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Early life impairment of thymus function: Effects on the developing immune system

Dissertation

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1. Introduction

1.1 The immune system protects the body from pathogens

The immune system encompasses a complex network of cells and soluble mediators that has evolved to protect the body from a vast number of pathogens. A main property of the immune system is the capacity to distinguish non-self from self, and between dead, malignant or faulty and functional viable cells. Environmental and genetic factors, and also medical interventions, can cause perturbations of the immune system resulting in hampered or excessive responses. Immunodeficiency compromises the potential to fight pathogens or malignant cells, increasing the susceptibility to infectious diseases and cancer. In contrast, overshooting immune responses may trigger autoimmune or allergic responses.

1.1.1 The rapid increase in the incidence of autoimmunity has a multifactorial origin

The prevalence of autoimmune diseases has drastically increased in industrial countries within the last seventy years (Bach, 2002, WHO, 2018a). Considering the very short period of seven decades, the fast rise in autoimmunity cannot be exclusively caused by genetic changes, but is rather a consequence of multifactorial triggers such as environmental, interventional, and (epi-) genetic factors (Theofilopoulos and Kono, 1999, Ermann and Fathman, 2001). Studies in monozygotic twins show that concordance rates for autoimmune diseases lay between 25-50%, and only one third of the variation in immunological traits in humans can be explained by inheritable factors (Orru et al., 2013, Brodin et al., 2015). In addition, most autoimmune diseases are caused by the interplay of several genes (multigenic), not by single variations (Fortune et al., 2015). Early in life, the immune system undergoes drastic developmental changes and interventions during this period are likely to affect immunity later in life. To name a few, early exposure to potential allergens such as allergenic food particles and proteins from house dust, crop, grass or animals reduces atopy (Reynolds and Finlay, 2017). Frequent early childhood infections in children that are genetically predisposed to type 1 diabetes indicate a role for infections in the pathogenesis of the disease (Mustonen et al., 2018). Thus, there is a high demand for research on early life factors altering function and composition of the immune system that may result in increased susceptibility of immune diseases, such as asthma and autoimmunity.

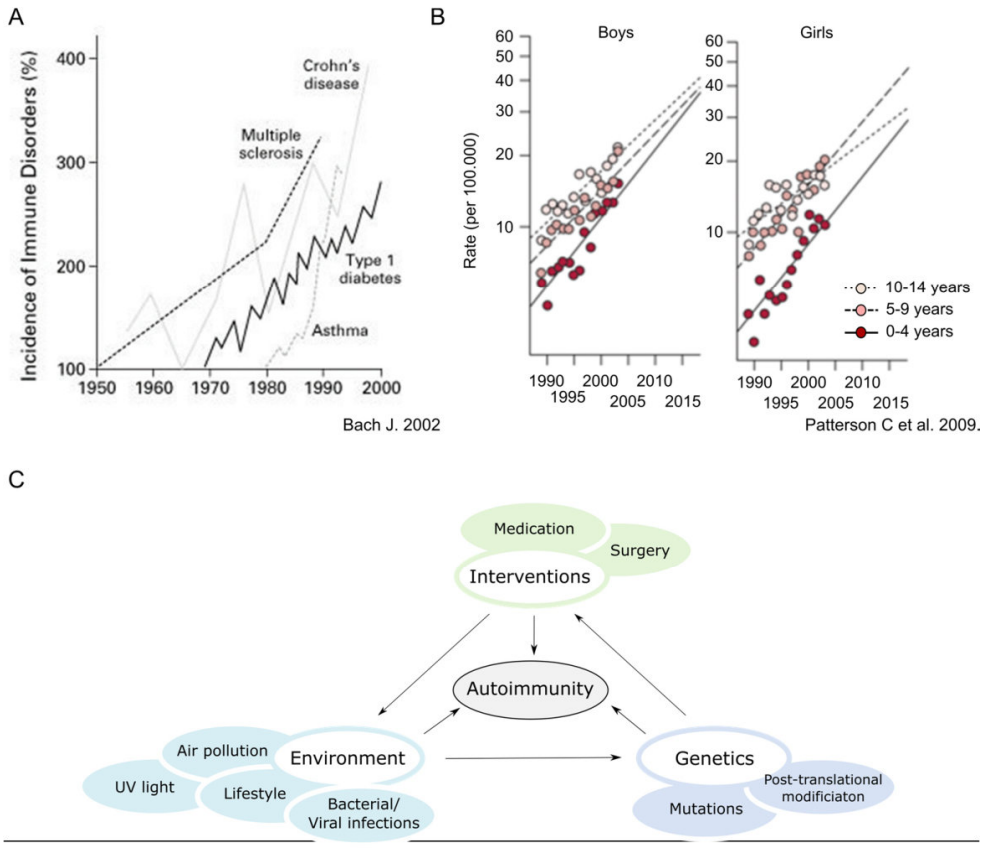

Figure 1. Rapid increase in immune diseases is most likely promoted by a multifactorial network. A) Increase of selected autoimmune diseases (multiple sclerosis, Crohn's disease, type 1 diabetes, asthma) from 1950 to 2000. Figure is adopted from (Bach, 2002). B) Rate of type1 diabetes in boys (left) and girls (right) from central European countries from 1990 to 2015. Different age-classes are represented by colors. Figure is modified from (Patterson et al., 2009). C) Multifactorial network of environmental factors, genetics and interventions influence the development of autoimmunity.

1.1.2 Diverse cell types facilitate effective and long-lasting immune responses

The immune system harbors two arms of defense (Figure 2A). The innate immune compartment consists of physical barriers, such as the skin or mucous membranes, and leucocytes. Innate immune cells, namely dendritic cells (DCs), neutrophils, eosinophils, basophils, mast cells, monocytes, or macrophages, rely on a limited repertoire of genetically conserved receptors (pattern recognition receptors) for the recognition of pathogen-associated molecular patterns present on pathogens. This general, non-specific recognition of pathogens, allows a rapid immune response within few hours.

On the other hand, the adaptive immune compartment consists of T and B lymphocytes harboring highly specific receptors for recognition of antigens. Each lymphocyte bears a unique receptor, the T cell receptor (TCR) or B cell receptor (BCR), arising from a random gene recombination process, called V(D)J recombination, during lymphocyte development. This somatic diversification leads to a highly flexible and diverse repertoire of lymphocytes capable of recognizing a vast number of different antigens. Activation of the adaptive immune system leads to the generation of long-lived memory cells that quickly react to pathogens upon a second encounter (Figure 2 B). While B cells react to native proteins, T cells rely on the

recognition of antigenic epitopes bound to major histocompatibility complexes (MHC) on antigen presenting cells, emphasizing the need of precisely orchestrated networking between the innate and the adaptive immune systems.

In addition to the conventional innate and adaptive cells, innate and innate-like unconventional lymphocytes, such as innate lymphoid cells (ILC), mucosal-associated invariant T cells (MAIT), natural killer T cells (NKT), innate-like B1, and TCRγδ cells, display structural and functional characteristics of both the innate and adaptive cell types. MAIT, NKT and TCRγδ cells express a limited TCR repertoire, and react to antigens presented by antigen-presenting cells (APCs), as well as to innate stimuli, such as cytokines or toll-like receptors (TLRs) (Pasman and Kasper, 2017). ILCs, in contrast, do not possess any antigen-specific receptor, and react exclusively to innate cues. B1 cells spontaneously secrete antibodies, mostly IgM, and harbor a less diverse, but broader, (auto)reactive repertoire of antibodies than conventional B cells (Gronwall et al., 2012). Shared features of all innate and innate-like lymphocytes are an early appearance during ontogeny, a tendency to localization in specialized tissue sites (mucosal membranes) and a fast immune response.

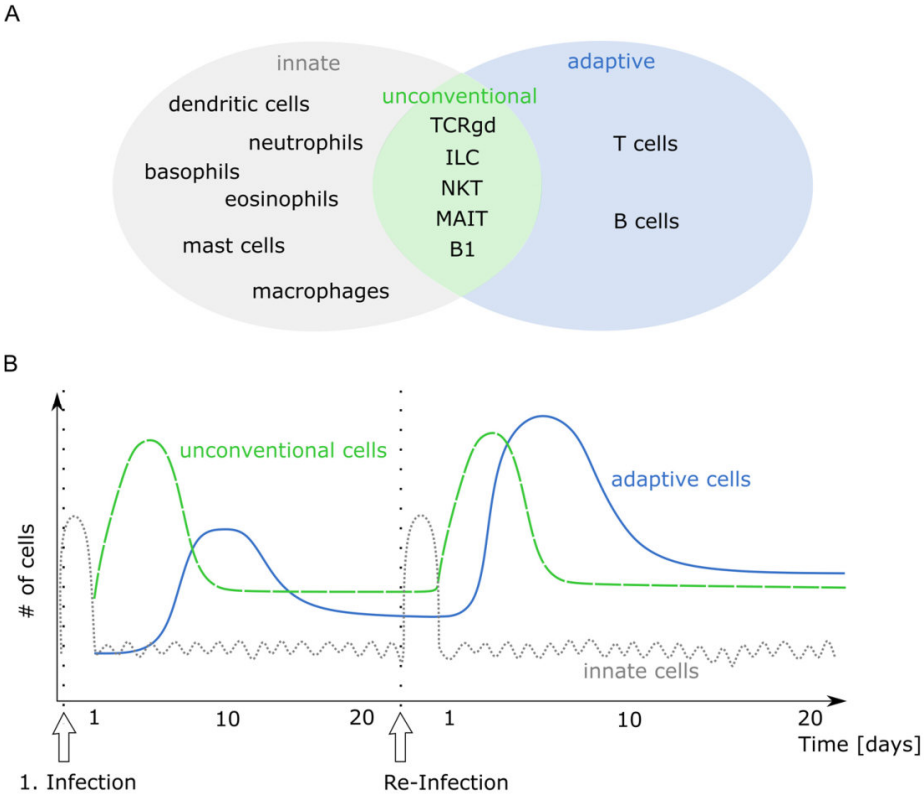

Figure 2. Composition of the immune system and kinetics of the response to pathogens. A) The immune system harbors an innate and an adaptive immune compartment. Unconventional cells show characteristics of both innate and adaptive traits. B) Response kinetics at first and second encounter of pathogens for innate, adaptive and unconventional cells. Adapted from (Godfrey et al., 2015)

1.2 The thymus provides the microenvironment for T cell development

1.2.1 Early thymic T cell development: from hematopoietic cells to T cells

Bone-marrow derived multipotent hematopoietic stem cells migrate through blood vessels to the thymus, where they undergo carefully orchestrated selection and maturation processes to generate an extensive repertoire of functional T cells. The thymus contains cortical and medullary thymic epithelial cells, each of them carrying out specific tasks during T cell development. Figure 3 summarizes the processes of human T cell development in the thymus. In brief, early CD34+ progenitor cells enter the thymus at the cortico-medullary junction. While they develop, they migrate following chemokine gradients first to the outer cortex and then through the cortex to the medulla (Yoganathan, 2019). Upon Notch signaling, early thymic progenitor cells (ETP) start to upregulate CD7 expression and lose myeloid and erythroid potential. Notch, GATA3 and Bcl11b signaling inhibits B cell differentiation, and leads to the expression of CD5 (Krueger et al., 2006, Taghon et al., 2005, Ikawa et al., 2010). Notch signaling induces the expression of critical transcription factors for T cell lineage fate, such as Tcf7, Bcl11b or E proteins. The potential for alternative lineages such as NK, DC or plasmacytoid DCs (pDCs) is lost upon upregulation of CD1α (Blom and Spits, 2006, Spits et al., 1998), finally leading to T cell commitment (Awong et al., 2009, Spits, 2002, Taghon et al., 2012). The developing T cells now begin to express CD4, but not CD8, and are termed intermediate single positive (ISP) cells (Galy et al., 1993, Kraft et al., 1993). Somatic recombination of the γ, δ, and β chain of the TCR leads to bifurcation of the T cell lineage: cells that successfully rearrange functional TCR γ and δ chains become $CD3^+TCR\gamma\delta$ cells (Blom et al., 1999, Ramiro et al., 1996). Functionally rearranged TCRβ chains pair with a pre-Tα (early double positive, DP), leading to strong proliferation, differentiation to $CD4^+CD8^+$ DP thymocytes, and rearrangement of the TCRα chain locus. Cells unable to rearrange a functional TCRβ undergo apoptosis (Spits, 2002). Recent studies in mice, however, suggest that it is rather the strength and the duration of the TCR signaling that controls the TCR$\alpha\beta$/$\gamma\delta$ lineage choice, with TCR$\gamma\delta$ cells arising from more potent and longer TCR signals (Zarin et al., 2014, Zarin et al., 2015). The $\gamma\delta$ lineage remains an option until shortly before positive selection (Carrasco et al., 1999, Joachims et al., 2006, Taghon and Rothenberg, 2008).

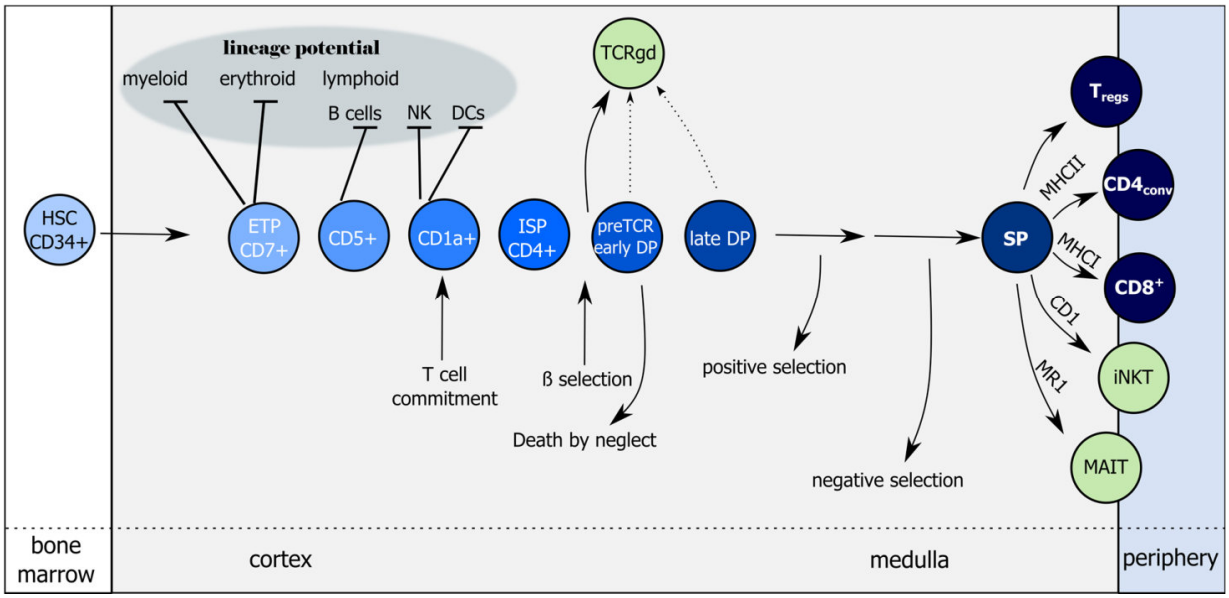

Figure 3. T cell development in the thymus. Hematopoietic stem cells from bone marrow enter the thymus and undergo precise maturation and selection processes facilitated by specialized epithelial cells in the cortex and the medulla of the thymus. Interaction with MHC and MHC-like molecules (CD1d, MR1) promotes T cell differentiation.

1.2.2 Unique processing of self-peptides by cortical thymic epithelial cells facilitates positive selection

Upon successful rearrangement of the TCRα chain locus, cells leave the outer cortex and spread inside the cortex. Subsequent selection processes depend on the recognition of self-peptides presented by MHC molecules on thymic epithelial cells (TEC). Thymus-specific proteases process endogenous proteins for presentation. The thymoproteasome, containing a unique catalytic subunit, β5t, generates a distinct set of self-peptides which are translocated to the endoplasmatic reticulum and loaded onto MHC I molecules on cortical TECs (cTECs). The peptides generated bind to the TCR with lower affinity, and the absence of β5t results in a polyclonal repertoire with loss of low affinity TCRs (Sasaki et al., 2015, Murata et al., 2007, Xing et al., 2013b). Of note, a single-nucleotide polymorphism (SNP) in β5t (G49S) is associated with a higher risk of developing Sjögrens syndrome, an autoimmune disease affecting exocrine glands, and impairs positive selection of $CD8^+$ cells in mice (Nitta et al., 2017). The loading of peptides onto MHCII molecules on cTECs is facilitated by an "unconventional" pathway: macroautophagosomes take up endogenous proteins and transport them to the lysosome, where they are processed by unique proteases, such as cathepsin V and thymus-specific serine protease (TSSP) (Tolosa et al., 2003, Viret et al., 2011). These uncommon processing machineries generate a unique set of peptides, distinct from the ones displayed by other thymic or peripheral APCs, and are thought to favor positive selection of cells with low affinity for self-antigens (Xing et al., 2013a). More than 90% of thymocytes are not able either to rearrange a proper TCR or to bind to any MHC:peptide complex, and undergo apoptosis (death by neglect), possibly mediated by endogenous glucocorticoids (GCs) in the absence of TCR signaling (Vacchio and Ashwell, 2000). In

contrast, successful binding to MHC:peptide induces Bcl-2 and other anti-apoptotic molecules, promoting survival (Linette et al., 1994, Punt et al., 1996). Positively selected thymocytes upregulate CD69 and CCR4 expression and translocate to the medullary region. The interaction of thymocytes with either MHC I or MHC II molecules determines CD8 or CD4 lineage commitment, respectively (Singer and Bosselut, 2004). Binding to MHC I leads to transcription of *Runx3* and a repression of *ThPOK,* facilitating the expression of CD8. Vice versa, binding to MHC II leads to the transcription of *ThPOK* and repression of *Runx3,* resulting in expression of CD4 (Luckey et al., 2014, Wang et al., 2008).

1.2.3 Negative selection eliminates self-reactive thymocytes

Approximately 5% of positively selected T cells bind with high affinity to self-antigens presented by MHC molecules on mTECs or DCs, and are eliminated to prevent autoimmunity (negative selection) (Figure ***5***). The promiscuous expression of antigenic peptides that are normally restricted to peripheral tissues, termed tissue-restricted antigens (TRA), is a unique feature of medullary thymic epithelial cells (mTEC) (Derbinski et al., 2001, Kyewski and Klein, 2006, Klein et al., 2014). TRA expression in mTECs is driven by the transcription factors AIRE (auto immune regulator) and Fezf2 (forebrain embryonic zinc finger-like 2), and their expression varies in the different stages of mTEC in the development (Anderson et al., 2002, Liston et al., 2003, Mathis and Benoist, 2009, Takaba et al., 2015, Takaba and Takayanagi, 2017). mTECs originate from the same progenitor as cTECs, the common thymic epithelial progenitor (TEP). The differentiation of mTECs is controlled by the expression of receptors of the TNF superfamily, namely LTβR, CD40, and RANK (Takaba and Takayanagi, 2017) (Figure 3). Subdivision of the mTEC population, according to their expression of MHCII and CD80, results in $mTEC^{low}$ ($MHCII^{low}CD80^{low}$) and $mTEC^{high}$ ($MHCII^{high}CD80^{high}$) (Gabler et al., 2007, Gray et al., 2007). Fezf2, regulated by LTβR, is expressed by both mTEC subsets, while AIRE is only present in the more mature $mTEC^{high}$ subset. Expression of AIRE in $mTEC^{high}$ is regulated through RANK and CD40 signaling. Ligands of RANK and CD40 are expressed on mature CD4 T cells, and their binding to mTECs induces AIRE expression and mTEC differentiation (Hikosaka et al., 2008). Approximately half of the $mTEC^{high}$ cells develop into post-AIRE mTECs. Post-AIRE cells down-regulate MHCII and CD80, express reduced levels of TRAs and are positive for involucin, a terminal maturation marker of keratinocytes (Wang et al., 2012). A subset of post-AIRE cells undergoes apoptosis and is cleared by phagocytosis, while the remaining cells lose their nuclei and become a component of Hassall's corpuscles (Mikusova et al., 2017).

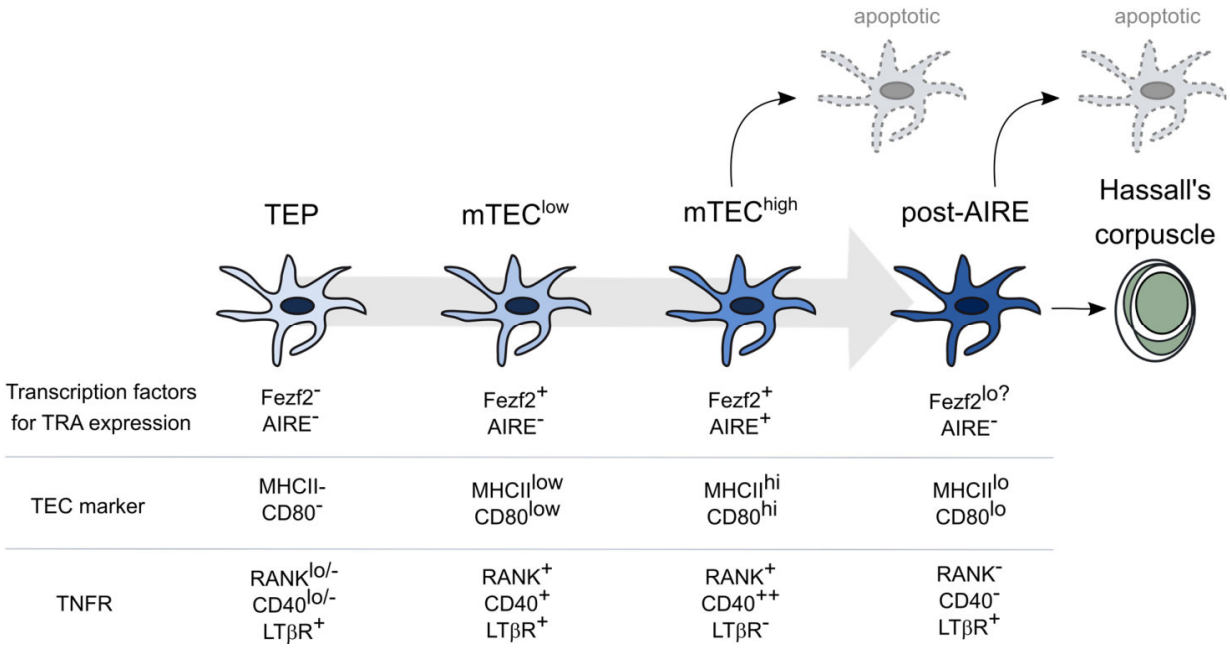

Figure 4. Development of medullary thymic epithelial cell (mTEC). mTEC originate from a common thymic epithelial progenitor (TEP) and differentiate into mTEC^{low}, mTEC^{high} and post-AIRE mTECs, which finally contribute to the formation of Hassall's corpuscles. Each developmental stage is characterized by a distinct signature of TEC markers and receptors of the TNF superfamily (TNFR). Adapted from (Takaba and Takayanagi, 2017).

