microorganisms and antigens after birth (Cornes 1965, Jung et al. 2010). The absence of T_{FH} cells in foetal intestinal tissues from 15 to 20 weeks gestational age in this study corresponds with the literature. Because T_{FH} cells are involved in the GC development, it can be assumed that they develop at similar times.

A study from Debock et al. (2013) demonstrated that T_{FH} cells in neonatal mice lymph nodes can be induced upon immunization but have an impaired migration towards the GC and consequently predominantly localize in the interfollicular region (IFR). The authors attributed this localization to a high CCR7 expression of these cells in neonates (Debock et al. 2013). In order to leave the T-cell zone and enter the B-cell zone, T_{FH} cells must downregulate the T-cell zone homing receptor CCR7 (Mastelic et al. 2012). Consequently, only T_{FH} cells with a $CCR7^-$ phenotype are able to migrate towards the GCs and provide help to B cells (Haynes et al. 2007). Campbell et al. (2001) showed in experiments with human intestinal tissue, that that $CCR7^-$ effector memory (EM) T cells are the predominant memory T-cell subset in the intestine.

However, in contrast to Debock et al.'s (2013) finding, the T_{FH} cells in human infant intestines predominantly downregulated CCR7 and presented an EM T_{FH}-cell phenotype. Consequently, infant T_{FH} cells in the human intestine are generally differentiated cells and should be capable of migrating towards the GCs. Nevertheless, localization in the IFR due to a low CXCL13 production cannot be ruled out. While infants displayed a consistent T_{EM} cell phenotype, in adults an additional proportion of T_{CM} cells were present. $CCR7^+$ T_{CM} cells migrate towards lymphoid tissues and provide less functionality at mucosal sites (Farber et al. 2014).

The finding of memory T_{FH} cells in infant intestinal tissues is consistent with Bunders et al. (2012), who previously detected frequent memory $CD4^+$ T cells in neonatal intestines. Thome et al. (2016) underlined that infant intestinal $CD4^+$ T cells present a naïve or an

effector memory phenotype, whereas adult cells predominantly show an effector memory phenotype. Within the pre-T_{FH} population in this study infants had significantly more naïve cells compared to adults, which is consistent with the results of total $CD4^+$ T cells from Thome et al. (2016). In general, the differentiation status of pre-T_{FH} cells was more diverse, which confirms a mixed cell population containing cells that are assumed to be recently activated, allowing plasticity and the conceivable differentiation in other T-cell lineages because differentiation is not completed.

In summary, the results demonstrate that infant intestinal T_{FH} cells have, comparable to the adult ones, a predominantly effector memory phenotype with no naïve cell differentiation. This indicates that T_{FH} cells are mature and functional in infant intestinal mucosae and might be ahead of the development of T_{FH} cells in infant lymphoid tissues and blood.

Recent concepts of compartmentalization of the immune system suggest that the local immune system at mucosal sites develops independently from other compartments, including lymphoid tissues or blood (Farber et al. 2014). T cells that provide unique site-specific functionalities show a tissue-resident phenotype and express the activation marker CD69 or CD103 (Sathaliyawala et al. 2013). CD103 is a marker mainly expressed by intestinal $CD8^+$ T cells and to a lesser extent by $CD4^+$ T cells (Thome and Farber 2015). It might be the explanation for the majority of $CD4^+$ T_{FH} cells not expressing CD103. Thome et al. (2016) could show that CD69 is expressed by the majority of general T_{EM} cells in all infant and adult tissues, whereas blood T_{EM} cells are negative for CD69. The results of this study demonstrated a consistent high CD69 expression on T_{FH} cells in infant and adult intestinal tissues. This finding suggests that T_{FH} cells in infants, as in adults, are not circulatory cells, but reside in tissues to fulfil their site-specific function including control upon reinfection.

Next to CXCR5 and PD-1 expression, Bcl-6 as the master transcription factor is supposed to be a critical characteristic. Nevertheless, some authors challenge this assumption (Ma et al. 2012b) and several studies could reveal lower levels of Bcl-6 in infants. Debock et al. (2013) have studied neonatal murine $IL\text{-}21^+$ $CD4^+$ T cells and detected a lower expression of the master transcription factor Bcl-6 compared to adult mice. A study from Mastelic-Gavillet et al. (2019) could confirm on $CXCR5^{high}$ $PD\text{-}1^{high}$ T_{FH} cells from murine lymph nodes that neonates presented lower levels of Bcl-6. The results in this study demonstrate

that Bcl-6 is expressed to a lesser extent on infants than on adult T_{FH} cells in human intestines as well.

The interfollicular localisation of neonatal T_{FH} cells as described by Debock et al. (2013) would be consistent with the assumption that T_{FH} cells increase Bcl-6 expression when entering the B-cell follicle (Liu et al. 2013). However, in this study it is assumed that the T_{FH} cells are able to migrate to the B-cell follicle due to their EM phenotype. Impaired cell interactions due to deficiencies in T_{FH} cells, B cells, or DC could account for the lower expression of Bcl-6.

As Bcl-6 is one of the characteristics defining the commitment of T cells to the T_{FH}-cell lineage, it must be considered, that the $CXCR5^{+}$ $PD\text{-}1^{++}$ T cells in infants are not mature and specialised T_{FH} cells. Nevertheless, different transcription factors, leading to the induction of Bcl-6, could be critical in infants. STAT3 is known to be critical for T_{FH}-cell development and increases Bcl-6 expression in T cells (Liu et al. 2013). Mastelic-Gavillet et al. (2019) describe T_{FH} cells presenting transcription factors biased by a T_H2-cell differentiation instead of Bcl-6 expression in neonatal murine lymph nodes upon immunization compared to adults.

ICOS signalling induces the expression of Bcl-6, supports the T_{FH}-cell differentiation and is essential for T_{FH} and DC interactions. The costimulatory molecules CD40L on T_{FH} cells mainly support their help to B cells (Linterman 2014). Different authors showed that CD40L and ICOS expression on mice neonatal T cells is limited compared to adult cells (Flamand et al. 1998, Debock et al. 2013). Results in this study revealed a trend towards a higher ICOS expression on infant T_{FH} cells, while CD40L is higher on adult cells. ICOS is required for the induction of Bcl-6, which is limited in infants, and the maintenance of T_{FH} cells and GCs. Consequently, ICOS might be more expressed in infants than in adults to enable T_{FH} cells to receive sufficient signals for further differentiation and maintenance. Mice blood $CD4^{+}$ T cells have a limited ICOS expression in higher age, which leads to limited numbers of T_{FH} cells due to less ICOS-specific signalling (Linterman 2014).

In addition to molecules for direct cell interaction, cell interactions also rely on the production of cytokines. The most important effector cytokine of T_{FH} cells is IL-21, which activates STAT3. The cytokine IL-4 is usually produced by T_H2 cells. Nevertheless, IL-4 can also be produced by T_{FH} cells to support the B-cell stimulation and the class switch to

IgG1 and IgE (Debock et al. 2013). Debock et al. (2013) also described that neonatal mice T_{FH} cells produce high levels of IL-21 and IL-4 compared to adults.

By stimulation of cells for twelve hours with PMA and ionomycin, the TNFα, IL-4 and IL-21 production by T cells could be determined. The high TNFα production upon stimulation indicates that infant T cells have the capacity to produce the pro-inflammatory cytokines, which is consistent with earlier findings (Schreurs et al. 2019) as they are involved in the process of tissue generation. Immune cells at the beginning of life focus in first place on avoiding indefensible inflammation (Kollmann et al. 2017). Therefore, infant immune cells usually express more T_H2-cell cytokines compared to adults (Renz et al. 2011), which are predominantly suppressive and anti-inflammatory. It is consistent with the finding of higher IL-4 production on $CD4^+$ T cells in infant intestinal tissues in this small study compared to adult intestines. A higher IL-21 production on $CXCR5^+$ cells than on $CXCR5^-$ $CD4^+$ T cells needs to be further determined. Meanwhile, infant and adult cells were able to produce IL-21 in approximately the same amount. IL-21 detection with intracellular staining is notoriously difficult and although the staining protocol provided good results in blood sample controls (data not shown), expression was relatively weak in intestinal $CD4^+$ T cells. In sum these data indicate that $CD4^+$ T cells in infant and adult intestines can produce hallmark cytokines.