AIRE and Fezf2 each drive the expression of a specific set of TRAs with relatively low overlap between them. TRA expression in individual mTECs is highly stochastic, with only 1-3% of mTECs expressing a particular set of TRAs (Takaba et al., 2015). Specific co-expression of different TRAs in discrete mTECs reflects conformational proximity of genes within open chromatin structures (Pinto et al., 2013). Proper thymic expression of TRA is necessary for central T cell tolerance (Klein et al., 2014). AIRE deficiency causes the autoimmune polyglandular syndrome type 1 (APS) in humans, characterized by autoimmunity affecting several endocrine organs (Betterle et al., 1998). An altered thymic expression of the major myelin sheat protein PLP (proteolipid protein) in SJL/J mice fails to induce sufficient tolerance and results in increased susceptibility to experimental autoimmune encephalomyelitis (EAE), the mouse model for multiple sclerosis (Klein et al., 2000). In addition, low expression of AIRE-dependent insulin transcripts in the thymus is associated with an increased risk of developing type 1 diabetes (Vafiadis et al., 1997, Fan et al., 2009). Since only 60% of known TRAs are regulated by AIRE and Fezf2, it is plausible that additional transcriptional regulators involved in the expression of TRAs are not yet identified (Takaba and Takayanagi, 2017). Dendritic cells are an additional source of antigen-presentation cells for negative selection of thymocytes. Thymus resident conventional DCs (cDCs) cross-present TRAs expressed by mTECs in close proximity (Gallegos and Bevan, 2004, Koble and Kyewski, 2009). A number of mechanisms for this unidirectional transfer are suggested, such as antigen-uptake upon clearance of apoptotic mTEC, and intercellular material transfer by exosomes or membrane exchange ("nibbling") (Klein et al., 2011, Skogberg et al., 2015). In addition, migratory cDCs and plasmacytoid DCs (pDCs) collect peripheral self-antigen and re-enter the

thymus, extending the repertoire of self-antigens, and contributing to the negative selection of self-reactive thymocytes (Villadangos and Young, 2008, Bonasio et al., 2006).

Engagement of TCRs with high avidity for MHC:peptide complexes promotes activation-induced apoptosis. In mice, activation-induced apoptosis during negative selection is mediated by Bim, a pro-apoptotic molecule downstream of TCR-signaling that promotes the mitochondrial apoptosis cascade (Hojo et al., 2019). Solely intermediate signaling via the TCR enables the thymocytes to survive. Activation-induced signaling by intermediate binding avidity to self-antigens is antagonized by GC signaling, resulting in the survival of cells (Vacchio et al., 1999, Stephens et al., 2003, Zacharchuk et al., 1990) (Figure ***5***). GCs are endogenously produced in the thymus, but it remains controversial whether they are actively produced by thymic epithelial cells or converted from inert GC metabolites (Vacchio et al., 1994, Lechner et al., 2000, Rocamora-Reverte et al., 2017). Reduced GC signaling shifts the threshold between positive and negative selection towards the survival of thymocytes with lower TCR avidity, resulting in the survival of cells which would normally die by neglect, and apoptosis of thymocytes with upper intermediate binding avidity to self-antigens (Mittelstadt et al., 2012, Tolosa et al., 1998). A minor fraction of cells with high self-affinity for TRA develops into regulatory T cells (Treg) instead of being eliminated (Figure ***5***). Treg cells play an important role in the control of autoimmunity and are described in more detail in chapter 2.2.5.

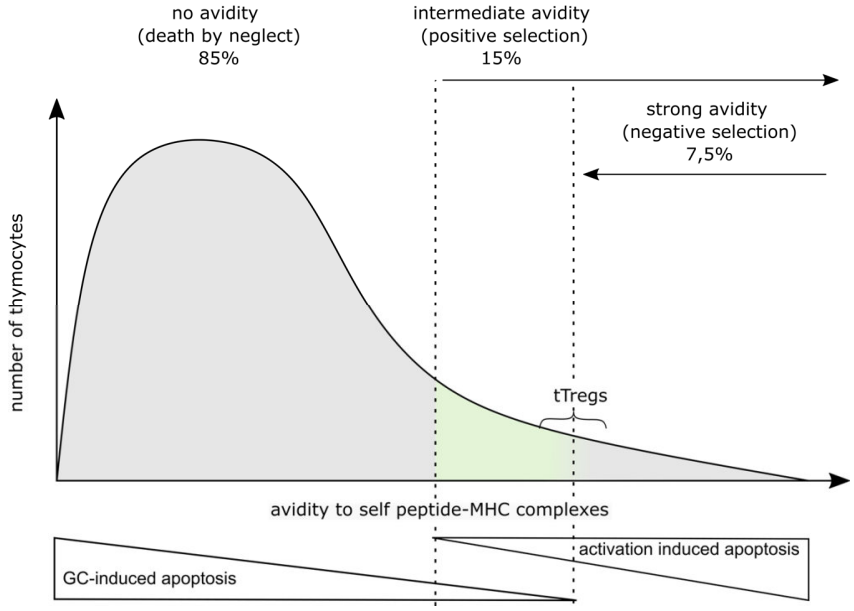

Figure 5. Selection processes in the thymus result in apoptosis of the majority of originally generated thymocytes. Thymocytes expressing TCRs with low avidity to MHC:peptide complexes die by neglect (left), and cells binding with high TCR avidity to self-peptide-MHC complexes are negatively selected to prevent autoimmunity (right). Only cells expressing TCRs with intermediate binding avidity for MHC:peptide complexes receive antagonizing signals from GR- and TCR-activation, promoting survival (middle). Thymic regulatory T cells (tTregs) present intermediate to high binding avidity corresponding to the margin of positive and negative selection. Adapted from (McDonald et al., 2015).

1.2.4 Single-positive thymocytes leave the thymus to populate the periphery

The final maturation steps of the surviving thymocytes relies on the FOXO1-regulated expression of Krüppel-like factor 2 (KLF2) that increases the transcription of sphyngosin-1-phospate receptor (S1PR), CD62L, CCR7 and interleukin (IL-) 7R (Gubbels Bupp et al., 2009, Carlson et al., 2006, Cyster and Schwab, 2012). Expression of S1PR on mature thymocytes is essential for the migration along a gradient of S1P between lymphoid tissue (low) and blood (high) to exit the thymus. CD69 forms a complex with S1P that leads to its internalization and degradation and impedes the release of T cells, requiring downregulation of CD69 for proper T cell egress (Carlson et al., 2006, Shiow et al., 2006). In parallel, upregulation of the homing receptors CCR7 and CD62L are required for the transition of T cells to secondary lymphoid organs. In addition, IL-7 signaling through the IL-7Rα is essential for promoting T cell survival and proliferation. IL-7 is produced by thymic and peripheral lymphoid stromal cells at a steady rate, and deprivation of IL-7 leads to T cell death within a few days (Fry and Mackall, 2005, Mazzucchelli and Durum, 2007). Thus, the available amount of IL-7 limits the size of the T cell pool. Lymphopenia leads to increased amounts of available IL-7, promoting homeostatic proliferation until an equilibrium is established, and vice versa, excessive T cell proliferation results in a lack of IL-7 and reduction of the T cell pool (Fry and Mackall, 2005). T cells which have recently migrated from the thymus are termed recent thymic emigrants (RTE). RTEs proliferate strongly in the presence of IL-7 (Hassan and Reen, 2001). In the absence of inflammation, they are prone to tolerance, with defects in proliferation, diminished cytokine production, and elevated expression of anergy-associated genes (Hendricks and Fink, 2011, Friesen et al., 2016). In addition, RTEs show a preference for trafficking to the liver and the gut, where they encounter many commensal-, food- and self-derived antigens, which are not efficiently displayed in the thymus (Staton et al., 2006). Localization in antigen-rich tissues, together with their tolerance-prone character, enables the post-thymic tolerance induction of RTEs to TRAs (Friesen et al., 2016). During inflammation, however, antigen-exposed RTEs convert into competent effector cells (Friesen et al., 2016). *S*timulation of RTEs results in a biased response towards the T_H2 type cytokines and promote allergic airway inflammation (Hendricks and Fink, 2011).

1.2.5 Cytokine environment mediates CD4 T-helper cell differentiation

Naive CD4 cells are activated upon recognition of their cognate antigen presented by DCs in the lymph nodes, and differentiate into different subsets depending on cytokine signals that they receive (Sallusto, 2016). Th1 cells develop in the presence of IL-12 and produce IFN (interferon)-γ to activate macrophages, promoting cell-mediated immunity to kill intracellular pathogens. IL-4 mediates the development of Th2 cells, which in turn produce IL-5 and IL-13 to attract eosinophils and mast cells in order to control parasitic infections. Th17 cells arise in the presence of IL-6, IL-1β and transforming growth factor (TGF)-beta (β), and produce IL-17 in response to fungi and extracellular bacteria. In addition to their function in controlling pathogens, Th1 and Th17 cells are major contributors to the development of autoimmunity, while Th2 cells are involved in allergic diseases. In addition, other types of helper subsets have been described, such as Th1*, Th22 and T follicular helper (Tfh) cells. Th1*, or unconventional Th1 cells, express chemokine receptors and transcription factors characteristic of Th17 cells, but do not produce IL-17 (Acosta-Rodriguez

et al., 2007). Instead, they secrete IFNγ in response to IL-12 and IL-23, and play a protective role in the response against *Mycobacterium tuberculosis* infections, where IFNγ production was found to be almost exclusively produced by Th1* cells (Acosta-Rodriguez et al., 2007, Zielinski et al., 2011, Martinez-Barricarte et al., 2018). Th22 cells are important for the regeneration of epithelial cells at mucosal barriers, and induce expression of antimicrobial peptides. They are involved in the pathogenesis of psoriasis and dermatitis. Tfh cells secrete IL-21 and IL-4 and are provide B cell help for the production of high affinity antibodies (Crotty, 2015). Finally, in the presence of TGFβ and retinoic acid, and in the absence of an infection (IL-6), Th cells upregulate FOXP3 and develop into peripherally-induced T regulatory cells (pTregs). Differentiated CD4+ cells retain the option to convert into other Th lineages, a feature termed T cell plasticity. Plasticity occurs in all T helper subsets, but is best studied for Th17 cells (Geginat et al., 2014). Upon chronic inflammation, Th17 cells acquire a Th1 phenotype, and can also convert into Treg cells (Harbour et al., 2015, Gagliani et al., 2015).

1.2.6 Peripheral tolerance controls autoreactive T cells and excessive immune activation

Negative selection in the thymus is referred to as central tolerance. A few autoreactive cells escape the stringent thymic selection processes and find their way to the periphery, where they may encounter and react to self-antigens. Different mechanisms in the periphery further ensure the control of autoreactive cells and excessive immune responses. Tregs, either naturally generated in the thymus (tTregs) or induced in the periphery by conversion of conventional CD4 cells (pTregs), are essential for peripheral tolerance. The thymic origin of human Treg cells is not completely understood yet. In mice, T cells with relatively high affinity for MHCII/self-peptide complexes are preferentially driven to the Treg lineage upon upregulation of FOXP3, the master regulator of Tregs. Unique structures in the human thymus, called Hassall's corpuscles, are suggested niches for human Treg development by providing thymic stromal lymphopoietin (TSLP), which is necessary for thymic $CD11c^+$ dendritic cells to induce FoxP3 expression in immature CD4 thymocytes (Watanabe et al., 2005). In addition to thymic Treg generation, peripheral conversion of CD4 cells to pTregs complements the natural pool of Treg cells. tTregs contribute to the prevention of autoimmunity, while pTregs control inflammatory responses and excessive immune reactivity upon pathogen clearance (LeGuern and Germana, 2019). Generation of pTregs in the periphery is stimulated by the production of retinoic acid and TGFβ (Chen et al., 2003).

Tregs harbor different mechanisms for the suppression of immune responses, namely sequestration of IL-2, secretion of immunosuppressive cytokines (IL-10, IL-35, and TGFβ), toleration of APCs by down modulation of CD80/CD86, and production of adenosine. Adenosine production is mediated by the enzymatic activity of ectonucleotidases CD39 and CD73, which degrade pro-inflammatory ATP into immune suppressive adenosine (Deaglio et al., 2007).

Other mechanisms of peripheral tolerance include immune ignorance, which refers to the limited entry and presence of lymphocytes in immune privileged organs, achieved by physical barriers (blood-tissue-barrier,

BTB). These BTBs include tight-junctions between epithelial cells, Fas-ligand expression in tissues leading to Fas-induced apoptosis of T cells, and a low-level release of tissue-specific antigens in conjunction with TGF-ß, which promote the generation of Tregs and thus increase tolerance to tissue antigens. In addition, recognition of self-peptides displayed on APCs in the absence of co-stimulatory molecules leads to sustained cell unresponsiveness (anergy) or clonal deletion.

1.3 Ontogeny of the immune system

1.3.1 First emergence of immune cells happens during early pregnancy

Immune system development in humans begins early during pregnancy. HSPCs arise from the yolk sac, the fetal liver or the bone marrow, depending on the developmental stage (Migliaccio et al., 1986, Christensen, 1989). The first myeloid cells to emerge are tissue resident macrophages in the central nervous system, present already at 4 weeks gestational age (GA). In contrast, neutrophils appear in blood as late as at 16 weeks GA, and mature neutrophils are scarce in fetal blood until shortly before birth, when they rapidly increase (Christensen, 1989). Innate-like lymphocytes such as fetal NK cells and ILCs were found in fetal blood at 9 weeks GA (Figure 6).T- and B cell maturation starts around 8 weeks of gestation and seeding of the periphery occurs at the end of the first trimester (Lobach et al., 1985). Diversity of the fetal T and B cell receptor repertoire increases during pregnancy with T lymphopoiesis lagging behind B cell development (Rechavi et al., 2015). The diversity of the Ig heavy chain (IGH) repertoire in B cells at 12 weeks GA is comparable to healthy control infants, while the T cell receptor β (TRB) repertoire slowly diversifies until it reaches an infant-like distribution at 20 weeks GA.

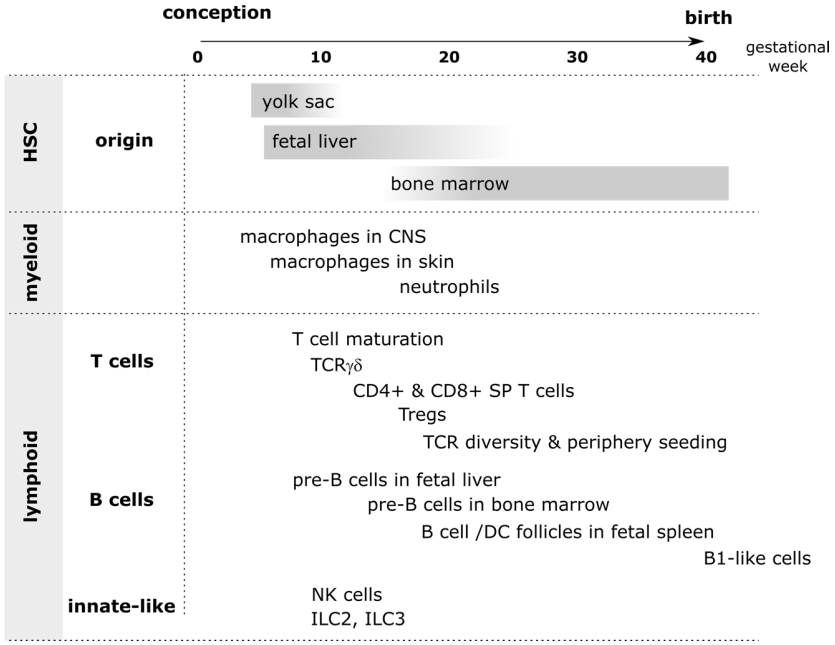

Figure 6. Ontogeny of the human immune system in the prenatal period. Origin of HSC (top) and chronological order of myeloid (middle) and lymphoid (bottom) immune cell appearance during embryonic and fetal development.

1.3.2 Distinct waves of progenitor cells populate the immune system

The fetal T cell compartment strongly differs from the one observed in adults. The source of progenitor cells transitions from fetal liver to bone marrow in mid gestation, correlating to changes observed in some human T cell populations, such as TCR$\gamma\delta$ subsets and Tregs. Changes in the frequency of cell populations during fetal development, and phenotypic and functional differences between fetal and adult cells, led to the hypothesis that progenitor cells enter the thymus in distinct waves to produce T cells adapted to developmental requirements (Herzenberg and Herzenberg, 1989). This type of immune cell development had already been postulated for mice and birds (Coltey et al., 1989, Carding and Egan, 2002). In mice, developmental waves were reported for TCR$\gamma\delta$ subsets, which are generated during different fetal periods, with each subset preferably homing to distinct tissue sites, such as skin, lung or lymph nodes (Prinz et al., 2013). Evidence for a similar pattern during TCR$\gamma\delta$ development in human is missing yet, but fluctuations of TCR$\gamma\delta$ subsets during development, with high frequency of TCRVδ2 cells during the second trimester of pregnancy, a decrease towards birth and a rapid increase throughout the first months of life, hint to different periods for the development of cells (Vermijlen and Prinz, 2014). Recently, TCR repertoire analysis in peripheral blood and thymocytes, strongly indicate different origins of fetal and postnatal Vδ2Vγ9 cells (Papadopoulou et al., 2019). In addition, Vδ2 cells are present only in low numbers in the pediatric thymus, and the VδJ-segment usage of Vδ2 cells strongly differs between cord blood and adult peripheral blood. This led to the hypothesis that fetal Vδ2 cells arise from a different wave of progenitors than postnatally generated cells (Willcox et al., 2018).

Treg cells are also abundantly present during mid-gestation, decrease toward end of pregnancy, and temporally increase shortly after birth, similar to the pattern of TCRVδ2 cells (Kim et al., 2012, Hayakawa et al., 2017). Fetal T cells preferentially convert into Tregs upon stimulation, particularly in a TGFβ rich environment such as the fetal lymph nodes (Mold et al., 2010, Mold et al., 2008). Gene expression analysis showed a greater similarity of fetal naïve cells to adult Treg cells than to adult naïve cells, indicating fetal naïve cells being predetermined to the Treg fate (Mold et al., 2010). Loss-of-function mutations in *FoxP3* results in fatal autoimmunity in humans and mice, while postnatal Treg depletion causes milder symptoms and slower disease progression (Kim et al., 2007, Hori et al., 2003, Fontenot et al., 2003). Altogether, fetal Treg cells feature characteristics which are distinct from adult Treg cells.

1.3.3 Perinatal tolerance mechanisms

Transition from the sterile *in utero* setting to the environment poses another great challenge for the fetal and neonatal immune system (Figure 7). In the past, it was assumed that the neonatal immune system is dysfunctional or deficient, however, the finding of active immune regulation disproved the hypothesis of the immature, non-functional immune compartments. Now it has become clear that neonates feature mechanisms to provide a tolerogenic state of the immune system that prevents overshooting immune responses upon encounter with commensal bacteria, food antigens, and pathogens.

$CD71^+CD235a^+$ erythroid progenitor cells are abundant in cord blood and virtually absent in adult blood. These cells efficiently prevent immune cell activation in murine tissues, such as intestine, spleen, and lung, which are rapidly colonized postpartum with commensal microbes (Elahi et al., 2013). Specific depletion of these immunosuppressive cells restored adult-like immune functions in human cord blood. The suppressive properties of $CD71^+$ $CD235a^+$ cells required arginase-2 which was recently also described to be the driving force in dampening the immune function of fetal DCs by preventing TNFα production (McGovern et al., 2017). Another study, however, reported no effect of $CD71^+CD235a^+$ cell transfer or depletion on neonatal survival upon endotoxin challenge or polymicrobial sepsis in mice, questioning their role in neonatal immunosuppression and their clinical relevance (Wynn et al., 2015).

A bias toward Th2 responses contributes to the tolerogenic immune status in neonates. Neonatal T cells show diminished production of Th1 cytokines and a bias toward Th2 responses. Hypomethylation of the loci for Th2 cytokines in neonatal CD4 cells favors gene transcription, while adult T cells feature methylated loci for Th2 cytokines (Webster et al., 2007). High expression of an unglycosylated isoform of IL-4 in resting and activated cord blood cells, and elevated GATA3 levels, promote the rapid development into Th2 cells and represses IFNγ secretion by Th1 cells (Hebel et al., 2014, Zaghouani et al., 2009). In mice, re-stimulation with antigen results in apoptosis of neonatal Th1 and promotion of Th2 survival, mediated by the heteroreceptor IL-4Rα and IL-13Rα (Li et al., 2004). Besides the beneficial contribution of the Th2 bias to the tolerogenic neonatal status, the reduced Th1 response also renders the neonate susceptible to infections. In addition, several studies reported that increased ratios of Th1:Th2 or Treg:Th2 increase the risk for the development of atopic dermatitis, atopic allergy and asthma (Baek et al., 2014). Of note, the dominance of the Th2 responses can be reshaped *in vivo* depending on environmental conditions. Vaccination against Bacillus Calmette-Guerin results in strong and long-lasting Th1 reactions in newborns and infants aged two to four months (Marchant et al., 1999).

During pregnancy, abundant Tregs prevent the rejection of the fetus and enable the generation of tolerance to antigens not encountered in the thymus, such as maternal antigens or antigens originating from food, amniotic fluid or microbes (Aagaard et al., 2014, Campbell et al., 2015, Underwood et al., 2005). The frequency of Tregs decreases from mid-pregnancy towards birth, resulting in comparable frequencies in cord blood and adult peripheral blood (Kim et al., 2012, Pagel et al., 2016). In contrast, Treg frequency in mucosal tissues is constantly enriched during the first two years of life (30-40% of T cells) compared to adult tissues (1-10%), stressing their role in tolerance induction and the control of immune responses at sites with high antigen exposure (Thome et al., 2016). During the first week of life, Treg frequency and absolute counts in peripheral blood are transiently increased by twofold compared to birth (Hayakawa et al., 2017). This transient rise in Treg, in combination with their enhanced suppressive function, are suggested to ensure adaptation to environmental changes after birth (Mold et al., 2008, Hayakawa et al., 2017).

Additional regulatory mechanisms present in the perinatal period are summarized in Figure 7. Although neonatal T cells hardly produce any cytokines, IL-8 is abundantly expressed by naïve ($CD45RA^+$) cord blood

T cells of term and preterm babies upon stimulation. IL-8 efficiently activates neutrophils and IFNγ production by γδ T cells (Gibbons et al., 2014).

In conclusion, the predominance of tolerance during the perinatal period enables a sustained pregnancy, the development and population of immune compartments, and a transition from the sterile *in utero* setting to the outside environment without excessive inflammation. The disadvantage of higher susceptibility to infections in early life is not due to an immature immune system, but rather the result of precise regulation mechanisms which are not yet completely understood (Zhang et al., 2017, Jennewein et al., 2017).

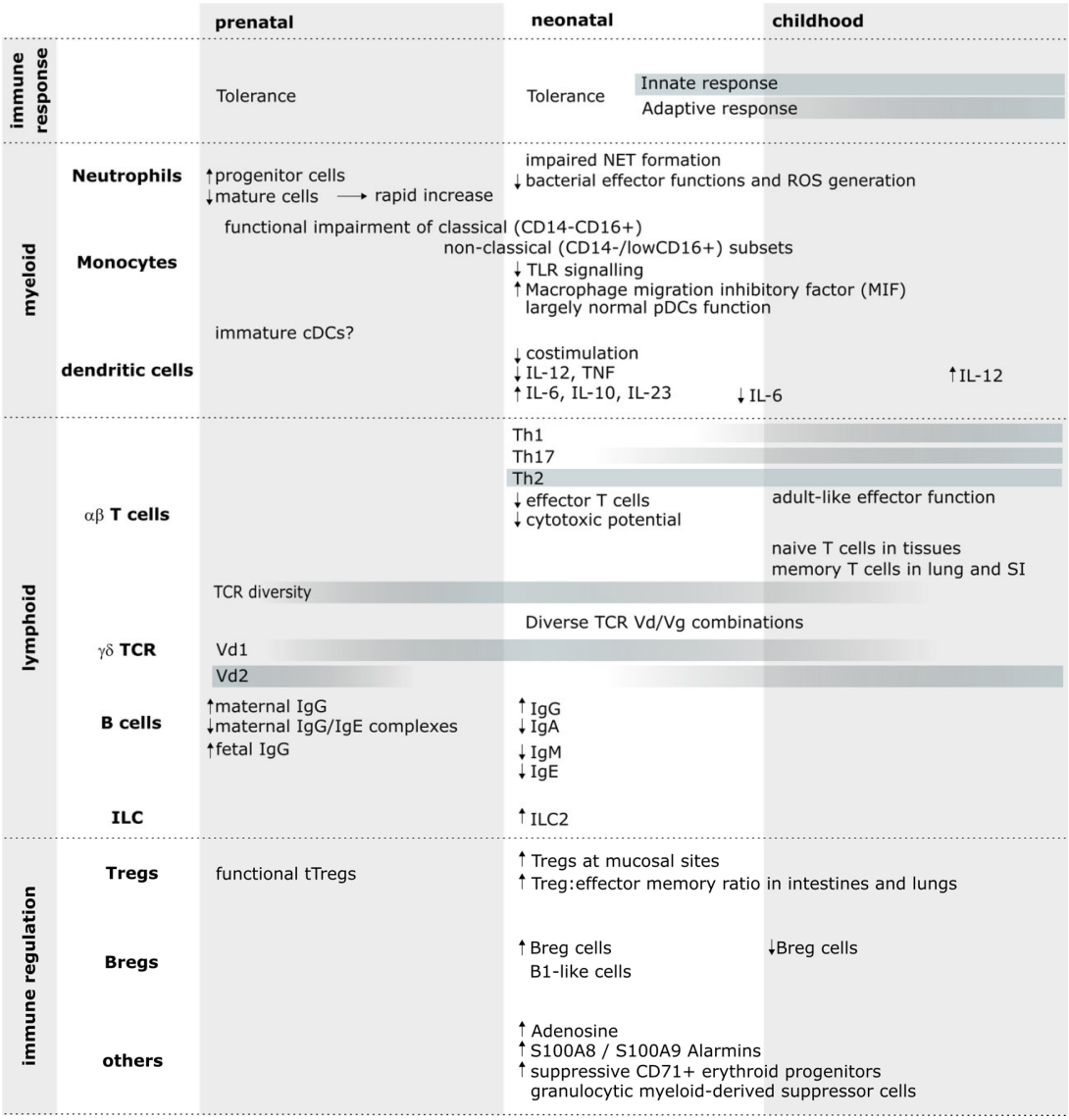

Figure 7. Changes of the immune system from the prenatal period to childhood. Progression of the myeloid and lymphoid compartments in the perinatal period. Mechanisms for achieving tolerance are summarized at the bottom. NET – neutrophil extracellular trap; ROS – reactive oxygen species; TLR – toll-like receptor; MIF – macrophage inhibitory factor; SI – small intestines.

1.3.4 Profound age-related changes of the immune system lead to increased susceptibility to infections and autoimmunity in the elderly

At birth, most cells are naïve, the diversity of the adaptive immune system is limited, and tolerance predominates. Passive protection by maternal immunoglobulins, transferred *in utero* and through breastfeeding, fades away within weeks or months after birth (Baxter, 2007). As a consequence, neonates and infants are susceptible to infections. These infections - even if subclinical - are crucial for the generation of memory T cells for life-long protection (Farber et al., 2014, Hayward et al., 2014). In addition, Treg cells decrease during childhood and helper T cell subsets (Th1, Th2 and Th17) increase, diversifying the T cell response (Shearer et al., 2003). The immune system in young adults efficiently protects and responds against pathogens, leading to fewer and milder infections. The transformation of the immune system continues beyond adulthood with profound changes, a process known as immune senescence or immune aging, which has major implications for health and survival (Weiskopf et al., 2009).

Infections are rated among the top global causes of death in elderly individuals (Ritchie, 2019). Innate immune responses decline with age, even though hematopoiesis becomes skewed towards the myeloid lineage and causes an increase in innate cell numbers (Tang et al., 2013, Wang et al., 2011). Similar to neonates, macrophages and neutrophils in the elderly show diminished phagocytic function and production of reactive oxygen species (ROS) (Simon et al., 2015). In addition, neutrophils exhibit impaired neutrophil extracellular trap (NET) formation, macrophages show reduced TLR signaling, and serum levels of macrophage inhibitory factor (MIF) are increased in both cord blood and peripheral blood of the elderly (Ventura et al., 2017, Olivieri et al., 2013). The impaired function results in an inefficient recognition and clearance of misfolded proteins, apoptotic cells and cell debris in the elderly (Fulop et al., 1985, Weiskopf et al., 2009). The accumulation of these components promotes the chronic production of pro-inflammatory cytokines (IL-1ß, IL-6, IL-18 and TNFα) leading to continued low-grade inflammation (inflammaging) (Franceschi et al., 2007, Franceschi et al., 2018). The impaired identification and disposal of faulty cells or peptides, and the constant low-grade pro-inflammatory cytokine signaling foster the susceptibility to infections and the development of cancer and autoimmunity. However, the greatest effects of aging are detected in the T cell compartment (Miller, 1996, Cambier, 2005). Thymic T cell output declines gradually starting in puberty, and homeostatic proliferation maintains the existing T cell pool, but causing a reduction in the diversity (Nikolich-Zugich, 2008, Nikolich-Zugich, 2014, Goronzy et al., 2015, Farber et al., 2014). Age-related loss of the co-stimulatory molecule CD28 leads to incomplete T cell activation, resistance to apoptosis, and decreased proliferative capacity (Saurwein-Teissl et al., 2002, Almanzar et al., 2005, Sansoni et al., 2008, Moro-Garcia et al., 2013). Enhanced co-production of cytokines (polyfunctionality) in CD4 and CD8 T cells upon stimulation is associated with age, and contributes to the pro-inflammatory state of the immune system in elderly (Van Epps et al., 2014). The quality of memory cells decreases with age. Memory cells produced during young age have long-term protective potential, in contrast to memory cells generated at older age (Hammarlund et al., 2003, Hammarlund et al., 2005, Haynes et al., 2003, Weinberger et al., 2008, Valkenburg et al., 2012). One quarter of age-related changes arise from intrinsic changes in transcription and genetic regulation, therefore the majority of alterations result from lifestyle and

environmental factors, such as nutrition, medical history, or physical activity, which either directly influence immune cells or affect their epigenetic regulation (Ponnappan and Ponnappan, 2011, Chen et al., 2013, de la Fuente, 2013).

The entity of changes in the elderly, with latent pro-inflammatory signals, reduced adaptive diversity and impaired function of cells, contributes to the increased susceptibility to and severity of infectious diseases such as influenza, pneumonia, sepsis and an increased risk for cancer (Chattopadhyay and Al-Zahawi, 1983, Derhovanessian et al., 2008, Gorse et al., 1984, LaCroix et al., 1989, Sprenger et al., 1993). Excessive homeostatic proliferation to maintain the peripheral T cell pool with low thymic output, decreased numbers of regulatory T cells, and impaired clearance of apoptotic cells by innate cells, might result in incomplete tolerance to self-antigens and increase the risk of autoimmunity (Goronzy and Weyand, 2003).

1.4 Genetic defects and early life perturbations of the immune system affecting the thymus

The thymus size increases during childhood and is greatest in puberty, decreasing thereafter and turning into fatty tissue (Steinmann, 1986). Importantly, thymic size in relation to body weight is maximal during the perinatal period, coinciding with the highest rate of thymic output. Interventions affecting the thymus size or function in the fragile perinatal window can thus have long-lasting effects. The premature reduction of the thymic tissue is a consequence of psychological stress, infections, genetic diseases, or surgical interventions early in life, and has been associated with augmented incidence of autoimmunity, cancer or opportunistic infections (van Baarlen et al., 1988, Gelfand et al., 1972, Savino, 2006, Cromi et al., 2009, Abe et al., 2015, Taub and Longo, 2005, Chinn et al., 2012, Gudmundsdottir et al., 2018).

1.4.1 Prenatal steroid treatment

In 1969 Liggins discovered that prenatal treatment with synthetic steroids (glucocorticoids) improved the breathing capability of lambs born preterm (Liggins, 1969). This breathing advantage is a consequence of rapid maturation of the fetal lungs induced by the production of surfactant proteins. Surfactant reduces the surface tension of the alveoli and facilitates their inflation and the exchange of oxygen. Naturally occurring surfactant is detectable in low amounts in the amniotic fluid starting the 24^{th} week of gestation and increases as pregnancy progresses, in parallel to the rise in endogenous glucocorticoids (Taeusch, 2005). Until the 34^{th} week of gestation, however, the fetal lung is functionally immature, and babies born earlier are at high risk for respiratory complications. The first clinical trial in humans using glucocorticoids to treat respiratory distress syndrome (RDS) was launched in 1972, and pioneered the prophylactic use of prenatal steroids for women at risk of preterm birth between gestational weeks 24^{th} and 34^{th} (Roberts and Dalziel, 2006, Crowley et al., 1990, Liggins and Howie, 1972). Currently, prenatal steroids are routinely administered to 8-10% of pregnant women in industrialized countries. Three quarter of the pregnant women who receive steroids remained pregnant beyond the effective window of seven days, and 50% of treated women deliver near term, thus unnecessarily exposing the child to steroids (Razaz et al., 2015).

In contrast to endogenous glucocorticoids (GC), Betamethasone or Dexamethasone, the synthetic GC of choice for treating mothers at risk of preterm delivery, are poor substrates for the inactivating enzyme 11ß-Hydroxysteroid dehydrogenase type 2 (HSD2) in the placenta. In addition, they feature reduced binding to the plasma proteins albumin and corticosteroid binding globulin (CBG), which improves their accessibility for the GR (Solano et al., 2016). Thus, application of exogenous Betamethasone or Dexamethasone to the pregnant mother easily reaches the fetus. GC bind to the GR, which is ubiquitously expressed in almost all cell types. Prenatal steroid treatment therefore has many off-targets in the neonate in addition to the desired effect on lung maturation. Inactive GR is located in the cytoplasm and bound to chaperones which are released upon engagement of the GR with steroids. Upon binding, the GR is translocated to the nucleus where it either homodimerizes and binds directly as transcription factor to the glucocorticoid response elements (GRE) in the DNA, or interacts as a monomer or a dimer with other transcription factors activating or repressing gene expression (Oakley and Cidlowski, 2013).

Beyond the undisputed beneficial effects on lung maturation, surprisingly little is known about possible side effects of prenatal steroid treatment that affect the child's physical and cognitive development. Recent reports suggest a reduced birth size, long-lasting alterations of the hypothalamic-pituary-adrenal (HPA) axis and response to stress of children and the development and growth of the heart, brain and kidney (Uno et al., 1990, Zhang et al., 2010, Rodriguez et al., 2019, Alexander et al., 2016, Alexander et al., 2012). Importantly, steroids are potent immune-suppressive drugs, and epidemiological studies show that excessive prenatal exposure to glucocorticoids is associated with higher risk of infections and the development of asthma or type 1 diabetes (Chen et al., 2017, Greene et al., 2013, Solano et al., 2016).

Steroids have a dramatic effect on thymocytes, causing apoptosis especially in immature thymocytes (Wyllie, 1980). In the thymus, endogenously produced steroids antagonize the TCR signaling in thymocytes, expressing TCRs with intermediate avidity to MHC:antigen complexes, rescuing thymocytes from negative selection (Vacchio et al., 1999, Stephens et al., 2003, Zacharchuk et al., 1990). A reduction in the GC signaling in murine thymocytes shifts the selection threshold for positive and negative selection toward less TCR avidity (Ashwell et al., 2000, Tolosa et al., 1998, Lu et al., 2000). In contrast, higher levels of GCs might lead to the survival of cells that would normally undergo negative selection, and promote the escape of autoreactive cells from negative selection. Findings from mouse models show that prenatal steroid treatment results in apoptosis of developing thymocytes, a decrease in thymic size and alterations of the peripheral TCR repertoire (Diepenbruck et al., 2013, Gieras et al., 2017). In humans, X-Ray measurements of the chest from prenatally treated children showed an absence of the thymic shadow that lasted several weeks after birth (Michie et al., 1998).

1.4.2 Early childhood thymectomy

One in one hundred children is born with congenital heart defects and 25% of them require surgical correction during the first year of life (Hoffman and Kaplan, 2002, Reller et al., 2008, Bauer, CDC, 2018). The thymus is located in the upper chest and its size relative to body size is greatest during infancy, preventing access to the heart. Thus, complete or partial removal of the thymus (thymectomy) is a common

practice during infant heart surgery. Considering the important role of the thymus in the generation of the T cell repertoire, in particular at the beginning of life, thymectomy in infancy might lead to changes in immunity later in life. Alterations of the peripheral T cell repertoire in thymectomized children encompass lower numbers of T cells and recent thymic emigrants, a less heterogeneous TCR repertoire and a delayed humoral immune response to tick-borne encephalitis vaccine (Halnon et al., 2005, Torfadottir et al., 2006, Madhok et al., 2005, Prelog et al., 2008, Gudmundsdottir et al., 2017, Gudmundsdottir et al., 2016, Zlamy et al., 2010). The reduction of the thymus niche – and thus the decrease of the thymus output – leads to compensatory peripheral homeostatic proliferation of naïve T cells in order to maintain the peripheral T cell repertoire. This results in a less diverse T cell repertoire and premature aging of the T cell compartment, promoting susceptibility to disease (Gudmundsdottir et al., 2016, Sauce et al., 2009, Zlamy and Prelog, 2009, Appay et al., 2010). A recent study showed an increased risk for the development of autoimmune diseases, cancer and infectious diseases in children thymectomized before the age of five years (Gudmundsdottir et al., 2018). Other studies, however, reported no differences in the prevalence of disease, but a longer recovery period and more hospitalizations after infectious diseases in thymectomized children compared to controls (Mancebo et al., 2008, Kurobe et al., 2013, Cao et al., 2011). Compared to age-matched controls, T cells from thymectomized children showed a significantly different phenotype, which corresponds to the phenotype of murine extrathymically produced T cells (Torfadottir et al., 2006). Extrathymic generation of T cells in mice takes place in the liver and the intestines, while the origin of human extrathymic T cells is not yet clear (Abo, 2001, Oida et al., 2000, Sato et al., 1995, Guy-Grand et al., 2003). The generation of T cells at extrathymic sites is less efficient for CD4 cells than for CD8 cells resulting in a bias towards CD8 cells in thymectomized children (Torfadottir et al., 2006). Apart from epidemiological studies and the investigation of the main T cell subsets in the blood, there are no studies on the detailed immune cell composition of the blood of children who were thymectomized in infancy.

The overall changes of immune cells were greater after a complete thymectomy than after a partial thymectomy (Kurobe et al., 2013). In addition, some individuals with partial thymectomy showed a regeneration of the thymus more than five years after surgery which resulted in a restoration of the naive and the effector T cell pool (van den Broek et al., 2016, van Gent et al., 2011). These findings emphasize the importance of preserving as much thymus tissue as possible to improve immune function in young children after thymectomy.

1.4.3 DiGeorge Syndrome

A typical feature of patients with DiGeorge Syndrome (DGS) is aplasia or of the thymus. Most DGS patients carry a microdeletion of chromosome 22q11.2, a common microdeletion in humans occurring in 1:3000 live births (Devriendt et al., 1998, McDonald-McGinn et al., 2015). The deletion affects 2.4 - 3 Mb, which affect about 50 genes and 7 microRNAs (McDonald-McGinn et al., 2015). It mainly occurs *de novo*, with approximately 10% being inherited from an affected parent. TBX1, a key transcription factor for the development of the fetus' body structures and for the thymic architecture, is missing in patients with 22q11.2 microdeletion. This might explain many of the clinical features in DiGeorge patients such as the absence or

atrophy of the thymus, a mild to moderate immunodeficiency, cardiac, renal and facial anomalies, hypoparathyroidism, skeletal defects and developmental delay (McDonald-McGinn et al., 2015, Kobrynski and Sullivan, 2007, Sullivan, 2019). There is a wide phenotypic heterogeneity and the etiologies range from teratogens, medications, metabolic conditions (such as maternal diabetes), to other chromosomal anomalies (Sullivan, 2019). The T cell compartment in patients with DiGeorge syndrome is affected to varying degrees, ranging from a phenotype which is indistinguishable from controls to severe immune deficiencies. Common characteristics are low CD3 cell counts, reduced numbers of recent thymic emigrants, and naïve CD4 and CD8 cells, an oligoclonal T cell pool and low numbers of regulatory T cells (Piliero et al., 2004, McLean-Tooke et al., 2011). The great variability of symptoms complicates diagnosis, thus many cases might not be diagnosed or be mixed with other disorders. DiGeorge patients have an increased risk for the development of allergy, atopic disease and autoimmunity early in life, stressing the demand for improved diagnosis (Jawad et al., 2001, Morsheimer et al., 2017). The changes in the immune system of patients with DGS are similar to those of thymectomized children, underlining the importance of an intact thymus for the prevention of allergy, autoimmunity and atopic diseases.

1.5 Aims of this thesis

The thymus provides the microenvironment for the generation of an extensive repertoire of functional T cells and for the elimination of autoreactive cells. The size of the thymus in relation to body weight is maximal around birth, marking a period of high activity for the generation of the naïve T cell pool and establishment of repertoire diversity in humans. Premature thymic atrophy as a consequence of stress (i.e. perinatal infection), genetic disease or surgical interventions is associated with higher risk for autoimmunity, cancer or opportunistic infections (van Baarlen et al., 1988, Gelfand et al., 1972, Savino, 2006, Cromi et al., 2009, Abe et al., 2015, Taub and Longo, 2005, Chinn et al., 2012, Caissutti et al., 2018, Gudmundsdottir et al., 2018).