T regulatory (T_{reg}) cells are supposed to induce self-tolerance and immune homeostasis by suppressing and controlling T-cell activity. The high frequency of T_{reg} cells in infants suggests that immune responses are more suppressed early in life than at higher age. Thome et al. (2016) could show that infants have a higher frequency of T_{reg} cells in blood than adults and showed a six- to ten-fold increased frequency in all tissues compared to adult tissues. In infant tissues, T_{reg} cells are predominantly distributed in mucosal and lymphoid tissues, while in adults they appear at higher frequencies in lymphoid tissues than at mucosal sites. The ratio of T_{reg}:T_{EM} is lower in infant intestinal mucosae than in infant blood (Thome et al. 2016). The predominance of regulatory cells in the non-T_{FH}-cell population in infant intestinal mucosa in comparison to the adult non-T_{FH}-cell population could be confirmed by this study.

T follicular regulatory (T_{FR}) cells express Bcl-6 to a lower amount than T_{FH} cells and instead express the transcription factor FoxP3 (Sage and Sharpe 2016). Since infants have higher frequencies of normal T_{reg} cells (Thome et al. 2016), it has been investigated whether infants

also have higher frequencies of T_{FR} cells compared to adults. However, the proportion of T_{FR} cells was similar for infants and adults. In lymph nodes, T_{FR} cells were described by Sage and Sharpe (2016) to be 50% of all T_{FH} cells, whereas in Peyer's patches they were, most likely because of constant antigen exposure in the intestine, present to a lesser extent. In this study, the frequency of FoxP3 expression on T_{FH} cells from LPL was 5 to 10% and thus consistent with the earlier finding of diminished T_{FR} cell proportions in intestinal tissues (Sage and Sharpe 2016). Reduced numbers of T_{FR} cells in the intestine compared to other tissues could suggest that the intestinal mucosa might be a potential site to induce vaccine responses. T_{FR} cells are the counterpart of T_{FH} cells and would diminish antibody production by B cells.

This study aimed to test the hypothesis that infants harbour mature T_{FH} cells in the intestine. In summary, our results provide evidence that at least infants with four months of age show effector T_{FH} cells with a tissue-resident phenotype in intestinal mucosae which are more frequent than in adult intestinal mucosae. The intestinal mucosa is exposed to several new antigens at the beginning of life, including vaginal and environmental antigens. Consequently, the intestinal immune system is for the first time massively stimulated, which may explain the abundance of T_{FH} cells in infant intestines. However, future studies are necessary to investigate the effect of a limited Bcl-6 expression on the T_{FH}-cell functionality in infants and their ability to interact with other cell types important for antibody production. As T_{FH} cells are a critical player for immune response induction upon vaccination, the abundance of T_{FH} cells in infant intestinal mucosa might indicate that there could be a potential to induce a sufficient vaccine response through oral vaccination.

5.3 Strengths and limitations

This study provides insight into the distribution and phenotypic characteristics of T_{FH} cells in the intestinal mucosa of infants. Earlier findings from mice tissues or human blood are less appropriate representatives of the human infant intestine.

Infant and adult tissue samples were obtained from patients with indications to surgery. T-cell frequencies in tissues can alter due to inflammatory processes from disease (Renz et al. 2011). However, only tissues from patients without infectious or immunological diseases were obtained and only non-inflamed tissues were included in order to minimize bias from disease or surgery.

Another bias could be the sex, as it also influences the immune system. Men typically exhibit a higher susceptibility towards infectious diseases and lower cell-mediated and humoral immune responses upon vaccination than women. A main contributor to this effect are the sex hormones. Oestradiol has an enhancing effect on the immune response, while testosterone is suppressive (Giefing-Kröll et al. 2015). The levels of sex hormones change with age, but differences in response to vaccines remain throughout every age. Therefore, most likely also genetic and other factors including the microbiome and the behaviour impact the immune response (Klein et al. 2015). To reduce bias caused by sex-differences, in this study, male and female patients were selected nearly equally for the infant (male n = 8, female n = 6) as well as the adult (male n = 8, female n = 6) group.

Deficiencies in the infant immune system stay at least up to 18 months of life (Vosters et al. 2010). The median age of infants in this study of seven months is representing the age where infants are vulnerable to infections and vaccination schedules are not completed for full protection. Unfortunately, tissues from neonates, infants younger than four months, or after 20 weeks of pregnancy are missing. The reason is that neonates barely undergo surgery where non-inflamed intestinal tissues remain and the possibility to obtain tissue from foetuses in the third trimester is rare. Consequently, it remains unclear at which age T_{FH} cells in the intestinal tissue are first present between the second trimester and the fourth month of life. It is very likely that T_{FH} cells develop soon after birth with the introduction of several new antigens. As it is still controversial whether microbiome is present in the placenta in utero (Aagaard et al. 2014, Theis et al. 2020), it could be possible that T_{FH} cells develop in the third trimester of pregnancy.

Additionally, it is unknown at which age the frequency of T_{FH} cells declines between the oldest infant with 30 months and the youngest adult with 50 years of age in this patient cohort. The adult group in this study with a median age of 64 years might inadequately represent the whole adult population as it does not represent young adults. At an age of 65 to 70 years the susceptibility to pathogens increases and immune responses to vaccination decline over time (Farber et al. 2014, Linterman 2014). In older adulthood GC become smaller, the amount of naïve T cells, which are available for activation, is decreased and T cells get less activated upon weaker T-cell receptor (TCR) stimulations, accompanied by a reduction in the number of T_{FH} cells (Linterman 2014). Because young adults predominantly undergo surgery due to chronic inflamed bowel diseases, which is a criterion for exclusion in this study, older adults are the best available comparison group.

The technique for mononuclear leukocytes isolation from epithelial and lamina propria layers is adapted from Schreurs et al. (2017, 2019) and Sagebiel et al. (2019). In several studies on tissues, the isolation of intraepithelial cells with EDTA and DTT is consistent. However, different isolation techniques were used for the isolation of mononuclear leukocytes from lamina propria layers, which impacts the results. Schreurs et al. (2017) previously showed that the isolation with Collagenase D had advantages over the isolation with Medimachine, an enzyme-free mechanical disaggregation method, and over the isolation with the Lipase enzyme. The advantages concerned cell yield and expression of surface molecules (Schreurs et al. 2017). Therefore, it can be assumed that the isolated cells accurately represent the cells in vivo.

The luminal content, anatomy, and bacterial composition changes along the intestinal tract (Brown et al. 2013, Mowat and Agace 2014). Consequently, the distribution, phenotype, and functionality of cell types vary according to their anatomical location. Whereas the frequency of T_{reg} cells and IgA producing plasma cells increases in the colon compared to the small intestine, the frequency of T_H1 and T_H2 cells does not alter significantly in the different parts of the intestine (Mowat and Agaace 2014). The adaptation of T_{FH}-cell frequencies over the length of the intestinal tract was not investigated yet and would be an interesting subject to future studies. Due to the limited number of human tissue samples, the samples from different parts of the intestine were merged to obtain an overall impression of the infant compared to adult intestinal tissues.

Power and sample size calculations adapted form Bunders et. al (2012) suggested an aim of seven to ten samples per patient cohort. This number was reached for most of the experiments so that the main results are representative and indicate significant differences. Although only three foetal samples were obtained, T_{FH} cells are undoubtedly not present in the second trimester. For the cell surface markers CD103, ICOS, CD40L, and cytokine production a detailed statement regarding the distribution and statistical analysis was not feasible due to limited numbers of samples.

Peyer's patches are located in the lamina propria layer and submucosa of the intestinal wall (Schünke et al. 2012, Mowat and Agace 2014). Since the submucosa had been cut when taking off the mucosa from the underlying muscular layer, it is not certain whether, next to isolated lymphoid aggregates, cells from the PPs were also included in the LPL after isolation. Therefore, it would be interesting to immunohistochemically investigate the

mucosa after the removal of submucosal fibres to see whether it contains PPs. Immunohistochemistry could additionally reveal whether T_{FH} cells in infants are localized in the interfollicular region as Debock et al. (2013) suggest.

The fact that adult mucosa is thicker than infant mucosa might distort the results regarding the absolute numbers of cells. In the calculating of the absolute cell number, only the area of the mucosa was included. Preliminary calculations that took the weight of the tissues into account, showed less consistent data (data not shown). The underlying reason is most likely fluids diffusing into the tissues during surgery that dramatically change the sample weights. Nevertheless, the difference between the absolute number of cells calculated with the area of the mucosa in infants and adults might rather be under- than over-estimated as the infant mucosa is thinner but still contains more T_{FH} cells.

In conclusion, the study cohort was chosen advisedly to reduce bias. Considering that the subject of research is human tissues, the cohort size is sufficient for most of the experiments. The advantage of this study in comparison to other studies is the investigation of human infant tissues.