Prenatal steroid therapy and early life thymectomy represent common perinatal interventions influencing the thymus. Prenatal steroids are routinely administrated to pregnant women at risk of preterm delivery to promote lung maturation. Previous work from our lab showed that transient thymic athrophy and dramatic death of developing T cells have been shown in a mouse model upon prenatal steroid treatment (Diepenbruck et al., 2013). Furthermore, approximately 0.25% of children every year are born with congenital heart defects. Many of these children undergo corrective heart surgery early in life, which involves complete or partial removal of the thymus gland (thymectomy) (Hoffman and Kaplan, 2002, Reller et al., 2008, Bauer, CDC, 2018). In contrast to these two extrinsic factors affecting the development or function of the thymus, DiGeorge syndrome, caused by a chromosomal microdeletion in chromosome 22, occurs in 1:3000 live births and results in apo- or hypoplasia of the thymus (McDonald-McGinn et al., 2015).

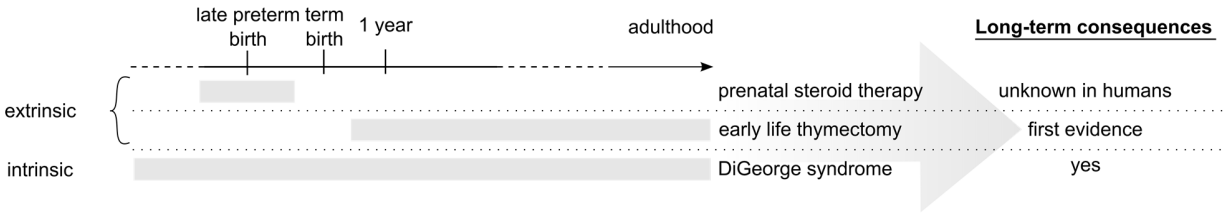

Figure 8. Extent and length of thymic reduction caused by prenatal steroid therapy, early life thymectomy or DiGeorge syndrome are different. Grey bars represent the period of thymic reduction.

The aims of this doctoral thesis are

I. To establish a flow cytometry-based method for detailed immune phenotyping of human blood.

II. To assess the effects of prenatal steroids on the thymus.

III. To examine the effects of thymic reduction caused by prenatal steroid treatment, early life thymectomy or DiGeorge syndrome on the “immune signature” of an individual, reflecting a detailed phenotypic and functional analysis of the immune cells in the blood, particularly the T cell compartment.

IV. To evaluate the combined effects of the conditions described above on the immune system in individual donors.

2. Material and Methods

2.1 Material

2.1.1 Human Blood samples

Sample	Source	Ethic approval
Peripheral blood from healthy adults	Volunteers	PV5139
Peripheral blood from children	Department of Obstetrics and Pediatrics, University medical center Hamburg-Eppendorf (UKE)	PV5482
	Department of Orthopedy, Children's Hospital Altona (AKK)	PV5482
	Department of Pediatric Cardiology, University Heart Center Hamburg (UHZ)	PV5482
Frozen peripheral mononuclear cells from children	Department of Pediatrics, Hannover Medical School (MHH)	
Cord blood	Department of Obstetrics and Pediatrics, University medical center Hamburg-Eppendorf (UKE)	PV5139
	Department of Neonatology and Pediatrics, University medical center Schleswig-Holstein (UKSH)	IRON AZ 15-304

2.1.2 Mouse strains

Strain	Source	Animal ethics
$GR^{fl/fl}$ Lck-Cre	Prof. Dr. Holger Reichardt (University Medical School, Göttingen)	N18/087; ORG884

2.1.3 Reagents and Solutions

Material	Company
Anti-CD28, clone 10F3, LEAF™ purified	BioLegend, San Diego (CA), USA
Anti-CD3, clone OKT3, LEAF™ purified	BioLegend, San Diego (CA), USA
Betamethasone	Sigma-Aldrich, St. Louis (MO),USA
Biocoll separation medium	Merck, Darmstadt, Germany
Brefeldin A Solution, 1000X	eBioscience, San Diego (CA), USA
CELL-DYN Emerald Calibrator/Controls	Abbott, Abbott Park (IL), USA
DEPC-H_20	Invitrogen, Carlsbad (CA), USA
Dithiothreitol 0.1 M	Invitrogen, Carlsbad (CA), USA
DNaseI	Qiagen, Hilden, Germany
dNTPs (dATP/dCTP/dGTP/dTTP; 100mM)	Invitrogen, Carlsbad (CA), USA
Dulbecco's Phophate Buffered Saline (PBS)	Thermo Fisher Scientific, Waltham (MA), USA
eBioscience™ 10x RBC Lysis Buffer	Invitrogen, Carlsbad (CA), USA
Ethanol ≥99,8%	Roth, Karlsruhe, Germany
Fetal bovine Serum	Biochrom, Berlin, Germany
First Strand Buffer 5x	Invitrogen, Carlsbad (CA), USA
GeneRuler 100 bp DNA Ladder	Thermo Fisher Scientific, Waltham (MA), USA
Human recombinant IL-2	Hoffmann-La Roche, Basel, Schweiz

Material	Company
Human recombinant IL-7	R&D Systems, Inc., Minneapolis (MN), USA
Ionomycin	Sigma-Aldrich, St. Louis (MO), USA
Live/Dead Dye AF750 succinimidyl ester	Thermo Fisher Scientific, Waltham (MA), USA
Live/Dead Dye PacificOrange Succinimidyl Ester	Invitrogen, Carlsbad (CA), USA
Maxima Probe/ROX qPCR Master Mix	Thermo Fisher Scientific, Waltham (MA), USA
M-MLV Reverse Transkriptase	Invitrogen, Carlsbad (CA), USA
Paraformaldehyde	Morphisto GmbH, Frankfurt, Germany
Phorbol-12-myristat-13-acetat	Sigma-Aldrich, St. Louis (MO), USA
Proteinkinase K	Macherey-Nagel, Düren, Germany
Random Primers	Invitrogen, Carlsbad (CA), USA
RNA*later*®	Sigma-Aldrich, St. Louis (MO), USA
RPMI 1640	Thermo Fisher Scientific, Waltham (MA), USA
RU-486 (Mifepriston)	Sigma-Aldrich, St. Louis (MO), USA
Sodium azide	Sigma-Aldrich, St. Louis (MO), USA
ß-mercaptoethanol, 50 mM	Invitrogen, Carlsbad (CA), USA
Streptomycin (10.000U/mL) / Penicillin (10.000U/mL)	Thermo Fisher Scientific, Waltham (MA), USA
Trypan blue solution, 0.4%	Sigma-Aldrich, St. Louis (MO), USA
Tuerck's solution	Sigma-Aldrich, St. Louis (MO), USA
X-Vivo 15 medium	Lonza, Basel, Switzerland

2.1.4 Media and Buffer

Buffer	Composition
Annexin buffer	H_20 with 1.4 M NaCl 25 mM $CaCl_2$ 0.1 M Hepes
FACS buffer	PBS with 0.1 % BSA 0.02 % NaN_3
Freezing medium I	RPMI with 10% FCS
Freezing medium II	RPMI with 40 % FCS (heat inactivated) 20 % DMSO
Full medium	RPMI with 10 % FCS (heat inactivated) 1 % penicillin/streptomycin 2 mM L-glutamine
Lysis buffer for DNA extraction	H_2O with 0.1 Mol/L Tris Base 5.0 mMol/L EDTA 0.2 Mol/L NaCl 0,2% SDS pH: 8.5

2.1.5 Kits

Kit	Company
IOTest® Beta Mark	Beckmann Coulter, Brea (CA), USA
LEGENDplex™ Human Th Panel (13-plex)	BioLegend, San Diego (CA), USA
QiaShredder Kit	Qiagen, Hilden, Germany
RNeasy Plus Mini Kit	Qiagen, Hilden, Germany

2.1.6 Human antibodies

Human antibodies were used in 13 multicolor flow cytometry panels as described in . The use of the antibodies listed below in the various panels is indicated as follows: 1) deep immunophenotyping approach (panels 1-9); 2) preterm cohort Lübeck (panel 13); 3) proliferation assay and exhaustion markers (panel 10-11) and 4) virtual memory cells (panel 12).

specificity	fluorochrome	clone	company	Panels
CCR10	APC	6588-5	BioLegend, San Diego (CA), USA	1, 2
CD10	BV711	HI10A	BioLegend, San Diego (CA), USA	1
CD10	PE-Dazzle	HI10A	Becton Dickinson, Franklin Lakes (NJ), USA	1
CD117 (c-kit)	APC	104D2	BioLegend, San Diego (CA), USA	1, 2
CD11c	PE-Dazzle	3.9	BioLegend, San Diego (CA), USA	1
CD122	PE	Mik-ß3	Becton Dickinson, Franklin Lakes (NJ), USA	4
CD123	PE-Cy7	6H6	BioLegend, San Diego (CA), USA	1
CD127 (IL-7Rα)	BV650	A019D5	BioLegend, San Diego (CA), USA	1, 2, 3
CD127 (IL-7Rα)	BV785	A019D5	BioLegend, San Diego (CA), USA	1, 4
CD127 (IL-7Rα)	PE	MB15-18C9	Miltenyi Biotec, Bergisch Gladbach, Germany	1
CD127 (IL-7Rα)	PerCPCy5.5	A019D5	BioLegend, San Diego (CA), USA	1, 2
CD133	PE	AC133	Miltenyi Biotec, Bergisch Gladbach, Germany	1
CD135	APC	BV10A4H2	BioLegend, San Diego (CA), USA	1
CD14	BV605	M5E2	BioLegend, San Diego (CA), USA	2
CD14	BV711	M5E2	BioLegend, San Diego (CA), USA	1
CD14	FITC	M5E2	BioLegend, San Diego (CA), USA	1
CD141	APC	M80	BioLegend, San Diego (CA), USA	1
CD15	PCP	W6D3	BioLegend, San Diego (CA), USA	1
CD158 (panKIR)	PE-Cy7	HP-MA4	BioLegend, San Diego (CA), USA	4
CD16	APC-Cy7	3G8	BioLegend, San Diego (CA), USA	2
CD16	BV605	3G8	BioLegend, San Diego (CA), USA	1
CD161	BV421	DX2	BioLegend, San Diego (CA), USA	2
CD161	BV605	HP-3G10	BioLegend, San Diego (CA), USA	1, 2
CD161	PE	HP-3G10	BioLegend, San Diego (CA), USA	1
CD183 (CXCR3)	AF488	1C6/CXCR3	Becton Dickinson, Franklin Lakes (NJ), USA	1, 2
CD183 (CXCR3)	BV650	G025H7	BioLegend, San Diego (CA), USA	4
CD185 (CXCR5)	PE-Dazzle	J252D4	BioLegend, San Diego (CA), USA	1

specificity	fluorochrome	clone	company	Panels
CD19	BV785	HIB19	BioLegend, San Diego (CA), USA	1, 2
CD19	PE-Cy7	HIB19	Becton Dickinson, Franklin Lakes (NJ), USA	1
CD194 (CCR4)	PE-Cy7	L291H4	BioLegend, San Diego (CA), USA	1, 2
CD194 (CCR4)	PerCPCy5.5	L291H4	BioLegend, San Diego (CA), USA	1
CD196 (CCR6)	PerCPCy5.5	G034E3	BioLegend, San Diego (CA), USA	1, 2
CD197 (CCR7)	AF647	G043H7	BioLegend, San Diego (CA), USA	1, 2
CD197 (CCR7)	BV711	G043H7	BioLegend, San Diego (CA), USA	1
CD1c	APC-Cy7	L161	BioLegend, San Diego (CA), USA	1
CD20	AF700	2H7	BioLegend, San Diego (CA), USA	1
CD20	BV421	2H7	BioLegend, San Diego (CA), USA	1
CD21	APC	Bu32	BioLegend, San Diego (CA), USA	1
CD235a (Glycophorin A)	BV421	GA-R2 (HIR2)	Becton Dickinson, Franklin Lakes (NJ), USA	1
CD24	PerCPCy5.5	ML5	BioLegend, San Diego (CA), USA	1
CD244 (2B4)	FITC	C1.7	BioLegend, San Diego (CA), USA	3
CD25	BV421	BC96	BioLegend, San Diego (CA), USA	1, 3, 4
CD25	BV785	BC96	BioLegend, San Diego (CA), USA	1, 2, 3
CD25	PE	2A3	Becton Dickinson, Franklin Lakes (NJ), USA	1, 2
CD27	APC-Cy7	O323	BioLegend, San Diego (CA), USA	1, 3, 4
CD27	BV650	O323	BioLegend, San Diego (CA), USA	1
CD279 (PD-1)	APC	EH12.2H7	BioLegend, San Diego (CA), USA	3
CD28	PE-Cy7	CD28.2	BioLegend, San Diego (CA), USA	1, 2, 3
CD28	FITC	CD28.2	Becton Dickinson, Franklin Lakes (NJ), USA	4
CD294 (CRTH2)	BV421	BM16	BioLegend, San Diego (CA), USA	1, 2
CD294 (CRTH2)	PE-Dazzle	BM16	BioLegend, San Diego (CA), USA	2
CD3	AF700	OKT3	BioLegend, San Diego (CA), USA	2
CD3	BV510	OKT3	BioLegend, San Diego (CA), USA	1, 2, 3
CD3	BV650	OKT3	BioLegend, San Diego (CA), USA	1
CD3	BV785	OKT3	BioLegend, San Diego (CA), USA	1
CD3	PerCPCy5.5	OKT3	BioLegend, San Diego (CA), USA	1, 3
CD31	APC-Cy7	WM59	BioLegend, San Diego (CA), USA	1, 2
CD34	FITC	561	BioLegend, San Diego (CA), USA	1, 2
CD366 (Tim-3)	PE	F38-2E2	BioLegend, San Diego (CA), USA	3
CD38	AF488	HIT2	BioLegend, San Diego (CA), USA	1
CD38	AF700	HIT2	BioLegend, San Diego (CA), USA	1, 2
CD38	BV605	HIT2	BioLegend, San Diego (CA), USA	1
CD39	BV421	A1	BioLegend, San Diego (CA), USA	2
CD39	PE-Cy7	A1	BioLegend, San Diego (CA), USA	1, 3
CD4	AF700	OKT4	BioLegend, San Diego (CA), USA	1, 2, 4
CD4	BV421	RPA-T4	BioLegend, San Diego (CA), USA	3
CD4	APC-Cy7	RPA-T4	BioLegend, San Diego (CA), USA	1, 2
CD4	PE-Dazzle	RPA-T4	BioLegend, San Diego (CA), USA	1
CD4	PerCPC5.5	RPA-T4	BioLegend, San Diego (CA), USA	3

specificity	fluorochrome	clone	company	Panels
CD43	PE-Vio770	DF-T1	Miltenyi Biotec, Bergisch Gladbach, Germany	1
CD45	BV510	HI30	BioLegend, San Diego (CA), USA	1, 2
CD45RA	BV711	HI100	BioLegend, San Diego (CA), USA	1, 2, 3
CD45RA	PE-Cy7	HI100	BioLegend, San Diego (CA), USA	1
CD45RA	PE-Dazzle	HI100	BioLegend, San Diego (CA), USA	1, 2, 4
CD45RO	BV785	UCHL1	BioLegend, San Diego (CA), USA	1
CD49d	APC	9F10	BioLegend, San Diego (CA), USA	4
CD5	PE-Dazzle	L17F12	BioLegend, San Diego (CA), USA	1
CD56	BV421	HCD56	BioLegend, San Diego (CA), USA	1
CD56	BV650	HCD56	BioLegend, San Diego (CA), USA	2
CD57	FITC	HCD57	BioLegend, San Diego (CA), USA	1, 2
CD57	PE-Dazzle	HNK-1	BioLegend, San Diego (CA), USA	3
CD69	APC-Cy7	FN50	BioLegend, San Diego (CA), USA	1
CD71	BV711	M-A712	Becton Dickinson, Franklin Lakes (NJ), USA	1
CD73	PE	AD2	BioLegend, San Diego (CA), USA	1, 3
CD73	PerCPCy5.5	AD2	BioLegend, San Diego (CA), USA	2, 4
CD8a	AF700	HIT8a	BioLegend, San Diego (CA), USA	1, 3
CD8a	BV510	RPA-T8	BioLegend, San Diego (CA), USA	1, 3
CD8a	BV605	RPA-T8	BioLegend, San Diego (CA), USA	1, 4
CD8b	PE	2ST8.5H7	Becton Dickinson, Franklin Lakes (NJ), USA	1
CD90 (Thy-1)	PerCPCy5.5	5E10	BioLegend, San Diego (CA), USA	1
CD94	FITC	HP-3D9	Becton Dickinson, Franklin Lakes (NJ), USA	1
CD95 (Fas)	BV421	DX2	BioLegend, San Diego (CA), USA	1
CD95 (Fas)	BV711	DX2	BioLegend, San Diego (CA), USA	2, 4
HLA-DR	BV711	L243	BioLegend, San Diego (CA), USA	1, 2
HLA-DR	BV785	L243	BioLegend, San Diego (CA), USA	1, 2
HLA-DR	FITC	G46-6	Becton Dickinson, Franklin Lakes (NJ), USA	3
IFNγ	APC-Cy7	4S.B3	BioLegend, San Diego (CA), USA	1
IgD	PE	IA6-2	BioLegend, San Diego (CA), USA	1
IL-10	PE	JES3-9D7	BioLegend, San Diego (CA), USA	1
IL-17A	BV421	BL168	BioLegend, San Diego (CA), USA	1
IL-2	BV605	MQ1-17H12	BioLegend, San Diego (CA), USA	1
IL-4	PE-Cy7	MP4-25D2	BioLegend, San Diego (CA), USA	1
IL-8	APC	E8N1	BioLegend, San Diego (CA), USA	1
KLRG1	BV605	2F1/KLRG1	BioLegend, San Diego (CA), USA	3
NKG2A	PE-Cy7	Z199	Becton Dickinson, Franklin Lakes (NJ), USA	4
TCR Vα7.2	APC	3C10	BioLegend, San Diego (CA), USA	1
TCR Vα7.2	PE	3C10	BioLegend, San Diego (CA), USA	2
TCR Vδ1-Jδ2	FITC	TS-1	Thermo Fisher Scientific, Waltham (MA), USA	1
TCR Vδ2	APC	123R3	Miltenyi Biotec, Bergisch Gladbach, Germany	1
TCR Vδ2	PE	123R3	Miltenyi Biotec, Bergisch Gladbach, Germany	2
TCR Vγ9	FITC	IMMU 360	Beckman Coulter, Brea (CA), USA	1

specificity	fluorochrome	clone	company	Panels
TCR$\gamma\delta$	FITC	11F2	Becton Dickinson, Franklin Lakes (NJ), USA	1
TCR$\gamma\delta$	PE	11F2	Becton Dickinson, Franklin Lakes (NJ), USA	1, 2
TCR$\gamma\delta$	PE-Cy7	11F2	Becton Dickinson, Franklin Lakes (NJ), USA	1, 2
TNFa	BV650	MAb11	Becton Dickinson, Franklin Lakes (NJ), USA	1

2.1.7 Mouse antibodies

specificity	fluorochrome	clone	company
Annexin V	FITC		Becton Dickinson, Franklin Lakes (NJ), USA
CD25	PE	3C7	BioLegend, San Diego (CA), USA
CD3	eFluor450	500A2	Thermo Fisher Scientific, Waltham (MA), USA
CD4	AF700	RM4-5	BioLegend, San Diego (CA), USA
CD44	APC-Cy7	IM7	BioLegend, San Diego (CA), USA
CD8a	BV650	53-6.7	BioLegend, San Diego (CA), USA
TCRbeta	BV605	H57-597	BioLegend, San Diego (CA), USA
TCR$\gamma\delta$	APC		Thermo Fisher Scientific, Waltham (MA), USA

2.1.8 Primer for genotyping

primer	sequence
Lck-Cre 1	GCG GTCT GGC AGT AAA AAC TAT C
Lck-Cre 2	GTG AAA CAG CAT TGC TGT CAC TT
IPC-Act-Forward	CAA ATG TTG CTT GTC TGG TG
IPC-Act-Reverse	GTC AGT CGA GTG CAC AGT TT
GRflox 1	GGC ATG CAC ATT ACT GGC CTT CT
GRflox 2	GTG TAG CAG CCA GCT TAC AGG A
GRflox 3	CCT TCT CAT TCC ATG TCA GCA TGT

2.1.9 qPCR primer

Gene name	Assay ID	company
Polr2a	Mm00839502_m1	Thermo Fisher Scientific, Waltham (MA), USA
Keratin 5	Mm01305291_g1	Thermo Fisher Scientific, Waltham (MA), USA
Keratin 8	Mm04209403_g1	Thermo Fisher Scientific, Waltham (MA), USA
AIRE	Mm00477461_m1	Thermo Fisher Scientific, Waltham (MA), USA
Fezf2	Mm01320619_m1	Thermo Fisher Scientific, Waltham (MA), USA
Ins2	Mm.47.17321456	Integrated DNA Technologies, Leuven, Belgium
Klk1b16	Mm00658470_g1	Thermo Fisher Scientific, Waltham (MA), USA

2.1.10 General equipment

Equipment	Company, Location
Analytical balance LA 124i	VWR International, Radnor (PA), USA
CellCamper® Freezing container	neoLab Migge, Heidelberg, Germany
CELL-DYN Emerald Cell Counter	Abbott, Abbott Park (IL), USA
Centrifuge 5424R	Eppendorf, Hamburg, Germany
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Centrifuge Allegra X-30R	Beckmann Coulter, Brea (CA), USA
CO2 Incubator MCO-20AIC	Sanyo, Osaka, Japan
FACS CantoII	Becton Dickinson, Franklin Lakes (NJ), USA

Equipment	Company, Location
FACS Celesta	Becton Dickinson, Franklin Lakes (NJ), USA
FACS LSRFortessa	Becton Dickinson, Franklin Lakes (NJ), USA
Freezer -20°C	Liebherr, Bulle, Switzerland
Freezer -80°C	Thermo Fisher Scientific, Waltham (MA), USA
gentleMACS™ Octo dissociator with heater	Miltenyi, Bergisch Gladbach, Germany
Micro centrifuge Galaxy MiniStar	VWR, Radnor (PA), USA
Mr. Frosty™ Freezing container	Thermo Fisher Scientific, Waltham (MA), USA
Multi-channel pipette 20-300 µL	Mettler-Toledo Rainin LLC, Columbus (OH), USA
Multi-channel pipettes 100/300 µL	Eppendorf, Hamburg, GERMANY
Nanodrop 2000	Thermo Fisher Scientific, Waltham (MA), USA
Neubauer cell chamber	Marienfeld, Lauda-Königshofen, Germany
NucleoCounter® NC-200	Chemometec, Allerod, Denmark
PIPETBOY acu 2	Integra Biosciences, Biebertal, Germany
Pipettes 2.5/10/20/200/1000 µL	Eppendorf, Hamburg, Germany
Refrigerator/Freezer	Liebherr, Bulle, Switzerland
StepOne Plus Real Time PCR system	Applied Biosystems, Foster City (CA), USA
Sterile bank MSC-Advantage	Thermo Fisher Scientific, Waltham (MA), USA
Vacuum pump Vacusafe	Integra Biosciences, Biebertal, Germany
Via1-Cassette™	Chemometec, Allerod, Denmark
Vortex mixer Gene 2	Scientific industries, Bohemia (NY), USA
Vortex mixer REAX 2000	Heidolph, Schwabach, Germany
Water bath	GFL, Burgwedel, Germany

2.1.11 General consumables

Material	Company
Cellstar polypropylene tubes 15/50 mL	Greiner bio-one, Kremsmünster, Austria
epT.I.P.S 1000 µL	Eppendorf, Hamburg, Germany
gentleMACS™ M tubes	Miltenyi, Bergisch Gladbach, Germany
Pipette tips 10/200/1000 µL	Sarstedt, Nümbrecht, Germany
Polystyrene round bottom tubes 5 mL	Sarstedt, Nümbrecht, Germany
Reagent reservoir	Starlab, Hamburg, Germany
SafeSeal tubes 1.5/2 mL	Sarstedt, Nümbrecht, Germany
Serological pipettes	Sarstedt, Nümbrecht, Germany
S-Monovette Z-Gel/K3E	Sarstedt, Nümbrecht, Germany

2.1.12 Software

Material	Company
Adobe Illustrator CS2	Adobe Systems, San Jose (CA), USA
Endnote X7.8	Clarivate Analytics, Philadelphia (PA), USA
FACSDiva™ V.8.0.1	Becton Dickinson, Franklin Lakes (NJ), USA
FlowJo 10.5.3	FlowJo, LLC, Ashland, USA
GraphPad Prism 6.07	GraphPad Software, Inc., La Jolla, USA
Nanodrop 2000/ 2000c	Thermo Fisher Scientific, Waltham (MA), USA
R Studio Version 1.1.456	R Studio, Inc. Boston (MA), USA
R Version 3.5.1	R Core Team 2018 (RCoreTeam, 2018)
StepOne Software 2.3	Applied Biosystems, Foster City (CA), USA
TBase / 4D v14.4	4D SAS

2.2 Methods

2.2.1 Human blood samples

Informed consent was signed by all study participants or their parents. The study protocols were approved by the chamber of physicians of Hamburg or the local committee on research in human subjects at the University of Lübeck as listed in section 2.1.12.1.1 Human Blood samples.

2.2.1.1 Cord blood

Umbilical cord blood samples were provided by the Prenatal Identification of children's health (PRINCE) study of the Department of Obstetrics and Prenatal Medicine from the medical center Hamburg-Eppendorf (PD Dr. Anke Diemert / Prof. Dr. Petra Arck / Prof. Dr. Kurt Hecher) and by the IRoN (Immunoregulation of the Neonate) study of the Department of the Neonatology and Pediatrics from the medical center Schleswig-Holstein in Lübeck (Prof. Dr. Härtel / Dr. Julia Pagel).

2.2.1.2 Peripheral blood

Peripheral blood from immunologically healthy children was obtained in cooperation with the Department of Pediatrics (PD Dr. Robin Kobbe / Dr. Ulf Schulze-Sturm) of the medical center Hamburg-Eppendorf, the Department of Pediatric Orthopedics of the children's hospital Hamburg-Altona (Dr. Thomas Brich) and the Department of Pediatric Surgery of the Hannover Medical School (Dr. Christian Klemann).

Peripheral blood from children born preterm and from age-matched term born controls were provided by the PRINCE study.

Peripheral blood from children with known or suspected immune deficiency was obtained in cooperation with the Department of Pediatrics (PD Dr. Robin Kobbe / Dr. Ulf Schulze-Sturm) of the medical center Hamburg-Eppendorf.

Peripheral blood from children with an open-heart surgery in early childhood was obtained in cooperation with the Department of Pediatric Cardiology (PD Dr. Jörg Sachweh) of the University Heart Center Hamburg.

Peripheral blood from children with a mutation in the *Bcl11b* gene were recruited by the Department of Human Genetics of the medical center Hamburg-Eppendorf (Dr. Davor Lessel) from various clinical centers.

Peripheral blood from healthy adult volunteers was drawn at the Department of Immunology or at the Department of Cardiology (Dr. Sarina Schäfer).

2.2.2 Fetal thymus ultrasound

Fetal thymus ultrasound data was provided by the PRINCE study. Thymus size was measured as published previously (Diemert et al., 2016).

2.2.3 Isolation of mononuclear cells

Peripheral mononuclear cells (PBMCs) or cord blood mononuclear cells (CBMCs) were isolated by density gradient centrifugation using Biocoll density gradient medium. Blood was diluted with PBS and carefully layered onto separation medium as listed in Table 1.

Table 1. Volumes of diluted Blood and Biocoll used for PBMC isolation

Blood volume	Final volume diluted blood	Volume Biocoll	Tube size
≤ 1.5 mL	3 mL	1.5 mL	5 mL polystyrene tube
≤ 5 mL	10 mL	5 mL	15 mL polypropylene tube
5-15 mL	30 mL	20 mL	50 mL polypropylene tube

Upon centrifugation for 25 mins at 800 x g with slow acceleration and slow break at room temperature, mononuclear cells form a milky layer between plasma (on top) and Biocoll while erythrocytes deposit at the bottom of the tube. Layer containing mononuclear cells was carefully transferred to a new reaction tube and washed twice with cold PBS by centrifugation at 650 x g for 10 min and 450 x g for 5 min at 4 °C respectively. The total cell count was assessed using a Neubauer counting chamber or an automated cell counter (NucleoCounter® NC-200™) with Via1-cassettes. Tuerck's solution was used for counting lymphocytes with a Neubauer counting chamber, while the Via1-cassette includes immobilized fluorescent dyes (acridine orange and DAPI) for live cell detection.
Isolated PMBCs were immediately used for further experiments or frozen with freezing medium and stored at 80°C or in liquid nitrogen.

2.2.4 Cryopreservation of mononuclear cells

Isolated mononuclear cells were centrifuged and resuspended in freezing medium I. 500 µL of cell suspension was transferred to cryotubes on cooling rack or ice. Every 30 seconds 50µL cold freezing medium II was carefully added and the tube was gently swirled for ten times. The usage of Mr. Frosty or CellCamper® ensured a gentle and steady freezing rate of -1 °C/min for final storage at -80 °C.

2.2.5 Flow cytometry

Flow cytometry is a technique to determine the phenotype and function of cells based on light scattering and the emission of fluorescence. Using a steady fluid stream, cells are lined up to ensure that single cells are passing the beams of an instrument-specific array of lasers. The detection system consist of three different ways of detection. First, naturally scattered light from a flat angle beam allows the determination of the size (forward scatter, FSC), and second, the scattered light from a right-angled beam allows the detection of the granularity of the cells (SSC). In addition, fluorochrome-conjugated antibodies bound to specific molecular markers on the cell can be excited with the laser beam. Absorbing a photon from the

laser beam temporarily increases the energy state of the receptor protein, the fluorophore, which is reverted to its ground state upon emission of a photon, the fluorescence signal. The emitted light is then transmitted and ordered via dichroic mirrors and filters and finally collected by optical detectors which either use photomultiplier or avalanche photodiodes to convert the optical signal into an electronic pulse which in turn is converted from an analog to a digital signal and can be processed and analyzed in data analysis software such as FACSDiva™ or FlowJo. Fluorochromes do not have a precise wavelength for excitation and emission but a spectral range which overlaps between certain fluorochromes. Thus, using a set of antibodies can lead to spectral spillover into detectors from a fluorochrome with a similar emission spectrum. To correct for this, cytometers are equipped with bandpass filters which restrict the light that reaches the detectors. In addition, compensation calculates and subtracts the ratio of spillover and fluorochrome-specific signal.

Flow cytometry analysis was performed on a BD FACS Celesta, BD FACS CantoII or BD LSRFortessa using the FACS Diva software (BD Bioscience). The data was analyzed with FlowJo software (Tree Star, Inc.).

If not otherwise stated, all steps were performed at room temperature and in the dark.

2.2.5.1 Staining of surface molecules

Whole blood staining was performed for seven panels (panel 1-7, Table 3). 50 µL whole blood or cord blood for each staining were mixed with 50 µL antibody cocktail and incubated for 30 min. Subsequent red cell lysis was performed by addition of 1mL lysing solution (eBioscience) and incubation for 10 min. Samples were washed twice with PBS and either resuspended in FACS buffer for immediate use or fixed with 1% paraformaldehyde in FACS buffer. Fixed cells were stored up to 24 hours at 4 °C.

Staining for hematopoietic stem cells and $CD235a^+CD71^+$ cells were performed on freshly isolated CBMCs to enrich for mononuclear cells by exclusion of granulocytes and red blood cells. Furthermore, using isolated cells circumvents the capture of fluorochrome-labelled antibodies against Glycophorin A (CD235a) by red blood cells, thus allows the detection of $CD71^+CD235a^+$ cells. 0.5 – 1 Mio mononuclear cells were stained with 50 µL antibody cocktail (panel 8, Table 3) for 10min. For dead cell exclusion, an amine-reacting life/dead (L/D) fluorescent dye, Alexa Fluor 750 (AF759, 1 µg/mL), was added in a final dilution of 1:1000. Intact cell membranes prevent the dye from entering the cell, while membranes of dead cells are permeable and can be identified by a positive signal for the dye. After an additional incubation of 20 min, cells were washed twice with PBS and either resuspended in FACS buffer for immediate use or fixed with 1% paraformaldehyde in FACS buffer. Fixed cells were stored up to 24 hours at 4 °C.

2.2.5.2 Intracellular cytokine detection

0.5 – 1 Mio PBMCs were resuspended in serum free X VIVO-15 medium (Lonza) and stimulated with Phorbol-12-myristat-13-acetat (PMA, 50 ng/mL) and Ionomycin (Iono, 1 µg/mL) for 5 hours at 37°C. To

prevent the release of cytokines into the extracellular environment, the protein transport from the endoplasmic reticulum to the Golgi apparatus was blocked with Brefeldin A (10 mg/mL), 30 min after the addition of PMA/Iono. Cells were washed with PBS and resuspended in residual liquid and 2 µL hIgG to block unspecific binding. After an incubation for 5min, 50 µL antibody cocktail for surface marker (panel 9.1, Table 3) was added and cells were incubated for 10min. For dead cell exclusion, an amine-reacting life/dead (L/D) fluorescent dye, Pacific Orange (PacO, 1 µg/mL), was added in a final dilution of 1:1000. Intact cell membranes prevent the dye from entering the cell, while membranes of dead cells are permeable and can be identified by a positive signal for the dye. After an additional incubation of 20 min, cells are washed twice with cold PBS and PBS with 0.2% BSA. To prevent leakage of intracellular material after permeabilization of the cell membrane, cells were fixed with 100 µL IC-Fixation buffer and incubated for 20min. Cells were washed with permeabilization buffer twice and resuspended in 50 µL antibody cocktail for intracellular staining (panel 9.2, Table 3) and incubated for 30min. After a final washing step, cells were resuspended in FACS buffer and measured within 24 hours.

2.2.5.3 Gating strategy

Human samples were analyzed on a 15-color LSRFortessa (BD Biosciences) provided by the FACS core unit at the University Medical Center Hamburg-Eppendorf. Data was evaluated using FlowJo software V10.7 (FlowJo, LLC). Gating of all leucocytes (panel 1) or lymphocytes (panel 2-8) was performed according to granularity and size (FSC-A versus SSC-A), followed by removal of doublets with a FSC-A versus FSC-H diagonal gate. Representative hierarchical gating strategies for panels 1-9 are depicted in Figure S1-4 in the appendix.

Murine thymocytes were analyzed on a 12-color Celesta (BD Biosciences) at the institute of immunology at the University Medical Center Hamburg-Eppendorf. Data was evaluated using FlowJo software V10.7 (FlowJo, LLC). A representative hierarchical gating strategy is shown in Figure S5 in the appendix.

2.2.6 Proliferation of human PBMCs

Proliferation of human T cells upon stimulation was assessed by eFluor dye dilution. 0.1 - 0.2 Mio PBMCs per well were resuspended in 2µM eFluor670 and incubated for 10 min at 37°C. eFluor labelling was stopped by the addition of 4-5 volumes of cold full medium and incubation on ice for 5 min. Cells were washed twice and resuspended in RPMI for cell counting. Using a 96-well plate, 0.1 Mio cells were mixed with appropriate stimuli (Table 2) or buffer (control) in a final volume of 200µL. Cells were cultured for 3, 5 or 7 days according to Table 2. To detect proliferation and activation status of the cells, surface markers were stained with fluorochrome-conjugated antibodies (Table 3) as described in section 2.2.5.1 and measured on a FACS CantoII.

Table 2. Concentration of stimuli and read-out days in proliferation assay of human PBMCs.

Stimulus	Final concentration	Read-out day 1	Read-out day 2
Soluble CD3	0.5 µg/ mL	Day 3	Day 5
Soluble CD3	0.5µg /mL (sCD3)	Day 3	Day 5
+ soluble CD28	+ 5 µg/ mL (sCD28)		
Recombinant human IL-2	20 U/mL	Day 5	Day 7
Recombinant human IL-7	50 ng/mL	Day 5	Day 7

Table 3. Summary of flow cytometry panels. L/D – LiveDead dye, [1] Lin: CD14, CD34, CD94

Panel		BV421	BV500	BV605	BV650	BV711	BV785	FITC	PerCP-Cy5.5	PE	PE-Texas Red	PE-Cy7	APC	AF700	APC-Cy7
1	subsets	CD56	CD45	CD16	CD3	CD14	HLA-DR		CD15	CD127	CD11c	CD123	CD141	CD20	CD1c
2	T effector	CD95	CD8		CD127	HLA-DR	CD3	CD57		CD25	CD45RA	CD28	CCR7	CD38	CD4
3	T helper	CRTH2	CD3	CD161	CD127	CD45RA	CD25	CXCR3	CCR6	TCRγδ	CXCR5	CCR4	CCR10	CD4	
4	T regulatory	CD25	CD3	CD8	CD127	HLA-DR			CCR4	CD73	CD45RA	CD39		CD4	CD31
5	invariant	CD25	CD3	CD161	CD27	CCR7	CD45RO	Vδ1 + Vγ9	CCR6	CD8β	CD4	TCRγδ	Vα7.2 + Vδ2	CD8α	CD69
6	ILC	CRTH2	CD45		CD3		HLA-DR	Lin[1]	CD127	CD161		CD19	c-Kit		
7	BC	CD20	CD45			CD10	CD19	CD38	CD24	sIgD	CD5	CD43	CD21		CD27
8	HSC	CD235a	CD45	CD38		CD71		CD34	CD90	CD133	CD10	CD45RA	CD135		live/dead
9.1	Cytokines surface		L/D				CD45RO	TCRγδ	CD3		CD4			CD8	
9.2	Cytokines intracellular	IL-17A	L/D	IL-2	TNFα					IL-10	CD4	IL-4	IL-8		IFNγ
10	Proliferation	CD25	CD8					HLA-DR	CD4	CD73		CD39	eFluor		Live/dead
11	Exhaustion marker	CD4	L/D	KLRG1	CD127	CD45RA	CD25	2B4	CD3	Tim-3	CD57	CD28	PD-1	CD8	Cd27
12	Virtual memory	CD25	CD3	CD8	CXCR3	CD95	CD127	CD28	CD73	CD122	CD45RA	panKIR + NKG2A	CD49d	CD4	CD27
13	Lübeck frozen	CD25	L/D	CD38	CD127	HLA-DR	CD3	TCRγδ	CD4	Vδ2	Vα7.2	CD45RA	CD28	CCR7	CD8
14	Murine thymocytes	CD3	L/D	TCRβ	CD8α			AnnexinV		CD25			TCRγδ	CD4	CD44

2.2.7 Determination of the cytokine concentration in plasma and cell culture supernatants

Cytokine concentrations in plasma samples or cell culture supernatants from the proliferation assay (section 2.2.6) were determined by flow cytometry using the *LEGENDplex™ Human Th Panel (13-plex)* kit from BioLegend that allows the co-detection of 13 cytokines (IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17a, IL-17f, IL-21, IL-22, TNFa, IFNg) in cell culture supernatant or plasma samples (diluted 1:2 and 1:4). Assay was performed according to manufacturer's instructions. Data analysis was done with the LEGENDplex analysis software v8.0.

2.2.8 Thawing procedure of frozen PBMCs and surface staining

For analysis of previously frozen PBMCs, cells were thawed and rested for an hour at 37°C prior to staining. In brief, samples stored at -80°C or in liquid nitrogen were transferred to a 37°C water bath until just ice-less. DMSO from the freezing medium was diluted twice by adding 20 ml RPMI with 20 % FCS. Cells were resuspended in 1mL medium and were rested for an hour at 37°C before stained for surface molecules or used for an *in vitro* cytokine assay as described in section *2.2.5.1 Staining of surface* molecules and *2.2.5.2 Intracellular cytokine detection.*

This procedure was performed for a subset of samples stained for panels 2-7, 9 and 11 and for all samples that were stained for panel 12 (Table 3).

2.2.9 Animal experiments

Animal studies were performed according to the animal protection law and approved by the relevant German authorities (Behörde für Gesundheit und Verbraucherschutz Hamburg) as listed in section *2.1.2 Mouse strains*. Mice were housed in individually ventilated cages in the S1-barrier of the animal facility at the University Medical Center Hamburg-Eppendorf. $GR^{flox/flox}$ mice, Nr3c1tm2Gsc, were generated by Günther Schütz (German Cancer Research Center, Heidelberg) (Tronche et al., 1999). These mice were crossed to Lck-Cre mice to generate GR^{flox}Lck-Cre mice, which were kindly provided by Prof. Dr. Holger Reichardt (University Medical School, Göttingen).

2.2.9.1 Genotyping

Genotyping for GR^{flox}Lck-Cre was performed prior to all experiments. DNA was extracted from tail biopsies by isopropanol precipitation. In brief, 100µg Proteinkinase K and lysis buffer were added to the biopsy and incubated for three hours at 55°C with constant shaking. Supernatant was decanted onto isopropanol and inverted several times. Samples were centrifuged at 9000 rpm for 2 min. Pellet was resuspended in TE-buffer and incubated 2-3 hours at 55°C or overnight at room temperature. Genotyping was performed for $GR^{flox/flox}$ and Lck-Cre in two independent reactions (Table 4).

Table 4. PCR Master mix to determine the genotype of GR^{flox}-Lck-Cre or GRflox-Lck-wt mice

$GR^{flox/flox}$	Lck-Cre
H_2O	H_2O
Hot Start Taq Buffer 10x	10 x Hot Start Buffer
$MgCl_2$	MgCl2
dNTPs	dNTPs
Primer GRflox 1 [10µM]	Primer Cre forward [10µM]
Primer GRflox 2 [10µM]	Primer Cre reverse [10µM]
Primer GRflox 3 [10µM]	Primer B-Actin forward [10µM]
	Primer B-Actin reverse [10µM]
Hot Start Taq Polymerase	Hot Start Taq Polymerase

Amplification reaction was performed for 35 cycles with slight differences between $GR^{flox/flox}$ (Table 5 A) and Lck-Cre reactions (Table 5 B)

Table 5. PCR amplification reactions to determine the genotype of GR^{flox}-Lck-Cre or GRfloxLck-wt mice.

A

$GR^{flox/flox}$			
	Temperature	**Time**	**Cycles**
1	94°C	5mins	1
2	94°C	30 sec	35
3	63°C	1 min	
4	72°C	1 min	
5	72°C	10 mins	1
6	15°C	∞	

B

Lck-Cre			
	Temperature	**Time**	**Cycles**
1	94°C	5mins	1
2	94°C	30 sec	35
3	55°C	30 sec	
4	72°C	45 sec	
5	72°C	7 mins	1
6	15°C	∞	

Resulting amplicons and Marker (GeneRuler 100bp DNA ladder) were loaded onto 2.5 % agarose gels. Bands were detected at 390 base pairs (bp) for complete GR deletion and at 225 bp for wildtype GR. Successful amplification of Cre resulted in a band at 214 bp and actin B (housekeeping gene) at 150 bp.