5.4 Outlook

This study shows that infants have a substantial number of T_{FH} cells with a tissue-resident effector memory phenotype in intestinal tissues. The T_{FH} cells in the intestinal compartment are most likely ahead of the development of T_{FH} cells in blood or lymphoid tissues due to early environmental exposure. Nevertheless, infant T_{FH} cells exhibited a reduced Bcl-6 expression which could indicate that T_{FH} cells have not completed development. In connection with further experiments of our group a first manuscript will be submitted for publication soon to share these results with the scientific world. Infant T_{FH} cells in the intestine seem functional, based on their tissue-resident effector memory phenotype, but several questions remain unexplored and further investigation will be necessary on this important issue.

Because of the decreased Bcl-6 levels in infants other transcription factors might be critically involved in the T_{FH}-cell development and maintenance in the infant intestine, including STAT3, IRF4, BATF, c-Maf, and the negative regulator Blimp-1. These several transcription factors are known to be involved in the regulation of T_{FH}-cell development and could lead to an upregulation of Bcl-6 (Ma et al. 2012b, Liu et al. 2013, Qi 2016). A real-

time quantitative polymerase chain reaction (RT-qPCR) on, by using flow cytometry, sorted T_{FH} cells can help to further investigate the expression of these different transcription factors in infant T_{FH} cells. Results could provide an understanding regarding the developmental status of T_{FH} cells in infant intestinal mucosae.

Direct cell interactions must be further investigated by increasing the number of samples in order to receive significant results regarding the surface marker ICOS and CD40L. The panel could be further extended with different adhesion molecules, including signalling lymphocyte activation molecule (SLAM) and SLAM-associated protein (SAP), which are important for the T_{FH}-B-cell interaction and GC development (Crotty 2014). Other strategies must be considered to detect IL-21 production by T_{FH} cells. An adjuvant method could be a stimulation without Brefeldin A and a subsequent enzyme-linked immunosorbent assay (ELISA) in the supernatant (Gringhuis et al. 2014).

An experiment to determine the functionality of T_{FH} cells and their ability to activate B cells was established and will be subject to future research. A coculture stimulation of T_{FH} cells with B cells is supposed to show the ability of T_{FH} cells to provide help to B cells. T_{FH} cells, obtained by cell sorting, are cultured with B cells. The B cells are isolated via magnetic beads by negative selection from healthy PBMC and stimulated with anti-CD3 and anti-CD28 for several days, which imitates a stimulation by antigen-presenting cells. Staining of activation molecules on B cells and different immunoglobulins will show the B-cell activation status. An ELISA for immunoglobulins of the harvested supernatant will complement the results (Debock et al. 2013, Gringhuis et al. 2014).

In order to induce a protecting antibody response upon vaccination not only T_{FH} cells, but also interacting cell types must provide enough functionality. Deficiencies in the defence against pathogens in infants compared to adults and the reduced reaction to vaccines could be caused by impaired DC or B cells, as well as interfering maternal antibodies and a weaker mucosal barrier (Basu 2015). Granot et al. (2017) recently determined DCs in different human tissues. They found increased frequencies of classical DC (cDC) 1, which cross-present antigens to $CD8^+$ T cells, in infant intestinal tissues, whereas cDC2 and $CD14^+$ cell frequencies resembled the frequencies in adults. Human neonatal B cells in blood present lower expression of the co-stimulatory molecules CD40, CD80 and CD86 and their response to CD40L is impaired (Siegrist and Aspinall 2009). In future, research must be extended to these cell types involved in vaccine responses to determine their functionality in neonatal

and infant intestines as well. With this knowledge, it could be conceivable to address specific cells by adding improved adjuvants to the vaccine antigen. Adjuvants might not need to enhance the T_{FH}-cell function, but the B or DC cell function.

Adjuvants activate the immune system so that the safety of vaccines and adjuvants always needs to be guaranteed first. An excessive immune activation by adjuvants could lead to an uncontrollable inflammation especially in infants, who are naturally more susceptible. In addition, T_{FH} cells are involved in the process of autoantibody production (Vinuesa et al. 2005). Due to high frequencies of T_{FH} cells in infants and the little number of T_{FR} cells, found in the infant intestinal mucosa in this study, which ensure the homeostasis of T_{FH} activation, further immune activation through vaccination must be regarded with caution. These safety concerns need to be investigated to avoid autoimmunity, inflammatory reaction to food antigens or the physiological microbiome (Linterman and Hill 2016).

However, the abundance of mature EM T_{FH} cells in the intestinal mucosa could be in future addressed by oral vaccination in infants and might provide potential for vaccination early in life. Oral vaccination induces protection at the site where several pathogens enter the organism, reduces the risk of interference with maternal antibodies early in life and additionally provides socioeconomic advantages (Griebel 2009). The results of this study do not exclusively contribute to vaccine development, but also the development of strategies against HIV. T_{FH} cells are required for the generation of broadly neutralizing antibodies during chronic HIV infection (Cohen et al. 2014). Furthermore, as T_{FH} cells serve as a reservoir for the virus (Miles and Connick 2016), the abundance of T_{FH} cells in infant intestines could be an important factor for antiretroviral and cure therapies in infants.

Extensive research is necessary until efficient vaccines, which can be applied in a single dose shortly after birth, induce lifelong protection, while providing enough safety, against the fatal pathogens are available (Siegrist 2018). Nevertheless, this study is one step towards the sustainable development goal: “Ensure healthy lives and promote well-being for all at all ages” (United Nations 2018).

6 Abstract

The worldwide main cause for six million deaths of under-five year old infants in 2015 are infectious diseases. Vaccines could contribute to reduce this number. However, infants present a weaker response upon vaccination than adults, which is most likely associated with their immature and suppressive immune system early in life. T follicular helper (T_{FH}) cells are a pivotal actor in the induction of protective antibodies, due to their ability to activate and promote B cells. In this study, it could be demonstrated, that T_{FH} cells in infant intestinal mucosae are abundant in comparison to T_{FH} cells in adult intestinal mucosa. Compared to adult T_{FH} cells, the infant T_{FH} cells present a diminished expression of the master transcription factor Bcl-6. Nevertheless, the infant T_{FH} cells predominantly showed an effector memory tissue-resident phenotype and therefore appeared to be as mature as the adult T_{FH} cells. Whether the infant T_{FH} cells can sufficiently interact with other cells and properly induce B cells needs to be investigated in further studies.

Zusammenfassung

Die weltweit häufigste Ursache für sechs Millionen Todesfälle der unter 5-Jährigen im Jahre 2015 waren Infektionskrankheiten. Impfungen haben bereits einen Beitrag dazu geleistet diese Zahl zu reduzieren. Jedoch haben Kinder eine schwächere Immunantwort auf Impfungen als Erwachsene, was sehr wahrscheinlich mit deren unreifem und physiologisch supprimiertem Immunsystem zusammenhängt. T-follikuläre Helfer- (T_{FH}) Zellen fungieren als zentraler Akteur bei der Produktion von Antikörpern, da sie die Fähigkeit besitzen B-Zellen zu aktivieren und zu fördern. In dieser Studie konnte gezeigt werden, dass in der Darmschleimhaut von Kindern, im Vergleich zur Darmschleimhaut von Erwachsenen, reichlich T_{FH}-Zellen vorhanden sind. Im Vergleich zu T_{FH}-Zellen von Erwachsenen, zeigten die kindlichen T_{FH}-Zellen eine verminderte Expression des Schlüsseltranskriptionsfaktors Bcl-6. Jedoch zeigten die T_{FH}-Zellen von Kindern überwiegend einen Effektor-Gedächtnis-Phänotyp, waren gewebsständig und erschienen somit in demselben Maße reif wie die erwachsenen T_{FH}-Zellen. Ob die kindlichen T_{FH}-Zellen ausreichend mit anderen Zellen interagieren und B-Zellen induzieren können, muss in weiteren Studien untersucht werden.

7 List of Abbreviations

T_{FH}	T follicular helper
Hib	*Haemophilus influenzae* type b
DC	dendritic cell
PAMP	pathogen-associated molecular pattern
TLR	toll-like receptor
PRR	pattern recognition receptor
LN	lymph node
MHC-II	major histocompatibility complex class II
APC	antigen-presenting cell
T_H	T helper
T_{reg}	T regulatory
Ig	immunoglobulin
CCR7	C-C chemokine receptor type 7
GC	germinal centre
FDC	follicular dendritic cell
GBS	group B streptococcus
RSV	respiratory syncytial virus
PBMC	peripheral blood mononuclear cells
HIV	human immunodeficiency virus
cART	combined antiretroviral therapy
HBV	hepatitis B virus
BCG	Bacillus Calmette-Guérin
OPV	oral polio vaccine
CXCR5	C-X-C chemokine receptor type 5
CXCL13	C-X-C motif ligand 13

PD-1	programmed cell death protein 1
ICOS	inducible T-cell costimulatory
Bcl-6	B-cell lymphoma 6
IL	interleukin
STAT	signal transducer and activator of transcription
Blimp-1	B lymphocyte-induced maturation protein 1
BATF	basic leucin zipper transcription factor activation transcription factor-like
IRF4	interferon regulatory factor 4
c-Maf	musculoaponeurotic fibrosarcoma oncogene homolog
CD40L	CD40 Ligand
T_{FR}	T follicular regulatory
MLN	mesenteric lymph node
PP	Peyer's patch
IEL	intraepithelial lymphocytes
LPL	lamina propria lymphocytes
GALT	gut-associated lymphoid tissue
FAE	follicle associated epithelium
M-cell	microfold cell
MTCT	mother-to-child transmission
T_{RM}	tissue-resident memory T
TNFα	tumour necrosis factor alpha
GIT	gastrointestinal tract
MALT	mucosa-associated lymphoid tissue
RV	rotavirus
WHO	World Health Organization
PBS	Phosphate Buffered Saline

IMDM	Iscove’s Modified Dulbecco’s Medium
FBS	Foetal Bovine Serum
DTT	1,4-Dithiothreit
EDTA	Ethylenediaminetetraacetic acid
PMA	Phorbol 12-myristate 13-acetate
Pen-Strep	Penicillin-Streptomycin
DMSO	Dimethyl sulfoxide
SIP	Standard Isotonic Percoll
UKE	University Medical Centre Hamburg-Eppendorf
AKK	Altona Children’s Hospital
IQR	interquartile range
AMC	Academic Medical Centre
CO_2	carbon dioxide
SSC	side-scattered
FSC	forward-scattered
CS&T	Cytometer Setup and Tracking
fcs	flow cytometry standard
SSC-W	side-scattered-width
FSC-W	forward-scattered-width
FSC-A	forward-scattered-area
SSC-A	side-scattered-area
EM	effector memory
CM	central memory
MFI	median fluorescence intensity
IFR	interfollicular region
TCR	T-cell receptor

RT-qPCR	real-time quantitative polymerase chain reaction
SLAM	signalling lymphocyte activation molecule
SAP	SLAM-associated protein
ELISA	enzyme-linked immunosorbent assay
cDC	classical dendritic cell
T_{FH}	T follikuläre Helfer

8 References

Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J (2014) The placenta harbors a unique microbiome. Sci Transl Med. 6(237):237ra65.

Adkins B, Leclerc C, Marshall-Clarke S (2004) Neonatal adaptive immunity comes of age. Nat Rev Immunol. 4(7):553–564.

Ai W, Li H, Song N, Li L, Chen H (2013) Optimal Method to Stimulate Cytokine Production and Its Use in Immunotoxicity Assessment. Int J Environ Res Public Health. 10(9):3834–3842.

Alexander-Miller MA (2014) Vaccines against respiratory viral pathogens for use in neonates: opportunities and challenges. J Immunol. 193(11):5363–5369.

Allam A, Majji S, Peachman K, Jagodzinski L, Kim J, Ratto-Kim S, Wijayalath W, Merbah M, Kim JH, Michael NL, Alving CR, Casares S, Rao M (2015) TFH cells accumulate in mucosal tissues of humanized-DRAG mice and are highly permissive to HIV-1. Sci Rep. 5:10443.

Altfeld M, Bunders MJ (2016) Impact of HIV-1 infection on the feto-maternal crosstalk and consequences for pregnancy outcome and infant health. Semin Immunopathol. 38(6):727–738.

Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG (1999) In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J Exp Med. 190(8):1123–1134.

Arrieta M-C, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B (2014) The intestinal microbiome in early life: health and disease. Front Immunol. 5:427.

Bacchetta R, Gregori S, Roncarolo M-G (2005) CD4+ regulatory T cells: mechanisms of induction and effector function. Autoimmun Rev. 4(8):491–496.

Banchereau J, Bazan F, Blanchard D, Brière F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Saeland S (1994) The CD40 antigen and its ligand. Annu Rev Immunol. 12:881–922.

Basha S, Surendran N, Pichichero M (2014) Immune responses in neonates. Expert Rev Clin Immunol. 10(9):1171–1184.

Basu S (2015) Neonatal sepsis: the gut connection. Eur J Clin Microbiol Infect Dis. 34(2):215–222.

Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho I-C, Sharpe AH, Kuchroo VK (2009) The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nat Immunol. 10(2):167–175.

BioLegend (2018) Zombie Aqua™ Fixable Viability Kit. San Diego [Online im Internet.] URL: https://www.biolegend.com/en-us/global-elements/pdf-popup/zombie-aqua-fixable-viability-kit-8444?filename=Zombie%20Aqua%20Fixable%20Viability%20Kit.pdf&pdfgen=true [Stand: 06.09.2018, 15:51].

Black RE, Allen LH, Bhutta ZA, Caulfield LE, de Onis M, Ezzati M, Mathers C, Rivera J, for the Maternal and Child Unternutrition Study Group (2008) Maternal and child undernutrition: global and regional exposures and health consequences. Lancet. 371(9608):243–260.

Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Förster R (2000) Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med. 192(11):1545–1552.

Brown EM, Sadarangani M, Finlay BB (2013) The role of the immune system in governing host-microbe interactions in the intestine. Nat Immunol. 14(7):660–667.

Bunders MJ, van der Loos CM, Klarenbeek PL, van Hamme JL, Boer K, Wilde JCH, de Vries N, van Lier RAW, Kootstra N, Pals ST, Kuijpers TW (2012) Memory CD4(+)CCR5(+) T cells are abundantly present in the gut of newborn infants to facilitate mother-to-child transmission of HIV-1. Blood. 120(22):4383–4390.

Bundesministerium für wirtschaftliche Zusammenarbeit und Entwicklung (2018) Die Millenniumsentwicklungsziele. Berlin [Online im Internet.] URL: http://www.bmz.de/de/ministerium/ziele/2030_agenda/historie/MDGs_2015/index.html [Stand: 18.06.2018, 16:03].

Burt TD (2013) Fetal regulatory T cells and peripheral immune tolerance in utero: implications for development and disease. Am J Reprod Immunol. 69(4):346–358.

Campbell JJ, Murphy KE, Kunkel EJ, Brightling CE, Soler D, Shen Z, Boisvert J, Greenberg HB, Vierra MA, Goodman SB, Genovese MC, Wardlaw AJ, Butcher EC, Wu L (2001) CCR7 expression and memory T cell diversity in humans. J Immunol. 166(2):877–884.

Carlsen HS, Baekkevold ES, Johansen F-E, Haraldsen G, Brandtzaeg P (2002) B cell attracting chemokine 1 (CXCL13) and its receptor CXCR5 are expressed in normal and aberrant gut associated lymphoid tissue. Gut. 51(3):364–371.

Cebra JJ (1999) Influences of microbiota on intestinal immune system development. Am J Clin Nutr. 69(5):1046S-1051S.

Chen L, Cohen AC, Lewis DB (2006) Impaired allogeneic activation and T-helper 1 differentiation of human cord blood naive CD4 T cells. Biol Blood Marrow Transplant. 12(2):160–171.

Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, Lao C, Crotty S (2011) ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity. 34(6):932–946.

Cohen K, Altfeld M, Alter G, Stamatatos L (2014) Early preservation of CXCR5+ PD-1+ helper T cells and B cell activation predict the breadth of neutralizing antibody responses in chronic HIV-1 infection. J Virol. 88(22):13310–13321.

Cooper MD (2015) The early history of B cells. Nat Rev Immunol. 15(3):191–197.

Cooper NR, Nemerow GR (1984) The role of antibody and complement in the control of viral infections. J Invest Dermatol. 83(1 Suppl):121s–7s.

Cornes JS (1965) Number, size, and distribution of Peyer's patches in the human small intestine: Part I The development of Peyer's patches. Gut. 6(3):225–229.

Corr SC, Gahan CCGM, Hill C (2008) M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. FEMS Immunol Med Microbiol. 52(1):2–12.

Crotty S (2011) Follicular helper CD4 T cells (TFH). Annu Rev Immunol. 29:621–663.

Crotty S (2014) T follicular helper cell differentiation, function, and roles in disease. Immunity. 41(4):529–542.

Debock I, Jaworski K, Chadlaoui H, Delbauve S, Passon N, Twyffels L, Leo O, Flamand V (2013) Neonatal follicular Th cell responses are impaired and modulated by IL-4. J Immunol. 191(3):1231–1239.

Deenick EK, Ma CS (2011) The regulation and role of T follicular helper cells in immunity. Immunology. 134(4):361–367.