2.2.9.2 *In vivo* Betamethasone treatment and organ harvest

0.1mg Betamethasone or PBS (control) were injected i.p. into 4-5 weeks old GR^{flox}Lck-Cre mice. Three, five or ten days later, mice were sacrificed and organs harvested. One thymic lobe was used for flow cytometry staining and the second lobe was stored in RNA*later* at 4°C for subsequent qPCR analysis.

2.2.9.3 RNA isolation of thymic tissue

Thymic tissue disruption was performed using the gentleMACS™ Dissociator. Thymic lobe was transferred to gentleMACS™ M Tubes (Miltenyi) containing RLT lysis buffer provided by the RNeasy Midi Kit (QIAGEN)

and ß-Mercaptoethanol. The predefined program RNA_01 was used for tissue dissociation. Cell lysate was centrifuged and RNA Isolation was performed according to the manufacturer's instructions for the RNeasy Midi Kit (QIAGEN). RNA concentration was determined by spectrophotometry using the Nanodrop™ 2000.

2.2.9.4 cDNA synthesis

100ng mRNA from murine thymi were reverse transcribed into complementary DNA (cDNA) in a two steps reaction. First, mRNA was incubated with dNTPs and hexamers for 5min at 65°C, chilled on ice and afterwards incubated with first strand buffer, Dithiothreitol (DTT), H_2O and M-MLV reverse transcriptase for 50min at 37°C and 15min at 70°C. cDNA was stored at -20°C.

2.2.9.5 Real time polymerase chain reaction

Quantification of gene expression of *Keratin 5, Keratin 8, AIRE, Fezf2, Ins2* and *Klk1b16* (2.1.9 qPCR primer) in thymic tissue was performed using the StepOnePlus Real-Time PCR System. PCR reactions were executed in 96-well plates. cDNA (1:10 dilution) was mixed with 2x Maxima Probe/ROX qPCR Master Mix (Thermo Scientific™) and a corresponding TaqMan™ probe to yield 20µL reaction volume. Reaction was run for 40 cycles according to Table 6.

Table 6. qPCR reaction to detect gene expression of *Keratin 5, Keratin 8, AIRE, Fezf2, Ins2* and *Klk1b16*.

	Temperature	Time	cycles
1	50°C	2 min	1
	95°C	10 mins	1
2	94°C	15 sec	45 cycles
3	60°C	40 sec	

2.2.9.6 FACS analysis of thymocytes

Single cell suspension of thymocytes was yielded by mechanical disruption and filtering through a 70 µm nylon mesh. 0.5 Mio cells were used for staining with antibody cocktail (Table 3, panel 14) prepared in AnnexinV buffer. After 10 min, live-dead dye (Pacific Orange) was added and the samples were incubated for another 20min. Samples were washed with Annexin buffer and resuspended in FACS buffer.

2.2.10 Visual representation of data

2.2.10.1 t-distributed stochastic neighbor embedding (tSNE)

Cell counts of manually gated cell populations from flow cytometry data were exported from FlowJo. Percentages of mother populations were calculated in Excel and used for further analysis in R (v 3.5.1). Different subsets (all parameters, T cells, B cells, innate-like cells and innate cells) of the data table were selected to perform t-SNE analyses using R-package Rtsne (v0.15). The resulting two-dimensional

representations were plotted using R-package ggplot2 (v 3.2.0) and dots were colored by according age classes and shaped by sample groups.

2.2.10.2 Ultrasound data

Fetal thymus transverse diameter was measured as described up to three times per pregnancy at weeks 23-24, 28-29 and 35-36 of gestation. Using R (v 3.5.1) and the R-package ggplot2 (v3.2.0) thymus transverse diameter was related to gestational week at measurement and to the estimated weight of the fetus. Mean thymic growth was calculated per individual as the slope between two consecutive ultrasound measurements. Plots were created using R-packages ggplot2 (v 3.2.0) and ggpubr (v0.2.1).

2.2.10.3 Distance quantification between healthy donors and children with multiple hits

To quantify homogeneity inside age classes, the center of each age class in the two-dimensional t-SNE map (created using R-package Rtsne v0.15) was calculated as the median of x- and y-values of all healthy individuals belonging to that particular age class. For each individual the Euclidean distance to the center of the corresponding age class was calculated. The computation including generation of the two-dimensional embedding was performed three times to account for variabilities in the creation of the embedding and to increase robustness of the analysis. The mean of the triplicated analysis was calculated and used for comparisons.

To assess the influence of non-healthy individuals to the distribution of points in the t-SNE representation, the t-SNE map was generated using healthy controls only and healthy controls plus non-healthy individuals. Cluster centers and distances of healthy controls to the corresponding cluster centers were calculated and compared for both t-SNE representation using a Wilcoxon rank-sum test. Plots were created using R-packages ggplot2 (v 3.2.0) and ggpubr (v0.2.1).

2.2.11 Statistics

Statistical analysis was performed using the GraphPad Prism Version 6.07 or R. Statistical significance of normally distributed data was analyzed by Student's *t* test (two groups) or two-way ANOVA (multiple groups) with Tukey's post-hoc test for correction of multiple comparisons. Mann Whitney *U* test was performed to compare two groups of not normally distributed data. Wilcoxon test was used for assessment of statistical significance of distance distribution between different tSNE calculation runs. Significance levels are indicated as * < 0.05, ** < 0.01 or *** < 0.001. Non-significant differences were not indicated.

3. Results

The result section is subdivided in two major parts. First, I introduce a comprehensive flow cytometry-based immune phenotyping approach that is tested in healthy human donors spanning a wide age range. Here, I analyzed the dynamics of the progression of the different immune cell subpopulations with age. In a second part, I used this highly detailed immune phenotyping approach to investigate the effects of prenatal steroid treatment and thymic reduction caused by extrinsic (early childhood thymectomy) and intrinsic (chromosome 22 deletion/DGS) factors on the developing immune system.

3.1 Detailed assessment of the immune composition in human blood

3.1.1 Establishment of a comprehensive immunophenotyping approach

The immune system composition undergoes age-related changes. Innate immune responses are dominant at birth, and T cells progress from a naïve phenotype and a predominance of Th2 responses to a diverse network of adaptive cells (Simon et al., 2015, Tu and Rao, 2016). Immunological studies in children are scarce. As a consequence, normality ranges are only defined for a few classical cell subsets. To assess the impact of early life events on all cellular components of the peripheral immune system, I established a comprehensive flow cytometry-based single cell immune phenotype approach. Nine 12 to 14- color flow cytometry panels, containing 62 different cell markers (section 2.2.5,), allowed us to investigate 141 immune cell populations covering the innate and the adaptive arms of the immune system using less than 1 ml of blood (Figure 9). This strategy encompassed the analysis of classical and well-studied cell subsets, such as $CD4^+$ and $CD8^+$ T cells, as well as more recently described cell populations that have not yet been analyzed in healthy children, such as innate lymphoid cells (ILCs).

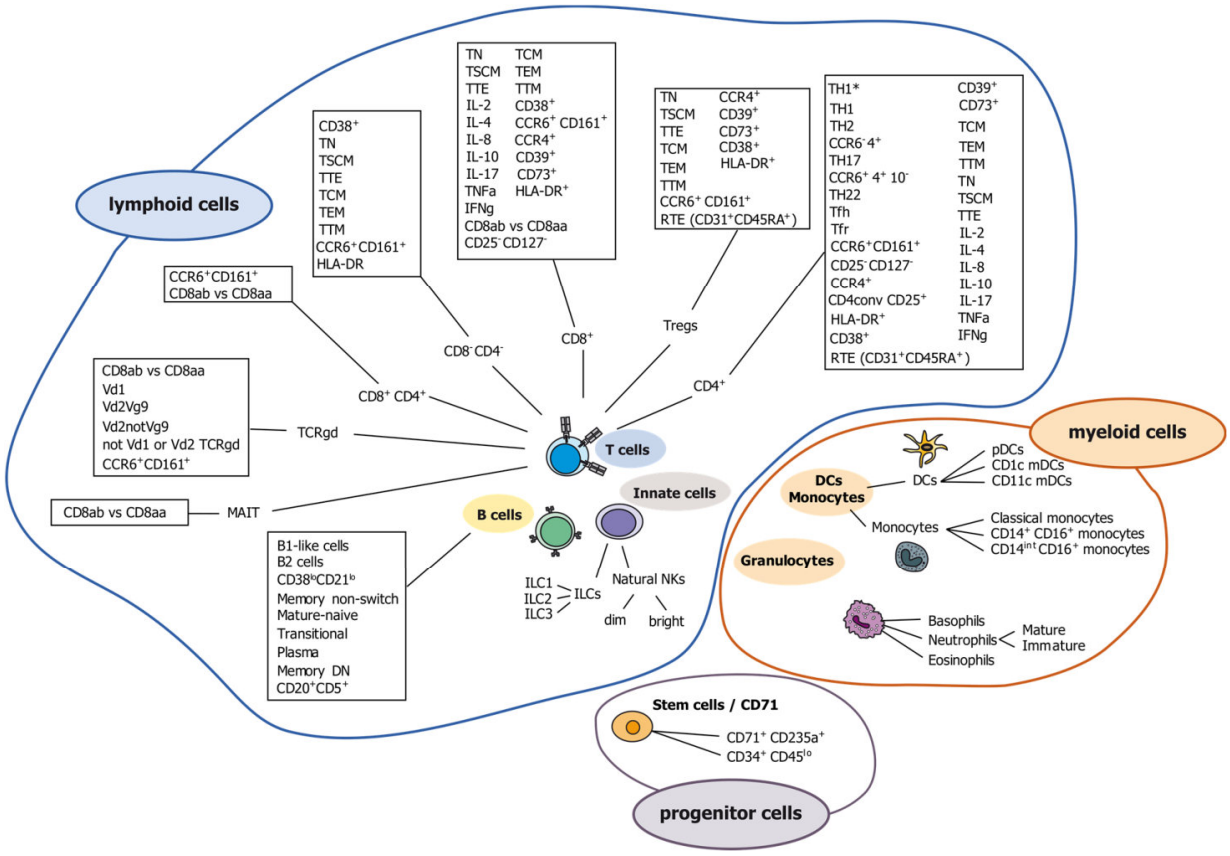

Figure 9. Cell populations analyzed in peripheral blood using a deep immune phenotyping approach. Multicolor flow cytometry enables the investigation of 141 immune cell populations in less than 1mL blood covering the innate and the adaptive immune system.

Changes with age occur in many different cell types and to varying degrees. To cover the entire span of changes throughout the analyzed populations, we sought to use an analysis method reflecting the complete immune phenotype for each sample (immune signature), instead of comparing single populations individually. In order to visualize the immune signature per sample, we used the dimensionality reduction algorithm t-SNE (t-distributed stochastic neighbor embedding) (van der Maaten, 2008), which embeds multi-dimensional data into two dimensions (t-SNE dimensions 1 and 2), while keeping local and global similarities of the original data. In these t-SNE plots, the distances between the points in the two-dimensional space represent differences and similarities within a group of samples. This method allowed us to generate an immune signature for each individual donor, representing all variables at a time. As a proof of principle, we plotted five cord blood and five adult peripheral blood samples using the t-SNE algorithm (Figure 10). As expected, cord blood samples cluster completely separated from peripheral adult blood samples, accounting for the phenotypic changes from birth to adulthood. Thorough testing indicated that variation among individual immune signatures was unaffected by fixation with 1% PFA or the origin of the sample (fresh blood or frozen PBMCs).

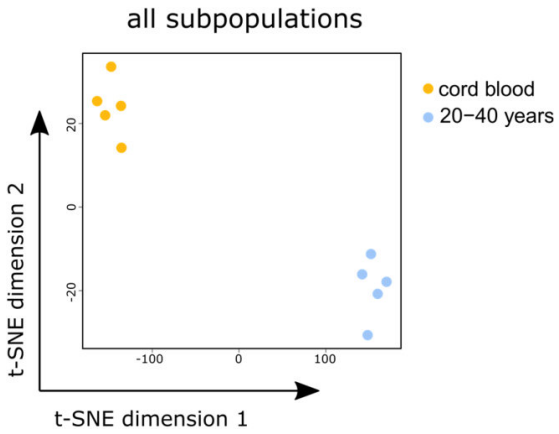

Figure 10. t-SNE plot representing immune signatures in cord blood (yellow) and adult peripheral blood (blue). Multidimensional reduction enables the representation of all subpopulations (141 subpopulations) in the 2D space. Each dot represents one donor. Distances between donors reflect the degree of inter-individual variation.

3.1.2 Dynamic age-related changes in the adaptive immune compartment

The combinatorial analysis of all 141 subpopulations shows an overall dynamic progression of the immune phenotype related to age (Figure 11 A). A progressive development is clearly appreciated when analyzing the T cell compartment (Figure 11 B), while an abrupt change in phenotype at an early age is observed for B cells (Figure 11 C). In contrast, the innate – including NK cells – and innate-like populations do not show a clear age-related progression (Figure 11 D-E).

The inter-individual variation increases with age. Considering all immune subpopulations, samples from cord blood and very young children cluster in close proximity, while the distribution of samples broadens with age (Figure 11A). The deviation of the immune signature among individuals starts during childhood, between four and twelve years of age, reflecting a higher degree of immune variation with age. The exclusive analysis of T cells (encompassing 104 parameters) (Figure 11B) reveals shows a clear age-related progression, again with increasing variation from young to old donors. Of note, Since T cell parameters account for 74% of the total populations, our analysis is strongly influenced by the age dynamics observed in the T cell compartment. The analysis of B cell parameters showed a distinct separation between samples from very young donors (< 3 years) and older donors, but we found no progression pattern within the two clusters (Figure 11 C). Innate-like lymphocytes (ILL, comprising MAIT, TCR$\gamma\delta$, and B1-like B cells) and innate lymphoid cells (ILCs, encompassing ILC1, ILC2, and ILC3) cluster together only for the cord blood samples, and divergence starts shortly after birth (Figure 11C). Remarkably, innate myeloid cells (granulocytes, monocytes and dendritic cells, NK cells) slightly separate between young donors (< 20 years) and adults. Within the clusters, there is no age-related segregation (Figure 11D).

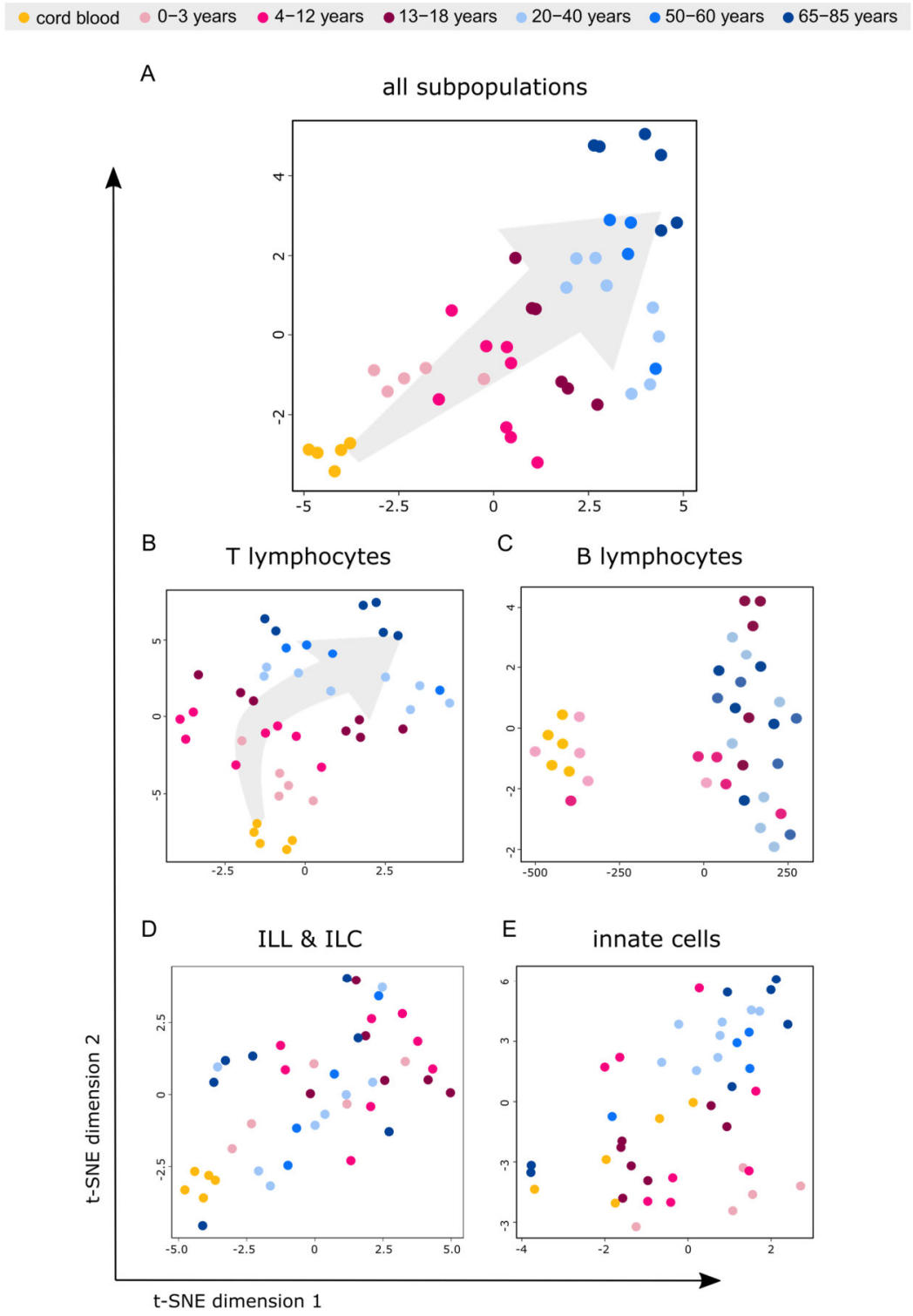

Figure 11. Age-related immune signatures in healthy donors. Analysis of all 141 subpopulations identified by our immunophenotyping approach (A) and sub-classification into T lymphocytes (104 subpopulations) (B), B lymphocytes (15 subpopulations) (C), innate-like lymphocytes and innate lymphoid cells (ILL & ILC) (MAIT, TCRγδ, B1-like B cells, ILC, NK) (22 subpopulations) (D) and innate cells (eosinophils, DCs, monocytes, NK, basophils, neutrophils) (16 subpopulations) (E). Each dot represents one healthy donor. Age-classes are indicated by colors. Manually drawn grey arrows reflect progression by age.

To ascertain which populations account for the differences in innate cells between young donors (<20 years) and adults, the relative frequencies of all innate subpopulations were plotted in a heat map (Figure 12 A). The most remarkable differences are an increased frequency of immature natural killer (NK) cells ($CD56^{bright}$), in young donors compared to adults and a rapid increase of mature neutrophils shortly after birth (Figure 12).

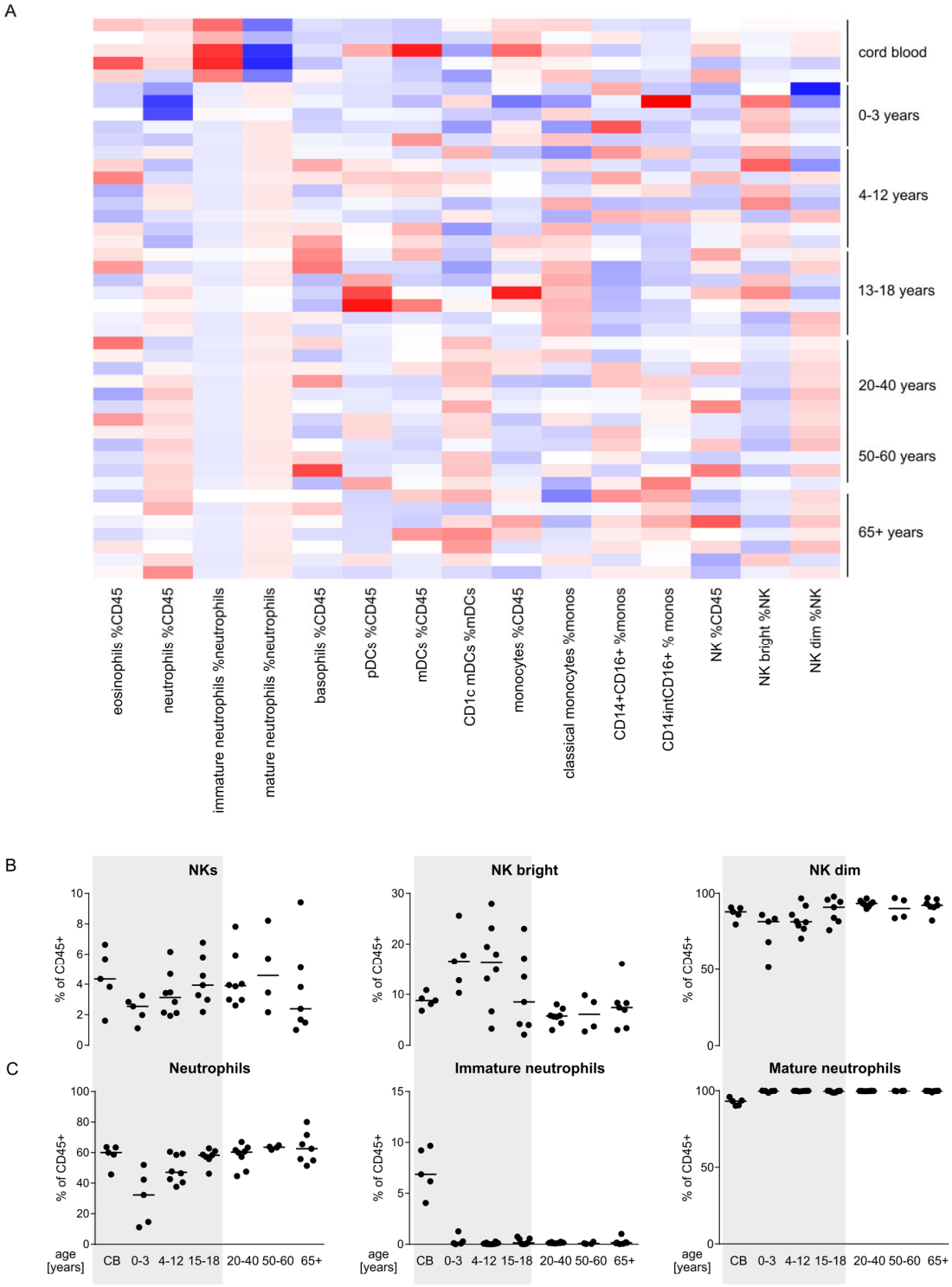

Figure 12. Age-dependent changes in NK cells and neutrophils account for separation between young children and adults in the innate compartment. A) Relative frequency of innate populations across all age-classes from high (red) to low (blue). B+C) Frequency of total NK cells and respective subpopulations (B) or neutrophils and respective subpopulations (C) by age-class. Line represents median. Grey shadow represents separation of young donors and adult according to separation shown in Figure 11 E.

A more detailed investigation of the age-related progression of all analyzed populations revealed that half of the populations are generally stable over a lifetime. Only 17% show a clear increase and 12% a clear decrease (Table 7). One quarter of the populations showed small oscillations in their frequencies.

The frequency of B and T cells decreased with age, while the frequencies of innate and innate-like lymphocytes remained predominantly stable over time (Figure 13 A). The frequencies of the major T cell subsets – CD4, CD8, Tregs and TCR$\gamma\delta$ – were stable, with only a slight decrease of TCR$\gamma\delta$ cells at the age of fifty and older (Figure 13 B). A decrease of the thymic output measured by the frequency of RTE ($CD45RA^+$ $CD31^+$) in the CD4 compartment is accompanied by the decrease of the naïve T cell pool, and an increase of cells with a memory phenotype. CD4conv and Treg cells show an accumulation of central memory (TCM, $CD45RA^-CCR7^+CD28^+$) and transitional memory (TTM, $CD45RA^-CCR7^-CD28^+$) cells with age, while the frequency of terminal effector cells (TTE, $CD45RA^+CCR7^-CD28^-$) increase in the CD8 compartment.

Functionally, the expression of chemokine receptors revealed that Th2 is the predominant phenotype at birth, while other subsets, namely Th1, Th17 and Th1*, increase later on (Figure 13 B+C). The high frequency of naive T cells and the low diversity of CD4 subsets in early infancy resulted in a reduced expression of cytokines early in life (Figure 13 D). At birth, both CD4 and CD8 cells produce exclusively IL-2, IL-8 and TNFα, while IL-10 and IL-17 production started during early infancy. IL-4 and IFNγ production increased with age, while IL-8 production strongly decreased during early childhood (> 4 years). The frequency of the other studied cytokines remained stable with age, and all of them showed a peak at puberty (15-16 years). We analyzed the co-expression of cytokines and their association with the memory marker CD45RO on CD4 cells using tSNE analysis of raw-data from flow cytometry (Figure 14). A heat map-like visualization of the expression of cytokines or CD45RO enabled us to investigate the co-expression of markers on a single cell level. We found that IL-8 was exclusively expressed in naïve ($CD45RO^-$) CD4 cells. Half of the naïve CD4 cells producing IL-8 also expressed IL-2 and TNFα. In addition, a second population that produced IL-2 and TNFα consisted of memory ($CD45RO^+$) cells and showed co-expression with IFNγ and IL-17. In contrast to IL-2, IL-8, and TNFα, which are already produced at birth, all other studied cytokines are exclusively expressed by memory ($CD45RO^+$) T cells.

Table 7. Overview of changes in immune cell frequencies during aging. Cell subpopulations are divided in clearly increasing (left), slightly increasing (middle left), stable (middle), slightly decreasing (middle right) and clearly decreasing (right) population frequencies by age.

	increasing		stable	decreasing	
innate & innate-like subsets	neutrophils $CD56^+CD16^{dim}$ NKs	mDCs	basophils NKs eosinophils mature neutrophils immature neutrophils pDCs ILC1 / 2 / 3 CD1c mDCs total monocytes CD14+CD16+ monocytes classical monocytes	$CD14^{int}CD16^+$ monocytes	total ILCs $CD56^+CD16^{bright}$ NKs
B cells	naive B cells memory B cells	switched B cells non-switched B cells $sIgD^-CD27^-$ B cells memory $CD20^+CD5^+$ B cells $CD38^{low}CD21^{low}$ B cells	B1-like B cells B2 B cells plasma B cells plasma $CD20^+CD5^+$		total B cells transitional B cells $CD20^+CD5^+$ B cells transitional $CD5^+$ B cells
main T cell subsets		TCRαβ $CD4^+CD25^+$	CD4 T cells CD8 T cells $CD4^+CD8^+$ $CD4^-CD8^-$ MAIT TCRγδ Vδ1 Vδ2 Vδ2notVγ9	Tregs other TCRγδ	total T cells
T helper	Th1 Th17 Tfr	Th1* Th22 Tfh	Th2		
memory differentiation	central memory CD4 central memory Tregs central memory CD8 central memory $CD4^+CD25^+$ transitional memory CD4 transitional memory Tregs transitional memory CD8 terminal effector CD4 terminal effector CD8	stem cell memory CD4 stem cell memory CD8 effector memory CD8	naive CD4conv $CD25^+$ stem cell memory Tregs stem cell memory $CD4^+CD25^+$ effector memory Tregs effector memory $CD4^+CD25^+$ terminal effector Tregs terminal effector $CD4^+CD25^+$	transitional memory $CD4^+CD25^+$	naive CD4 naive CD8 naive Tregs
Cytokine production	IL-4 IFNγ		IL-2 IL-10 IL-17 TNFα		IL-8
CD8α/β chain distribution	CD8 $CD8\alpha\beta^{int}$	$CD4^+CD8^+$ $CD8\alpha\beta^{int}$ MAIT $CD8\alpha\beta^{int}$	$CD8\ CD8\alpha\alpha$ $CD4^+CD8^+$ $CD8\alpha\beta$ TCRγδ $CD8\alpha\alpha$ TCRγδ $CD8\alpha\beta^{int}$ TCRγδ $CD8\alpha\beta$ MAIT $CD8\alpha\beta$	MAIT $CD8\alpha\alpha$ $CD4^+CD8^+$ $CD8\alpha\alpha$	CD8 $CD8\alpha\beta$
ectoenzyme expression	$CD73^+$ CD4	$CD39^+$ CD4 $CD39^+$ Tregs $CD39^+CD73^+$ Tregs	$CD39^+CD73^+$ CD4 $CD39^+CD73^+$ CD8 CD39+CD73+ CD4CD25+ $CD39^+$ CD8 CD39+ $CD4^+CD25^+$ CD73+ $CD4^+CD25^+$	$CD73^+$ Tregs	$CD73^+$ CD8
others	activated Tregs		activated CD8 activated Tregs activated $CD4^+CD25^+$ $CD4^+CD25^+$ RTE $CD38^+$ Tregs $CD38^+$ $CD4^+CD25^+$ $CD25^-CD127^-$ CD4 $CD25^-CD127^-$ CD8	$CD38^+$ CD4 $CD38^+$ CD8	CD4 RTE Tregs RTE

*HLA-DR+ / CCR4+

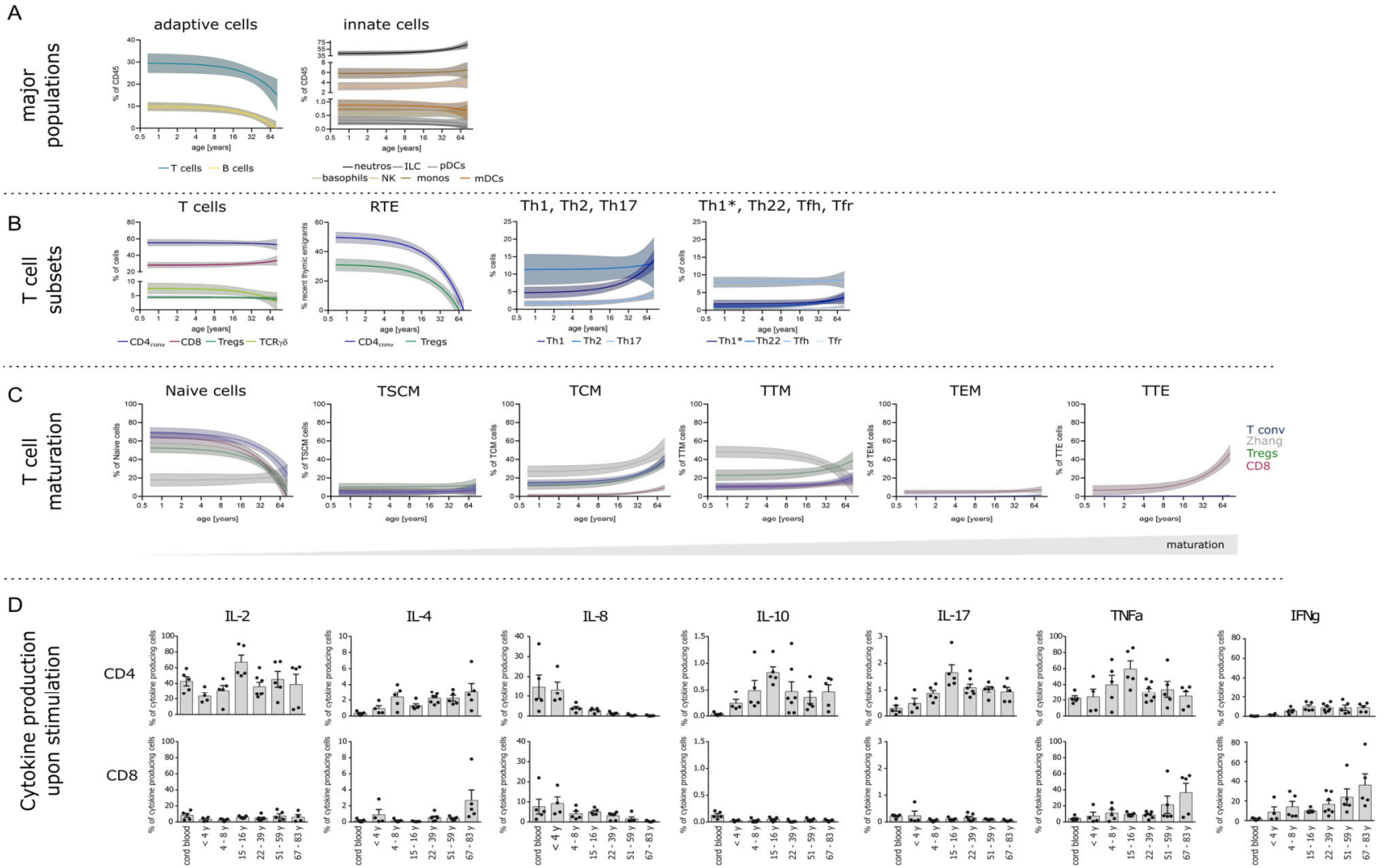

Figure 13. Major changes in the frequencies of immune populations of the adaptive immune system arise during aging. Regression band representation of immune cell frequencies from early age to 85 years for major immune cell populations. (A) T cell subsets: CD4, CD8, Tregs, TCRγδ, recent thymic emigrants and differentiated helper T cell. (B) T cell maturation states (C) and cytokine production by CD4 and CD8 T cells (D). Bars represent mean ± SEM.

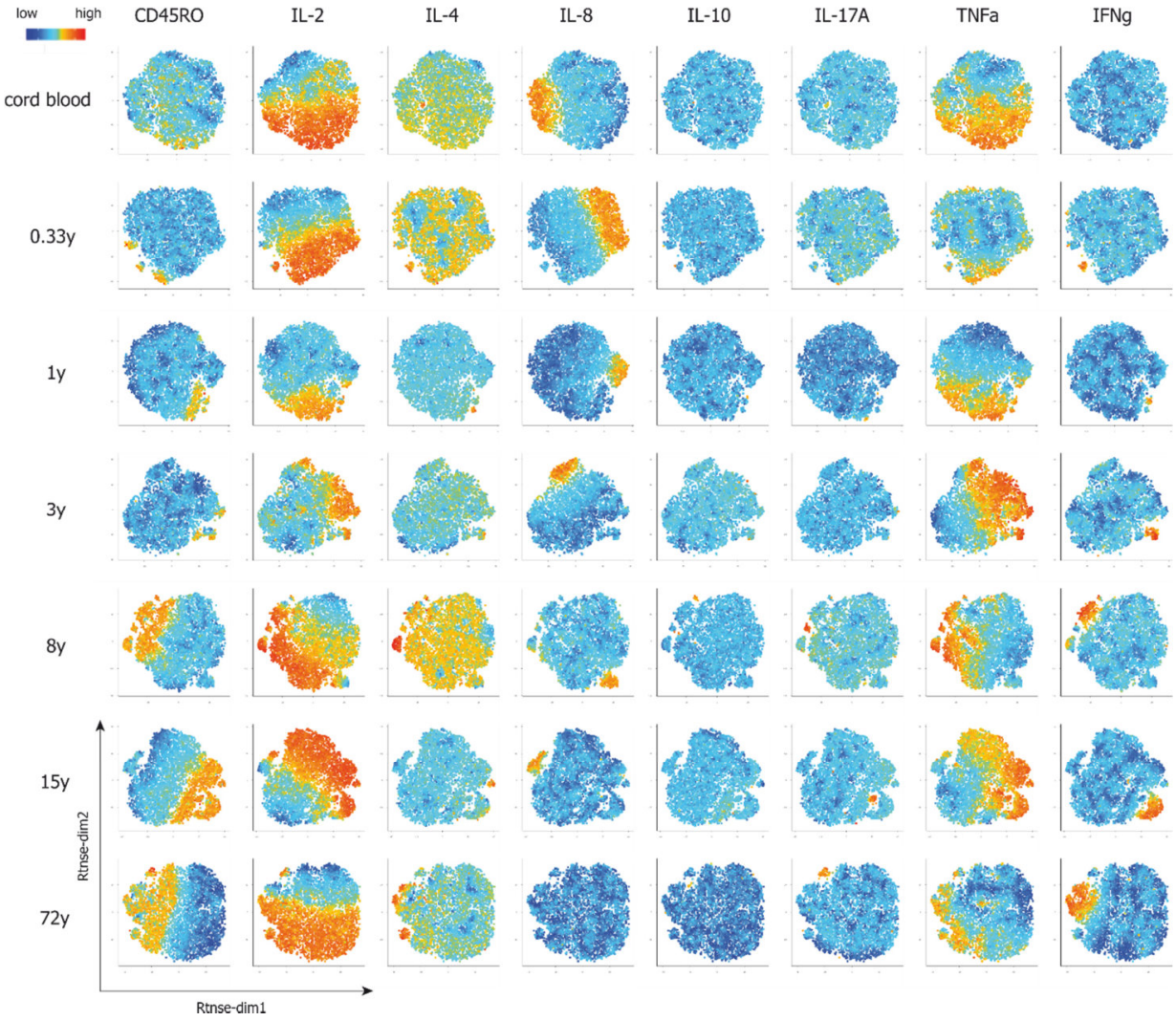

Figure 14. Cytokine production by CD4 cells of representative donors at different ages. tSNE representation of flow cytometry data on the expression of IL-2, IL-4, IL-8, IL-10, IL-17, TNFα, and IFNγ in CD4 cells after *ex vivo* stimulation with PMA/Iono. Each row represents the results for one donor. The age is indicated on the left. Marker (top) expression is color-coded from blue (low expression) to red (high expression).

3.1.3 Premature aging of the T cell compartment and drastic reduction of ILC2 in children with *de novo* mutations in *BCL11B*

Bcl11b is a transcriptional regulator playing an important role in T cell lineage development (Wakabayashi et al., 2003). Bcl11b-deficient mice show an early blockade during T cell development resulting in a reduced TCRαβ, but not TCRγδ compartment, and in an increase of NK cells (Wakabayashi et al., 2003, Li et al., 2010). In addition, Bcl11b regulates the development and function of murine ILC2 cells (Walker et al., 2015, Yu et al., 2015, Califano et al., 2015, Yu et al., 2016). I had the chance to analyze the immune compartment of a cohort of ten children with heterozygous mutations in *BCL11B* (assembled by Dr. D. Lessel, Department of Human Genetics) (Figure 15). All children presented in the clinic with cognitive and motor developmental delay, and trio exome analysis revealed *de novo* mutations in *BCL11B* in all cases, namely seven frameshift

mutations, three missense mutations and one translocation The precise location of the mutations is shown in Figure 7(Lessel et al., 2018).

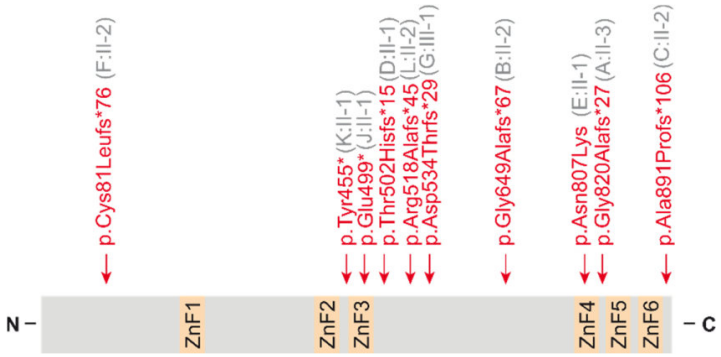

Figure 15. Location of the mutations in the protein structure of BCL11B. Schematic protein structure of BCL11B: the position of the mutations identified in this study are marked with vertical arrows and shown in red (ZnF- zinc-finger C2H2 domain, N- N terminus; C- C terminus). Modified from (Lessel et al., 2018).

I analyzed the immune composition in the peripheral blood of affected children, and compared it to controls of various ages. Differential blood counts were normal, except high eosinophil counts in three children (Table 8). Using my comprehensive immunophenotyping approach and t-SNE representation of the frequencies of each subpopulation, we found a clear shift of the T cell signature of children with mutation in *BCL11B* towards older age-classes (Figure 16 B). Considering innate-like lymphocytes (ILL, comprising MAIT, TCR$\gamma\delta$, and B1-like B cells), innate lymphoid cells (ILCs, encompassing ILC1, ILC2, ILC3) and innate myeloid cells (granulocytes, monocytes and dendritic cells, NK cells), we found most samples of children with mutation in *BCL11B* to cluster in close proximity, indicating some common phenotype (Figure 16 C+D).

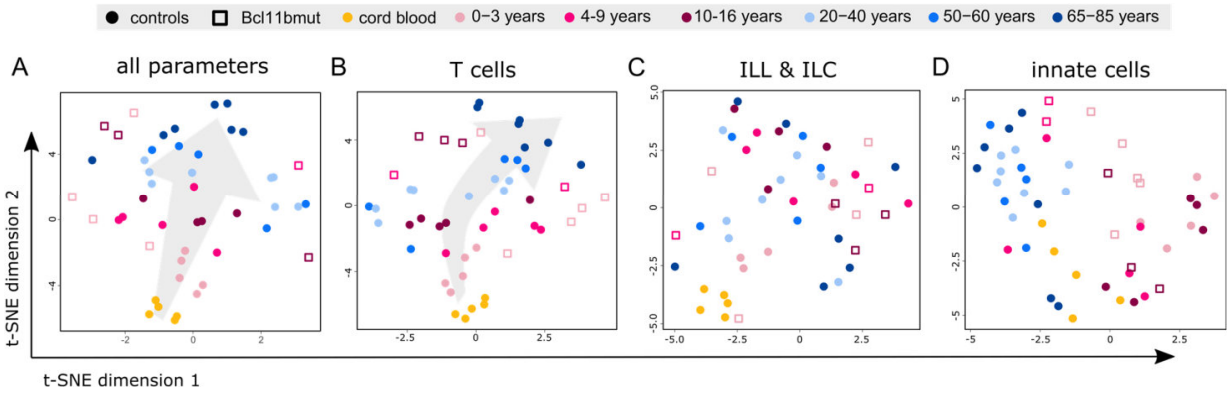

Figure 16. Combinatorial analysis shows premature aging of the T cell compartment in children with *BCL11B* mutations. Multidimensional reduction algorithm tSNE analysis for healthy controls (filled circles) and affected individuals (empty squares). A) Analysis of all of subpopulations identified by our comprehensive immunophenotyping approach and sub-classification into T cell populations (B), innate-like lymphocytes and innate lymphoid cells (C) and innate myeloid cells (C). Each symbol represents one donor. Age-classes are indicated by colors. Manually drawn grey arrows reflect the progression by age.

We found a severe reduction in RTE to less than half of the normal age-dependent values in eight out of ten children, in line with a bias towards a memory phenotype in CD4 and CD8 cells (Figure 17 A &). TCR

repertoire analysis, performed for two cases (L. Kuhlmann & Prof. I. Prinz, Hannover Medical School), resulted in normal diversity of the T cell compartment (not shown). In addition, the frequency of TCRγδ cells was 3.5 fold higher in children bearing a Bcl11b mutation (Figure 17 B, left). The distribution of the major TCRγδ subsets Vδ1 and Vδ2Vγ9 was slightly shifted to higher Vδ1 frequencies (Figure 17 B, right).