Dorfman DM, Brown JA, Shahsafaei A, Freeman GJ (2006) Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. Am J Surg Pathol. 30(7):802–810.

Farber DL, Yudanin NA, Restifo NP (2014) Human memory T cells: generation, compartmentalization and homeostasis. Nat Rev Immunol. 14(1):24–35.

Faucette AN, Unger BL, Gonik B, Chen K (2015) Maternal vaccination: moving the science forward. Hum Reprod Update. 21(1):119–135.

Flamand V, Donckier V, Demoor FX, Le Moine A, Matthys P, Vanderhaeghen ML, Tagawa Y, Iwakura Y, Billiau A, Abramowicz D, Goldman M (1998) CD40 ligation prevents neonatal induction of transplantation tolerance. J Immunol. 160(10):4666–4669.

Förster R, Emrich T, Kremmer E, Lipp M (1994) Expression of the G-protein--coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. Blood. 84(3):830–840.

Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M (1996) A Putative Chemokine Receptor, BLR1, Directs B Cell Migration to Defined Lymphoid Organs and Specific Anatomic Compartments of the Spleen. Cell. 87(6):1037–1047.

Fuss IJ, Kanof ME, Smith PD, Zola H (2009) Isolation of whole mononuclear cells from peripheral blood and cord blood. Curr Protoc Immunol. 85(1):7.1.1-7.1.8.

Giefing-Kröll C, Berger P, Lepperdinger G, Grubeck-Loebenstein B (2015) How sex and age affect immune responses, susceptibility to infections, and response to vaccination. Aging Cell. 14(3):309–321.

Granot T, Senda T, Carpenter DJ, Matsuoka N, Weiner J, Gordon CL, Miron M, Kumar BV, Griesemer A, Ho S-H, Lerner H, Thome JJC, Connors T, Reizis B, Farber DL (2017) Dendritic Cells Display Subset and Tissue-Specific Maturation Dynamics over Human Life. Immunity. 46(3):504–515.

Griebel PJ (2009) Mucosal vaccination of the newborn: an unrealized opportunity. Expert Rev Vaccines. 8(1):1–3.

Gringhuis SI, Kaptein TM, Wevers BA, van der Vlist M, Klaver EJ, van Die I, Vriend LEM, de Jong MAWP, Geijtenbeek TBH (2014) Fucose-based PAMPs prime dendritic cells for follicular T helper cell polarization via DC-SIGN-dependent IL-27 production. Nat Commun. 5:5074.

Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG (2007) Role of CXCR5 and CCR7 in Follicular Th Cell Positioning and Appearance of a Programmed Cell Death Gene-1High Germinal Center-Associated Subpopulation. The Journal of Immunology. 179(8):5099–5108.

Hodgins DC, Shewen PE (2012) Vaccination of neonates: problem and issues. Vaccine. 30(9):1541–1559.

Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med. 11(4 Suppl):S45-53.

Ibrahim SF, van den Engh G (2007) Flow cytometry and cell sorting. Adv Biochem Eng Biotechnol. 106:19–39.

Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. Nat Immunol. 5(10):987–995.

Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, Dent AL, Craft J, Crotty S (2009) Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science. 325(5943):1006–1010.

Jung C, Hugot J-P, Barreau F (2010) Peyer's Patches: The Immune Sensors of the Intestine. Int J Inflam. 2010:823710.

Kerfoot SM, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, Haberman, AM (2011) Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. Immunity. 34(6):947–960.

Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC (2001) Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. J Exp Med. 193(12):1373–1381.

Kim S-H, Jang Y-S (2017) The development of mucosal vaccines for both mucosal and systemic immune induction and the roles played by adjuvants. Clin Exp Vaccine Res. 6(1):15–21.

Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, Okada T (2011) Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. Immunity. 34(6):961–972.

Klein SL, Marriott I, Fish EN (2015) Sex-based differences in immune function and responses to vaccination. Trans R Soc Trop Med Hyg. 109(1):9–15.

Kollaritsch H, Kundi M, Giaquinto C, Paulke-Korinek M (2015) Rotavirus vaccines: a story of success. Clin Microbiol Infect. 21(8):735–743.

Kollmann TR, Kampmann B, Mazmanian SK, Marchant A, Levy O (2017) Protecting the Newborn and Young Infant from Infectious Diseases: Lessons from Immune Ontogeny. Immunity. 46(3):350–363.

Kovarik J, Siegrist C-A (1998) Immunity in early life. Immunol Today. 19(4):150–152.

Krumbiegel D, Zepp F, Meyer CU (2007) Combined Toll-like receptor agonists synergistically increase production of inflammatory cytokines in human neonatal dendritic cells. Hum Immunol. 68(10):813–822.

Küppers R (2005) Mechanisms of B-cell lymphoma pathogenesis. Nat Rev Cancer. 5(4):251–262.

Lavelle EC (2005) Generation of improved mucosal vaccines by induction of innate immunity. Cell Mol Life Sci. 62(23):2750–2770.

Levine MM, Dougan G (1998) Optimism over vaccines administered via mucosal surfaces. Lancet. 351(9113):1375–1376.

Levy O (2007) Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol. 7(5):379–390.

Linterman MA (2014) How T follicular helper cells and the germinal centre response change with age. Immunol Cell Biol. 92(1):72–79.

Linterman MA, Hill DL (2016) Can follicular helper T cells be targeted to improve vaccine efficacy? F1000Res. 5.

Liu X, Nurieva RI, Dong C (2013) Transcriptional regulation of follicular T-helper (Tfh) cells. Immunol Rev. 252(1):139–145.

Lycke N (2012) Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol. 12(8):592–605.

Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, Arkwright PD, Kreins AY, Averbuch D, Engelhard D, Magdorf K, Kilic SS, Minegishi Y, Nonoyama S, French MA, Choo S, Smart JM, Peake J, Wong M, Gray P, Cook MC, Fulcher DA, Casanova J-L, Deenick EK, Tangye SG (2012a) Functional STAT3 deficiency compromises the generation of human T follicular helper cells. Blood. 119(17):3997–4008.

Ma CS, Deenick EK, Batten M, Tangye SG (2012b) The origins, function, and regulation of T follicular helper cells. J Exp Med. 209(7):1241–1253.

MacLennan ICM, Toellner K-M, Cunningham AF, Serre K, Sze DM-Y, Zúñiga E, Cook MC, Vinuesa CG (2003) Extrafollicular antibody responses. Immunol Rev. 194:8–18.

Marrack P, McKee AS, Munks MW (2009) Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol. 9(4):287–293.

Mastelic B, Kamath AT, Fontannaz P, Tougne C, Rochat A-F, Belnoue E, Combescure C, Auderset F, Lambert P-H, Tacchini-Cottier F, Siegrist C-A (2012) Environmental and T cell-intrinsic factors limit the expansion of neonatal follicular T helper cells but may be circumvented by specific adjuvants. J Immunol. 189(12):5764–5772.

Mastelic-Gavillet B, Vono M, Gonzalez-Dias P, Ferreira FM, Cardozo L, Lambert P-H, Nakaya HI, Siegrist C-A (2019) Neonatal T Follicular Helper Cells Are Lodged in a Pre-T Follicular Helper Stage Favoring Innate Over Adaptive Germinal Center Responses. Front Immunol. 10: 1845.

Mathers C, Fat DM, Boerma JT, World Health Organization, eds. (2008) The Global Burden of Disease: 2004 Update. World Health Organization, Geneva.

Medzhitov R (2001) Toll-like receptors and innate immunity. Nat Rev Immunol. 1(2):135–145.

Meinelt E, Reunanen M, Edinger M, Jaimes M, Stall A, Sasaki D, Trotter J (2012) Standardizing Application Setup Across Multiple Flow Cytometers Using BD FACSDivaTM Version 6 Software. Franklin Lakes [Online im Internet] URL:

https://www.bdbiosciences.com/documents/BD_FACSDiva_Stndrd_App_Setup_TechBulletin.pdf [Stand: 16.11.2018, 16:22].

Michaëlsson J, Mold JE, McCune JM, Nixon DF (2006) Regulation of T cell responses in the developing human fetus. J Immunol. 176(10):5741–5748.

Miles B, Connick E (2016) TFH in HIV Latency and as Sources of Replication-Competent Virus. Trends Microbiol. 24(5):338–344.

Morris MC, Surendran N (2016) Neonatal Vaccination: Challenges and Intervention Strategies. Neonatology. 109(3):161–169.

Mowat AM, Agace WW (2014) Regional specialization within the intestinal immune system. Nat Rev Immunol. 14(10):667–685.

Murphy KM, Weaver C (2017) Janeway's immunobiology. 9th edition, Garland Science, Taylor & Francis Group, New York.

Neutra MR, Kozlowski PA (2006) Mucosal vaccines: the promise and the challenge. Nat Rev Immunol. 6(2):148–158.

Neutra MR, Mantis NJ, Kraehenbuhl JP (2001) Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat Immunol. 2(11):1004–1009.

Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, Wang Y, Watowich SS, Jetten AM, Tian Q, Dong C (2008) Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity. 29(1):138–149.

Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang Y, Dong C (2009) Bcl6 mediates the development of T follicular helper cells. Science. 325(5943):1001–1005.

Oxford University Press (2018) Definition of infant in English. Oxford [Online im Internet.] URL: https://en.oxforddictionaries.com/definition/infant [Stand: 23.07.2018, 09:13].

Oxford University Press (2018) Definition of neonate in English. Oxford [Online im Internet.] URL: https://en.oxforddictionaries.com/definition/neonate [Stand: 23.07.2018, 09:17].

Pabst O (2012) New concepts in the generation and functions of IgA. Nat Rev Immunol. 12(12):821–832.

Pashine A, Valiante NM, Ulmer JB (2005) Targeting the innate immune response with improved vaccine adjuvants. Nature Medicine.11(4s):S63–68.

Paulke-Korinek M, Kundi M, Rendi-Wagner P, de Martin A, Eder G, Schmidle-Loss B, Vecsei A, Kollaritsch H (2011) Herd immunity after two years of the universal mass vaccination program against rotavirus gastroenteritis in Austria. Vaccine. 29(15):2791–2796.

Pichichero ME (2014) Challenges in vaccination of neonates, infants and young children. Vaccine. 32(31):3886–3894.

Prelog M (2012) Differential approaches for vaccination from childhood to old age. Gerontology. 59(3):230–9.

Qi H (2016) T follicular helper cells in space-time. Nat Rev Immunol. 16(10):612–625.

Renz H, Brandtzaeg P, Hornef M (2011) The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nat Rev Immunol. 2(1):9–23.

Roider J, Maehara T, Ngoepe A, Ramsuran D, Muenchhoff M, Adland E, Aicher T, Kazer SW, Jooste P, Karim F, Kuhn W, Shalek AK, Ndung'u T, Morris L, Moore PL, Pillai S, Kløverpris H, Goulder P, Leslie A (2018) High-Frequency, Functional HIV-Specific T-Follicular Helper and Regulatory Cells Are Present Within Germinal Centers in Children but Not Adults. Front Immunol. 9:1975.

Sage PT, Sharpe AH (2016) T follicular regulatory cells. Immunol Rev. 271(1):246–259.

Sagebiel AF, Steinert F, Lunemann S, Körner C, Schreurs RRCE, Altfeld M, Perez D, Reinshagen K, Bunders MJ (2019) Tissue-resident Eomes+ NK cells are the major innate lymphoid cell population in human infant intestine. Nat Commun. 10(1):975.

Sallusto F, Lanzavecchia A, Araki K, Ahmed R (2010) From vaccines to memory and back. Immunity. 33(4):451–463.

Sánchez de Medina F, Romero-Calvo I, Mascaraque C, Martínez-Augustin O (2014) Intestinal inflammation and mucosal barrier function. Inflamm Bowel Dis. 20(12):2394–2404.

Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJC, Bickham KL, Lerner H, Goldstein M, Sykes M, Kato T, Farber DL (2013) Distribution and

compartmentalization of human circulating and tissue-resident memory T cell subsets. Immunity. 38(1):187–197.

Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B (2000) CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med. 192(11):1553–1562.

Schatorjé EJH, Gemen EFA, Driessen GJA, Leuvenink J, van Hout RWNM, de Vries E (2012) Paediatric Reference Values for the Peripheral T cell Compartment. Scandinavian Journal of Immunology. 75(4):436–444.

Schmitt N, Bentebibel S-E, Ueno H (2014) Phenotype and functions of memory Tfh cells in human blood. Trends Immunol. 35(9):436–442.

Schön MP, Arya A, Murphy EA, Adams CM, Strauch UG, Agace WW, Marsal J, Donohue JP, Her H, Beier DR, Olson S, Lefrancois L, Brenner MB, Grusby MJ, Parker CM (1999) Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. J Immunol. 162(11):6641–6649.

Schreurs RRCE, Drewniak A, Bakx R, Corpeleijn WE, Geijtenbeek THB, van Goudoever JB, Bunders MJ (2017) Quantitative comparison of human intestinal mononuclear leukocyte isolation techniques for flow cytometric analyses. J Immunol Methods. 445:45–52.

Schreurs RRCE, Baumdick ME, Sagebiel AF, Kaufmann M, Mokry M, Klarenbeek PL, Schaltenberg N, Steinert FL, van Rijn JM, Drewniak A, The S-MML, Bakx R, Derikx JPM, de Vries N, Corpeleijn WE, Pals ST, Gagliani N, Friese MA, Middendorp S, Nieuwenhuis EES, Reinshagen K, Geijtenbeek TBH, van Goudoever JB, Bunders MJ (2019) Human Fetal TNF-α-Cytokine-Producing CD4+ Effector Memory T Cells Promote Intestinal Development and Mediate Inflammation Early in Life. Immunity. 50(2):462-476.e8.

Schünke M, Schulte E and Schumacher U (2012) Prometheus LernAtlas Der Anatomie - Innere Organe. 3. Auflage, Georg Thieme Verlag, Stuttgart.

Sheridan BS, Lefrançois L (2011) Regional and mucosal memory T cells. Nat Immunol. 12(6):485–491.

Siegrist C-A (2003) Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. Vaccine. 21(24):3406–3412.

Siegrist C-A (2007) The challenges of vaccine responses in early life: selected examples. J Comp Pathol. 137 Suppl 1:S4-9.

Siegrist C-A (2018) 2 - Vaccine Immunology. In: Plotkin's Vaccines. Plotkin SA, Orenstein WA, Offit PA, Edwards KM (editors), Seventh Edition, Elsevier, Philadelphia, 16-34.e7.

Siegrist C-A, Aspinall R (2009) B-cell responses to vaccination at the extremes of age. Nat Rev Immunol. 9(3):185–194.

Sigma-Aldrich (2018a) Brefeldin A. St. Louis [Online im Internet.] URL: https://www.sigmaaldrich.com/catalog/product/sigma/b7651 [Stand: 15.08.2018, 11:16].

Sigma-Aldrich (2018b) Collagenase D. St. Louis [Online im Internet.] URL: https://www.sigmaaldrich.com/catalog/product/roche/colldro [Stand: 15.08.2018, 10:57].

Simister NE (2003) Placental transport of immunoglobulin G. Vaccine. 21(24):3365–3369.

Ständige Impfkommission (2019) Empfehlungen der Ständigen Impfkommission (STIKO) am Robert Koch-Institut. Epidemiologisches Bulletin. 34: 313-364.

Stark PL, Lee A (1982) The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. J Med Microbiol. 15(2):189–203.

Tangye SG, Ma CS, Brink R, Deenick EK (2013) The good, the bad and the ugly - TFH cells in human health and disease. Nat Rev Immunol. 13(6):412–426.

Tate JE, Parashar UD (2014) Rotavirus vaccines in routine use. Clin Infect Dis. 59(9):1291–1301.

Theis KR, Romero R, Greenberg JM, Winters AD, Garcia-Flores V, Motomura K, Ahmad MM, Galaz J, Arenas-Hernandez M, Gomez-Lopez N (2020) No Consistent Evidence for Microbiota in Murine Placental and Fetal Tissues. mSphere. 5(1).

Thome JJC, Bickham KL, Ohmura Y, Kubota M, Matsuoka N, Gordon C, Granot T, Griesemer A, Lerner H, Kato T, Farber DL (2016) Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. Nat Med. 22(1):72–77.

Thome JJC, Farber DL (2015) Emerging concepts in tissue-resident T cells: lessons from humans. Trends Immunol. 36(7):428–435.

Townsend MJ, Monroe JG, Chan AC (2010) B-cell targeted therapies in human autoimmune diseases: an updated perspective. Immunol Rev. 237(1):264–283.

United Nations (2015) The Millennium Development Goals Report 2015. New York [Online im Internet.] URL: http://www.un.org/millenniumgoals/2015_MDG_Report/pdf/MDG%202015%20Summary%20web_english.pdf [Stand: 19.06.2018, 12:35].

United Nations (2018) About the Sustainable Development Goals. New York [Online im Internet.] URL: https://www.un.org/sustainabledevelopment/sustainable-development-goals/ [Stand: 18.06.2018, 8:45].

Vinuesa CG, Tangye SG, Moser B, Mackay CR (2005) Follicular B helper T cells in antibody responses and autoimmunity. Nat Rev Immunol. 5(11):853–865.

Vosters O, Lombard C, André F, Sana G, Sokal EM, Smets F (2010) The interferon-alpha and interleukin-10 responses in neonates differ from adults, and their production remains partial throughout the first 18 months of life. Clin Exp Immunol. 162(3):494–499.

WHO (2013) Rotavirus vaccines. WHO position paper – January 2013. Wkly Epidemiol Rec. 88(5):49–64.

WHO (2017) Children: reducing mortality. Geneva [Online im Internet.] URL: http://www.who.int/news-room/fact-sheets/detail/children-reducing-mortality [Stand: 19.06.2018, 10:43].

WHO (2018) MODULE 2 – Route of administration - WHO Vaccine Safety Basics. Geneva [Online im Internet] URL: http://vaccine-safety-training.org/route-of-administration.html [Stand: 23.07.2018, 09:57].

WHO, Unicef (2018) Progress and Challenges with achieving Universal Immunization Coverage. New York [Online im Internet.] URL: http://www.who.int/immunization/monitoring_surveillance/who-immuniz.pdf?ua=1 [Stand: 15.07.2018, 10:05].

Wood N, Siegrist C-A (2011) Neonatal immunization: where do we stand? Curr Opin Infect Dis. 24(3):190–195.

Xu H, Li X, Liu D, Li J, Zhang X, Chen X, Hou S, Peng L, Xu C, Liu W, Zhang L, Qi H (2013) Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature. 496(7446):523–527.

Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, Srivastava M, Linterman M, Zheng L, Simpson N, Ellyard JI, Parish IA, Ma CS, Li Q-J, Parish CR, Mackay CR, Vinuesa (2009) The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. Immunity. 31(3):457–468.

Yu S, Jia L, Zhang Y, Zhong J, Yang B, Wu C (2015) IL-12 induced the generation of IL-21- and IFN-γ-co-expressing poly-functional CD4+ T cells from human naive CD4+ T cells. Cell Cycle. 14(21):3362–3372.

Yuki Y, Kiyono H (2009) Mucosal vaccines: novel advances in technology and delivery. Expert Rev Vaccines. 8(8):1083–1097.

Zhang X, Zhivaki D, Lo-Man R (2017) Unique aspects of the perinatal immune system. Nat Rev Immunol. 17(8):495–507.

9 List of figures

10 Appendix

Table 9. Data on which the figures are based. Expression of markers always have been measured on $CD4^+$ T cells. The values of boxes marked in dark grey were not acquired. The values of boxes marked in light grey were excluded due to a population size smaller than ten cells.

Donor no.	$CXCR5^+$ of $CD4^+$ (%)	# $CXCR5^+$ / cm^2	$CXCR5^+$ $PD\text{-}1^{++}$ of $CD4^+$ (%)	# $CXCR5^+$ $PD\text{-}1^{++}/cm^2$
Tissue	IEL			
1F				
2F				
3F				
4I				
5I	11.7	209	3.26	58
6I	2.89		1.73	
7I				
8I				
9I	2.40		0.31	
10I				
11I	4.26	521	1.93	236
12I	4.17	49	1.70	20
13I				
14I				
15I				
16I				
17I	7.86	321	6.22	254
18I				
19I	7.69	7758	0.31	311
20A				
21A	3.93	116	0.99	29
22A	32.1	2014	9.05	568
23A	0.73	13	0.06	1
24A	3.20	563	0.34	60
25A				
26A	1.29	20	0.03	<1
27A				
28A	1.82		0.04	
29A	2.33	188	0.48	39
30A	6.77	538	0.17	13
31A	3.07	244	0.01	1
32A	2.14		<0.01	
33A				

Continuation of Table 9. I

Donor no.	$CXCR5^+$ of $CD4^+$ (%)	# $CXCR5^+/cm^2$	$CXCR5^+$ $PD\text{-}1^{++}$ of $CD4^+$ (%)	# $CXCR5^+$ $PD\text{-}1^{++}/cm^2$	$CD45RA^+$ $CCR7^+$ of $CXCR5^+$ $PD\text{-}1^{++}$ (%)	$CD45RA^-$ $CCR7^+$ of $CXCR5^+$ $PD\text{-}1^{++}$ (%)	$CD45RA^-$ $CCR7^-$ of $CXCR5^+$ $PD\text{-}1^{++}$ (%)
Tissue	LPL						
1F			0.03				
2F			0.44				
3F			0.45				
4I							
5I	2.49	1167	1.15	539	0.10	5.34	93.6
6I	1.29		0.14		17.6	41.2	41.2
7I	6.07	1379	4.97	1130	0.23	4.00	94.9
8I							
9I	1.58		0.51		0.32	5.78	92.4
10I							
11I	5.41	3275	2.05	1243	0.34	9.59	89.6
12I	1.04	89	0.23	20			
13I	43.8		29.8		0.07	4.63	94.9
14I	4.67	1830	3.35	1312	1.50	3.32	94.7
15I	3.79		0.45				
16I							
17I	2.26	2828	1.80	2254	0.67	8.37	90.2
18I							
19I	4.31	5424	0.94	1177	0.27	10.4	88.7
20A							
21A	2.28	241	0.56	60	0.83	21.7	77.5
22A	2.45	1513	0.70	433	0.77	25.3	71.4
23A	0.63	68	0.01	1			
24A	4.20	1273	0.09	27	0.00	0.00	100.0
25A	0.76	478	0.48	307	1.15	30.5	68.0
26A	3.45	1218	0.12	43	0.37	8.24	88.0
27A							
28A	2.42	341	0.05	6			
29A	1.85	483	0.22	57	0.00	13.1	86.9
30A	4.39	5463	0.23	280	8.00	36.5	53.0
31A	2.16	1269	0.02	9			
32A	1.26		0.03		4.35	0.00	21.7
33A							

Continuation of Table 9. II

Donor no.	CD45RA$^+$ CCR7$^+$ of CXCR5$^+$ PD-1$^-$ (%)	CD45RA$^-$ CCR7$^+$ of CXCR5$^+$ PD-1$^-$ (%)	CD45RA$^-$ CCR7$^-$ of CXCR5$^+$ PD-1$^-$ (%)	CD69$^+$ of CXCR5$^+$ PD-1^{++} (%)	CD69$^+$ of CXCR5$^+$ PD-1$^-$ (%)	CD69$^+$ CD103$^+$ of CXCR5$^+$ PD-1^{++} (%)	CD69$^+$ CD103$^+$ of CXCR5$^+$ PD-1$^-$ (%)
Tissue	LPL						
1F							
2F							
3F							
4I							
5I	24.1	38.7	34.5	98.5	74.8		
6I	59.1	33.0	6.96	58.8	30.4	0.00	9.09
7I	30.0	18.7	48.8	96.0	70.0	1.55	1.93
8I							
9I	13.2	30.3	51.4	91.2	78.8		
10I							
11I	26.2	37.4	32.2	98.3	74.3		
12I	37.9	37.9	17.2		48.3		
13I	11.1	37.3	44.9	69.9	55.3		
14I	26.5	26.0	43.0	98.4	62.9	1.43	3.87
15I	90.9	9.09	0.00		18.2		
16I							
17I	38.8	21.5	29.0	96.1	70.6	0.23	9.86
18I							
19I	2.23	30.2	66.8	96.7	94.4		
20A							
21A	3.49	25.7	67.6	87.5	77.8		
22A	12.9	40.6	42.0	90.8	78.9		
23A	13.0	59.8	25.0		66.3		
24A	7.48	26.2	62.8	92.3	75.3		
25A	9.52	64.3	21.4	97.7	71.4	1.73	26.8
26A	0.95	10.1	83.5	89.1	89.9		
27A							
28A	3.04	60.5	35.0		90.2		59.6
29A	5.01	46.2	44.0	93.4	90.8		
30A	4.96	73.0	20.9	94.0	78.4	14.0	37.8
31A	15.6	2.93	6.51		99.0		90.1
32A	16.3	3.17	5.10	78.3	98.8	13.0	93.5
33A							

Continuation of Table 9. III

Donor no.	MFI Bcl-6 $CXCR5^+$	MFI Bcl-6 $CXCR5^-$	ratio MFI Bcl-6 $CXCR5^+/CXCR5^-$	Bcl-6^+ of $CXCR5^+$ $PD\text{-}1^{++}$ (%)	Bcl-6^+ of $CXCR5^+$ $PD\text{-}1^+$ (%)	Bcl-6^+ of $CXCR5^+$ $PD\text{-}1^-$ (%)	Bcl-6^+ of $CXCR5^-$ $PD\text{-}1^-$ (%)
Tissue	LPL						
1F							
2F							
3F							
4I							
5I	469	422	1.11	7.86	8.90	4.50	2.13
6I	1725	1193	1.45				
7I	261	293	0.89	6.56	16.4	6.61	2.53
8I							
9I	146	129	1.13	0.43	1.83	1.24	0.39
10I							
11I	561	350	1.60	11.5	32.2	32.7	5.43
12I	252	168	1.50			0.00	2.27
13I	1103	833	1.32	5.88	5.39	6.71	2.95
14I	424	347	1.22	9.04	16.5	14.7	5.76
15I	1293	724	1.79			27.3	27.1
16I							
17I	438	313	1.40	9.18	32.3	29.6	4.06
18I							
19I	337	454	0.74	8.58	14.5	4.88	5.51
20A							
21A	770	507	1.52	10.0	13.7	11.7	4.42
22A	1913	733	2.61	38.5	46.7	40.2	16.1
23A	599	183	3.27		16.7	22.8	2.64
24A	288	191	1.51	69.2	42.5	38.9	21.1
25A							
26A	134	114	1.18	1.50	3.57	0.56	0.12
27A							
28A	1295	355	3.65		80.2	49.5	12.1
29A	717	281	2.55	18.0	39.1	47.1	17.1
30A	875	405	2.16	30.5	68.9	43.0	13.5
31A	583	451	1.29		42.9	22.8	17.0
32A	573	437	1.31	17.4	29.5	25.6	13.0
33A							

Continuation of Table 9. IV

Donor no.	Foxp3^{+} of CXCR5^{+} PD-1^{++} (%)	FoxP3^{+} of CXCR5^{+} PD-1^{+} (%)	FoxP3^{+} of CXCR5^{+} PD-1^{-} (%)	FoxP3^{+} of CXCR5^{-} PD-1^{-} (%)	ICOS^{+} of CXCR5^{+} PD-1^{++} (%)	ICOS^{+} of CXCR5^{+} PD-1^{+} (%)	ICOS^{+} of CXCR5^{+} PD-1^{-} (%)
Tissue	LPL						
1F							
2F							
3F							
4I							
5I	0.87	14.6	6.90	12.3			
6I	41.2	38.1	32.2	27.9	34.1		27.3
7I	22.6	20.2	19.3	10.3			
8I							
9I	0.00	0.52	3.35	8.18			
10I							
11I	0.86	16.0	6.56	14.8			
12I			6.90	20.1			
13I	2.55	18.0	39.1	12.4			
14I	18.2	20.1	12.9	8.46	10.3	20.9	6.28
15I			0.00	11.8			
16I							
17I	6.60	11.8	7.40	1.81	28.6	40.0	13.7
18I							
19I	3.54	12.7	6.95	9.59			
20A							
21A	13.3	19.2	7.94	10.7			
22A	1.53	10.5	7.96	9.24			
23A			12.0	2.98			
24A	7.69	12.3	4.99	5.24			
25A					15.3	6.90	5.36
26A	0.00	0.27	0.00	0.03			
27A							
28A		4.40	11.0	4.18			
29A	9.84	28.1	13.9	5.10			
30A	13.5	8.51	6.91	5.53	23.2	16.6	3.26
31A		2.04	0.98	0.50	9.09	9.43	8.86
32A	8.70	22.9	12.6	4.34			
33A							

Continuation of Table 9. V

Donor no.	$ICOS^+$ of $CXCR5^-$ $PD\text{-}1^-$ (%)	$CD40L^+$ of $CXCR5^+$ $PD\text{-}1^{++}$ (%)	$CD40L^+$ of $CXCR5^+$ $PD\text{-}1^+$ (%)	$CD40L^+$ of $CXCR5^+$ $PD\text{-}1^-$ (%)	$CD40L^+$ of $CXCR5^-$ $PD\text{-}1^-$ (%)	ratio $ICOS^+/CD40L^+$ of $CXCR5^+$ $PD\text{-}1^{++}$	ratio $ICOS^+/CD40L^+$ of $CXCR5^+$ $PD\text{-}1^+$
Tissue	LPL						
1F							
2F							
3F							
4I							
5I							
6I	1.68	47.7		90.0	32.9	0.71	
7I							
8I							
9I							
10I							
11I							
12I							
13I							
14I	3.17	1.99	2.67	4.64	0.95	5.18	7.83
15I							
16I							
17I	7.05	3.41	9.94	8.65	1.89	8.39	4.02
18I							
19I							
20A							
21A							
22A							
23A							
24A							
25A	0.97	8.07	41.4	16.1	4.06	1.90	0.17
26A							
27A							
28A							
29A							
30A	2.27	35.1	78.2	44.6	14.7	0.66	0.21
31A	1.41	22.7	43.4	36.9	26.8	0.40	0.22
32A							
33A							

Continuation of Table 9. VI

Donor no.	ratio $ICOS^+/CD40L^+$ of $CXCR5^+$ $PD\text{-}1^-$	ratio $ICOS^+/CD40L^+$ of $CXCR5^-$ $PD\text{-}1^-$	TNFα produced by $CD4^+$ (-) (%)	TNFα produced by $CD4^+$ (p/i) (%)	IL-4 produced by $CD4^+$ (-) (%)	IL-4 produced by $CD4^+$ (p/i) (%)	IL-21 produced by $CD4^+$ (-) (%)
Tissue	LPL						
1F							
2F							
3F							
4I			0.15	13.9	18.6	1.18	
5I							
6I	0.30	0.05					
7I							
8I			2.10	16.6	8.80	1.71	0.62
9I							
10I			0.42	39.9	19.3	4.05	0.63
11I							
12I							
13I			1.28	24.8	1.84	4.00	0.65
14I	1.35	3.34					
15I							
16I			0.84	12.1	4.17	4.79	
17I	1.58	3.73					
18I			1.09	44.4	8.73	11.3	
19I							
20A			1.83	24.3	1.56	2.76	
21A							2.54
22A							
23A							
24A							
25A	0.33	0.24					
26A							
27A			0.34	75.2	0.73	2.88	
28A							0.44
29A							
30A	0.07	0.15					
31A	0.24	0.05					
32A							
33A			0.27	8.54	4.15	4.64	4.65

Continuation of Table 9. VII

Donor no.	IL-21 produced by $CD4^+$ (p/i) (%)	IL-21 produced by $CXCR5^+$ (-) (%)	IL-21 produced by $CXCR5^+$ (p/i) (%)	IL-21 produced by $CXCR5^-$ (-) (%)	IL-21 produced by $CXCR5^-$ (p/i) (%)
Tissue	LPL				
1F					
2F					
3F					
4I					
5I					
6I					
7I					
8I	1.90	5.00	6.54	0.58	1.84
9I					
10I	5.25	2.25	5.41	0.55	5.24
11I					
12I					
13I	9.46				
14I					
15I					
16I					
17I					
18I					
19I					
20A					
21A	3.18	7.48	4.80	2.33	3.13
22A					
23A					
24A					
25A					
26A					
27A					
28A	4.86	1.82	6.38	0.42	4.80
29A					
30A					
31A					
32A					
33A	10.3				

11 Acknowledgments

I want to thank everyone who was involved in the process of my study, even if not personally mentioned. In the first place, I thank the German Centre for Infection Research (DZIF) for the foundation of my MD project.

Furthermore, some special thanks go to Dr. Dr. Madeleine Altfeld-Bunders, for giving me the opportunity to be part of her fascinating project and for extensive support during the whole process of experimental work and writing. I am also glad for Prof. Dr. Marcus Altfeld being my supervisor, giving helpful input and including me into his research department "Virus Immunology". All the members of the research department welcomed me cordially and cooperative. Especially Adrian Sagebiel comprehensively supported me with the implementation of my experiments at the HPI and did answer my numerous questions all day long. I want to thank Renée Schreurs from the AMC for supporting me during my entire project and teaching me the necessary techniques with patience and affection. Furthermore, I am thankful for Dr. Glòria Martrus Zapater for taking on the responsibility for my project after I left the department.

Another special thanks go to all tissue donors, the adults as well as the infants and their parents for giving their consent and providing the remaining tissues. Additionally, I want to thank all the surgeons from the UKE and AKK, who extensively contributed to the organisation of the tissue acquisition, which included obtainment of the informed consents and taking care of the tissues during the surgeries. I especially want to thank Prof. Dr. Konrad Reinshagen and Priv.-Doz. Dr. Daniel Perez, who supervised the tissue acquisition and gave comprehensive support.

Finally, I want to thank my parents Maren and Dr. Bernd Steinert, as well as my sisters Sölve and Levke who were always backing me up and gave helpful advice in the process of writing.

12 Lebenslauf

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt

13 Eidesstattliche Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....