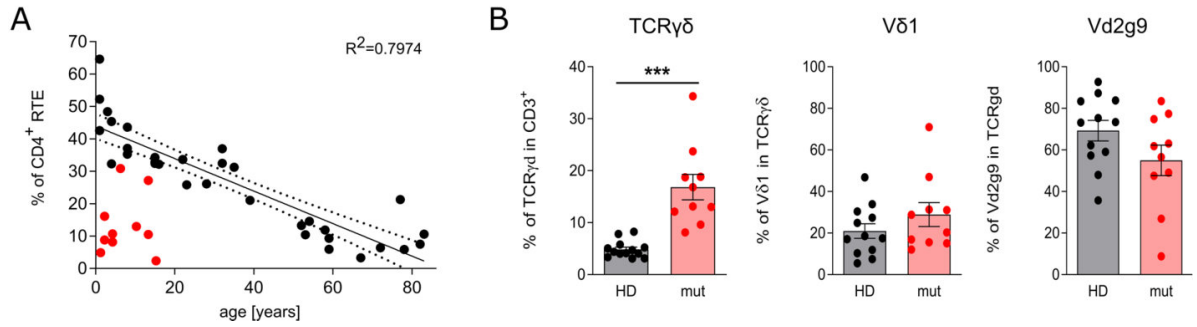

Figure 17. Increased frequency of TCRγδ cells and reduction of recent thymic emigrants in children with *BCL11B* mutations. A) Relative frequencies of recent thymic emigrants (RTE) for healthy controls (black) and children with *BCL11B* mutations (red). Regression line is plotted with 95% confidence interval. B) Reduced frequencies of total TCRγδ cells (left) in peripheral blood of children bearing a mutation in *BCL11B* (red) and healthy age-matched controls (black). Distribution of the major TCRγδ subsets Vδ1 (middle) and Vδ2Vγ9 (right) is slightly shifted towards Vδ1. Bars represent the mean ± SEM. ***p<0.001.

Considering the important role of Bcl11b in the development of murine ILC2 cells, we wondered whether we can see an effect on the ILC compartment in human. In all cases, ILC2 cells were greatly reduced in numbers and frequencies, while the overall ILC compartment as well as ILC1 and ILC3 were unaffected (Figure 18). In contrast to Bcl11-deficient mice, NK cell frequencies were in the normal age-range in children with mutations in *BCL11B*.

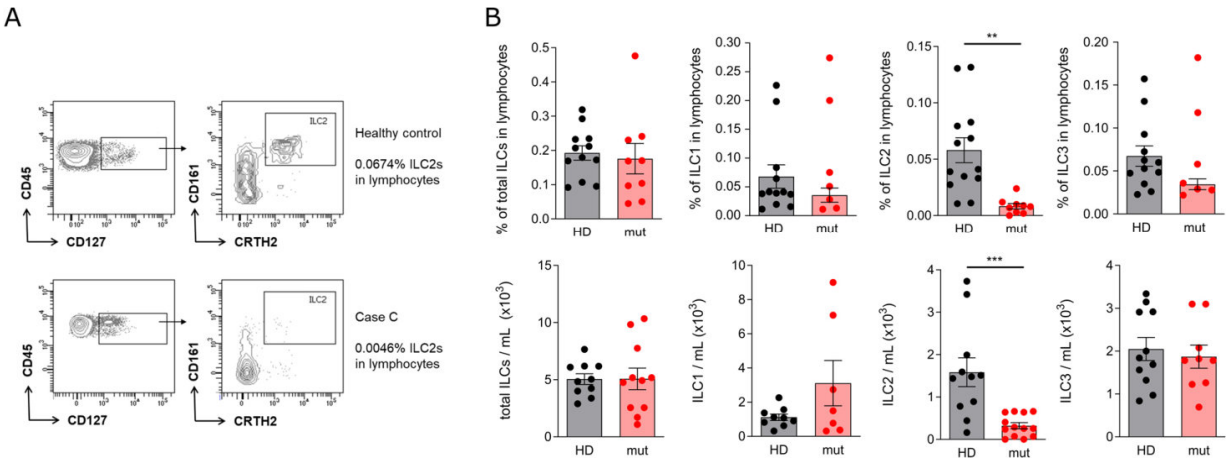

Figure 18. Drastic loss of ILC2 cells in blood from children with mutations in *BCL11B*. A) Representative gating strategy for the identification of total ILCs (CD45+lineage-CD161+CD127+) and, specifically, ILC2s ($CD45^+lineage^-CD161^+CD127^+CRTH2^+$) in peripheral blood from children with mutation in *BCL11B* (bottom) and healthy age-matched control (top). Selection for total ILCs is performed on a $CD45^+lineage^-HLA\text{-}DR^-$ gate. B) Relative frequencies (top) and absolute counts (bottom) of total ILCs, ILC1, ILC2 and ILC3. Bars represent mean ± SEM. **p<0.01, ***p<0.001.

In summary, using our extensive immunophenotyping approach, we found that children bearing a mutation in the *BCL11B* gene have an altered T cell compartment with a clear reduction of recent thymic emigrants, an increase in TCR$\gamma\delta$ cells and strongly diminished frequencies of ILC2 cells (Table 8) (Lessel et al., 2018). Multidimensional analysis showed a bias of the immune signature of children with *BCL11B* mutation towards older age-classes, indicating an aged immune signature. The clear separation of children with *BCL11B* mutation from age-matched controls, even in the absence of overt immune deficiency, stresses the resolution of our immune phenotyping approach.

Table 8. Cell blood counts and immune phenotyping of patients with alterations in *BCL11B*.

Individual	A:II-3	B:II-2	C:II-2	D:II-1	E:II-1	F:II-2	G:III-1	H:II-1	J:II-1	K:II-1	L:II-2	Normal range
Sex	Female	Male	Male	Female	Male	Male	Female	Male	Female	Male	Male	
Age (years)	4	15	1	4	2	18	13	13	6 6/12	9 11/12	10 5/12	
Haematocrit and cell counts												
Haematocrit (%)	35.3	43.2	47.6	31.2	39.9	40.4	40.0	43.2	36.3	37.6	39.9	32-43
WBC (10^6/mL)	4.50	15.0	15.0	6.07	-	8.7	10.8	8.1	2.4	7.5	7.2	5.5-15.5
Neutrophils (10^6/mL)	2.79	7.48	4.3	1.6	-	-	3.8	2.8	1.1	-	2.5	1.5-8
Eosinophils (10^6/mL)	0.24	1.58	0.12	0.36	0.05	0.97	0.1	0.7	0.5	-	1.16	< 0.5
Monocytes (10^6/mL)	0.27	0.92	1.05	0.73	0.22	0.59	0.46	0.7	0.2	-	0.7	0.2-0.9
Lymph. (10^6/mL)	1.51	5.34	9.45	3.22	2.4	3.52	4.5	3.4	0.5	1.95	2.9	1.5-7
Lymphocytes												
T cells (%)	71.5	70.1	65.8	37.9	42.9	79	65.4	72.3	-	77	30.7	49-83
B cells (%)	16.3	11.5	21.1	31.8	36.0	8	16.0	7.5	-	15	5.1	8-31
T-lymphocytes												
CD4+ (%)	44.9	41.8	26.3	65.5	52.5	38	59	44	28	59.7	35.4	27-53
CD8+ (%)	38.3	39.4	48.9	21.0	27,0	43	26.1	39.5	25	35.0	32.6	16-40
CD4/CD8 ratio	1.2	1.1	0.5	3.1	1.9	0,9	2.2	1.1	1.1	1.7	1.1	0.7-2.6
T-γδ (%)	13.0	13.1	18.8	8.1	18.7	19§	9.6	16.8	-	5.3§	23.9	< 6
Treg (% in CD4+)	6.8	5.1	7.3	4.9	9.4	-	4.0	5.8	-	-	3.78	4.5-10 *
Naïve CD4+ (%)	32.6	27.8	69.9	44.7	48.5	-	61.3	30.2	49	-	40.6	50-70 *
RTE in CD4+ (%)	8.2	2.4	4.9	10.7	8.8	-	27.1	10.5	16	-	13	age depend.
Naïve CD8+ (%)	20.9	27.7	48.0	43.2	73.2	-	47.1	9.8	-	-	22.1	50-90 *
DR+ CD4+ (%)	0.2	2.7	1.7	3.3	2.1	-	0.1	0.3	-	normal	1.3	0.1-1.8 *
DR+ CD8+ (%)	2.0	1.8	2.8	4.8	1.0	-	0.3	0.1	-	normal	0.6	0.3-2.6 *
TNFα+ IFNγ+ in CD4+ (%) IL17+	59.1 31.2 1.7	58 32.1 3.4	32.4 5.2 0.6	76.4 4.6 2.4	50.2 6.4 0.6	-	24.8 6.4 1.7	18.9 6.8 2.6	-	-	8.61 9.73 2.02	43-86 * 5-15 0.5-4
TNFα+ IFNγ+ in CD8+ (%)	35.7 48.8	40.8 46.6	8.4 25.9	22.3 55.3	17.2 3.6	-	8.6 17.3	7.7 12.5	-	-	4.18 12.1	10-20 * 7.5-20

Continuation Table 8. Cell blood counts and immune phenotyping of patients with alterations in *BCL11B*.

Individual	A:II-3	B:II-2	C:II-2	D:II-1	E:II-1	F:II-2	G:III-1	H:II-1	J:II-1	K:II-1	L:II-2	Normal range
Innate Lymphoid cells (% in lymphocyte gate)												
NK cells (%)	7.1	13.6	10.9	23.3	9.0	11	2.9	11.3	27.3	4	3.6	3-30
ILC (% in lymph.)	0.169	0.097	0.104	0.241	0.045	-	0.230	0.050	-	-	0.144	0.169-0.621 *
ILC1 (% in lymph.)	0.051	0.028	0.075	-	0.013	-	0.200	0.011	-	-	-	0.016-0.433 *
ILC2 (% in lymph.)	0	0.012	0.007	0.008	0.003	-	0.009	0.002	-	-	0.008	0.027-0.131 *
ILC3 (% in lymph.)	0.118	0.058	0.022	-	0.029	-	0.028	0.036	-	-	-	0.027-0.158 *
***BCL11B* alteration**	p.Gly820Ala*fs**27	p.Gly649Ala*fs**67	p.Ala891Pro*fs**106	p.Thr502His*fs**15	p.Asn807Lys	p.Cys81Leu*fs**76	p.Asp534Thr*fs**29	Positional effect	p.Glu499*	p.Tyr455*	p.Arg518Alafs*45	

3.2 Effect of prenatal steroid treatment on the thymus and the peripheral T cell compartment

3.2.1 Transient reduction in fetal thymic size after prenatal steroid treatment

Using a mouse model for prenatal GC treatment, we found that administration of betamethasone to the pregnant dam one day before delivery resulted in a dramatic reduction of the thymic size in the newborn pups (Diepenbruck 2013), and changes in the TCR repertoire that persisted later in life (Gieras et al., 2017, Diepenbruck et al., 2013). To investigate potential alterations in thymus size in human fetuses after prenatal steroid treatment, we used ultrasound data on fetal thymus size from the PRINCE (Prenatal Identification of Children's Health) cohort obtained by PD Dr. Diemert and colleagues in the Department of Gynecology and Obstetrics at the UKE (Diemert et al., 2016). In this cohort, the transverse diameter of the fetal thymus was measured at three time points, namely at weeks 23-24, 28-29 and 35-36 of gestation in 573 pregnant women, including seven cases treated with prenatal steroids. Prenatal steroid treatment resulted in a strongly reduced or even negative thymic growth progression observed in the measurement after treatment (Figure 19 A). This decrease was compensated by an increase in thymus growth observed in the subsequent measurement, yielding thymus sizes close to untreated controls. To exclude child weight as a bias for our analysis, we assessed the relation between estimated fetal weight at time of measurement and thymus size, and confirmed the reduced or negative thymic growth progression after prenatal steroid treatment (Figure 19 B).

To determine how long does fetal thymic atrophy persist after steroid administration, we analyzed the mean thymic growth per week in each fetus in relation to the time elapsed between treatment and measurement. Remarkably, reduced thymic growth was evident up to six weeks after prenatal steroid treatment (Figure 19 C). Comparing the thymic growth from treated and untreated pregnancies, we found a clear clustering of fetal thymi from pregnancies who had recently (< 40 days before measurement) received prenatal steroid treatment in the lower 10^{th} percentile of the normal growth range. In contrast, thymic growth rates from fetuses that had received prenatal steroids more than six weeks before measurement clustered within the top 50% of the growth ranges, thus outperforming untreated controls (Figure 19 D). Altogether, these data demonstrate a decreased rate of thymic growth after prenatal steroid treatment that may last for several weeks, followed by a compensatory increase during a recovery period.

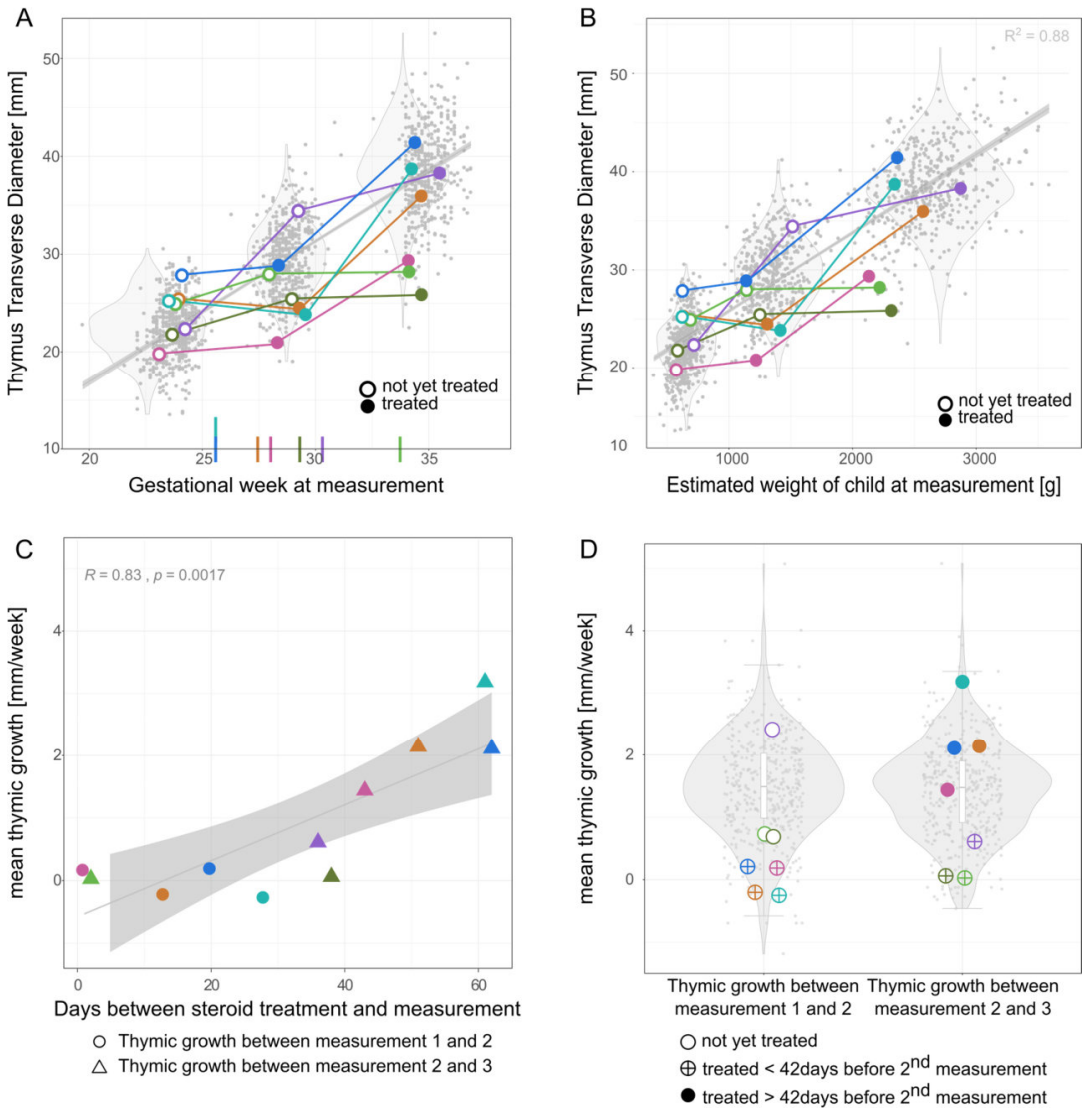

Figure 19. Transient reduction in fetal thymic size after prenatal steroid treatment A-B) Thymus transverse diameter measured by fetal thymus ultrasound in healthy controls (grey) and fetuses prenatally treated with steroids (colored) correlated to gestational age (A) or to estimated fetal weight at measurement (B). Each colored line represents consecutive measurements for one fetus. Lines at the bottom indicate the time point of steroid treatment. Open circles (○) represent measurements before treatment, and filled circles (●) represent the time points after treatment. The grey shadow represents the distribution of the data. The Regression line is based on data from untreated healthy controls. C) Calculated mean of the fetal thymic growth after prenatal steroid treatment in relation to the elapsed time between treatment and measurement. Each color represents one individual. Thymic growth was calculated between the first and the second (circle) or the second and the third (triangle) measurements. The regression band is shown in grey. D) Comparison of the mean fetal thymic growth of healthy controls (grey) and individuals receiving prenatal steroids. Open circles represent cases before treatment (○); while fetal thymic weight from fetuses treated with steroids within six weeks after treatment are shown by circles with a cross (⊖), and measurements taken more than six weeks after treatment are indicated by filled circles (●). Each color represents one individual. The distribution of the data is shown in grey.

3.2.2 Transient reduction of murine thymus size and altered expression of tissue restricted antigens in the thymus of steroid treated mice

Thymocytes are not the only cell type in the thymus sensitive to steroids. The injection of steroids, at a dose five times higher than in our prenatal steroid model, resulted in a transient loss of murine thymic epithelial cells (TEC), particularly medullary thymic epithelial cells (mTEC) (Fletcher et al., 2009). mTEC, expressing tissue-restricted antigens (TRA), are required for proper negative selection of thymocytes. We wondered whether treatment-relevant doses of steroids could lead to changes in the TEC compartment.

To assess the integrity of the TEC compartment, we used qPCR to measure gene expression of transcription factors, structural proteins and autoantigens in the thymus, with or without steroid treatment. In our established mouse model for prenatal steroid treatment, we injected 10µg Betamethasone i.p. to pregnant dams on day E18.5. The concentration of bet in serum of the pups within a litter was however, highly variable (Diepenbruck et al., 2013). To ensure an equal dose and to avoid masking a potentially mild effect, we directly injected four week old C57BL/6 mice. Gene expression analysis performed on mRNA isolated from thymi of betamethasone-treated mice and controls (PBS) resulted in unreliable data due to the massive thymocyte death. To circumvent this issue, we used mice lacking the GR in T cells ($GR^{fl/fl}$Lck-Cre). In these animals, thymocytes should not be affected by betamethasone injection due to the thymocyte-specific knock-down of the glucocorticoid receptor (Tronche et al., 1999). Indeed, *in vitro* steroid treatment resulted in steroid resistance of thymocytes from $GR^{fl/fl}$Lck-Cre mice, detected by the frequency of living cells after overnight incubation with Betamethasone (0-1 µM) (Figure 20). Inhibition of the GR in thymocytes from $GR^{fl/fl}$Lck-Cre mice with mifepristone (RU486) did not change the overall survival of thymocytes. As expected, we found a strong reduction in the frequency of living cells in control mice with an intact GR on thymocytes ($GR^{fl/fl}$), while the addition of RU486 prevented thymocyte apoptosis.

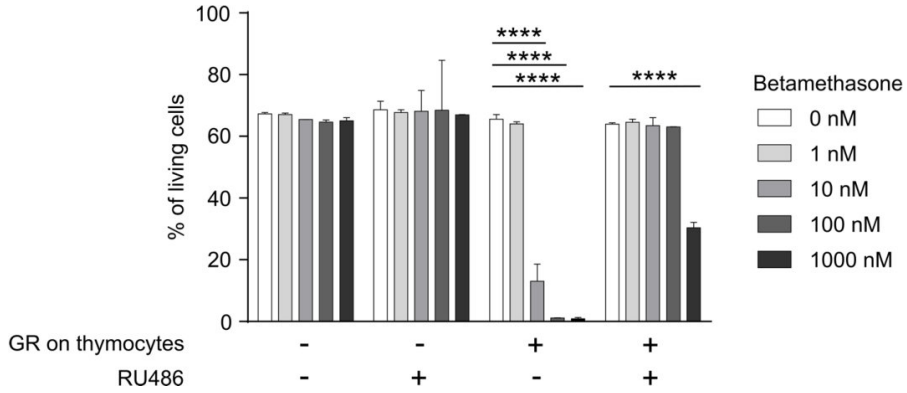

Figure 20. Thymocytes of $GR^{fl/fl}$Lck-Cre mice are resistant to steroid treatment *in vitro*. Thymocytes from $GR^{fl/fl}$Lck-Cre or $GR^{fl/fl}$ were incubated overnight with betamethasone (0-1µM) in the presence (+) or absence (-) of the glucocorticoid receptor antagonist mifepristone (RU486). ****p<000.1.

Surprisingly, *in vivo* injection (i.p.) of the treatment-relevant dose of Betamethasone (10µg) to $GR^{fl/fl}$Lck-Cre resulted in a transient reduction in thymic weight of 25% compared to PBS-treated $GR^{fl/fl}$Lck-Cre mice three days after treatment (Figure 21). The effect was still noticeable after correction for body weight (thymic

index). At day five after treatment, the thymus was still small per age, and at day ten, the difference to the control had disappeared. Glucocorticoids did not affect the weight of the spleen in relation to the body weight.

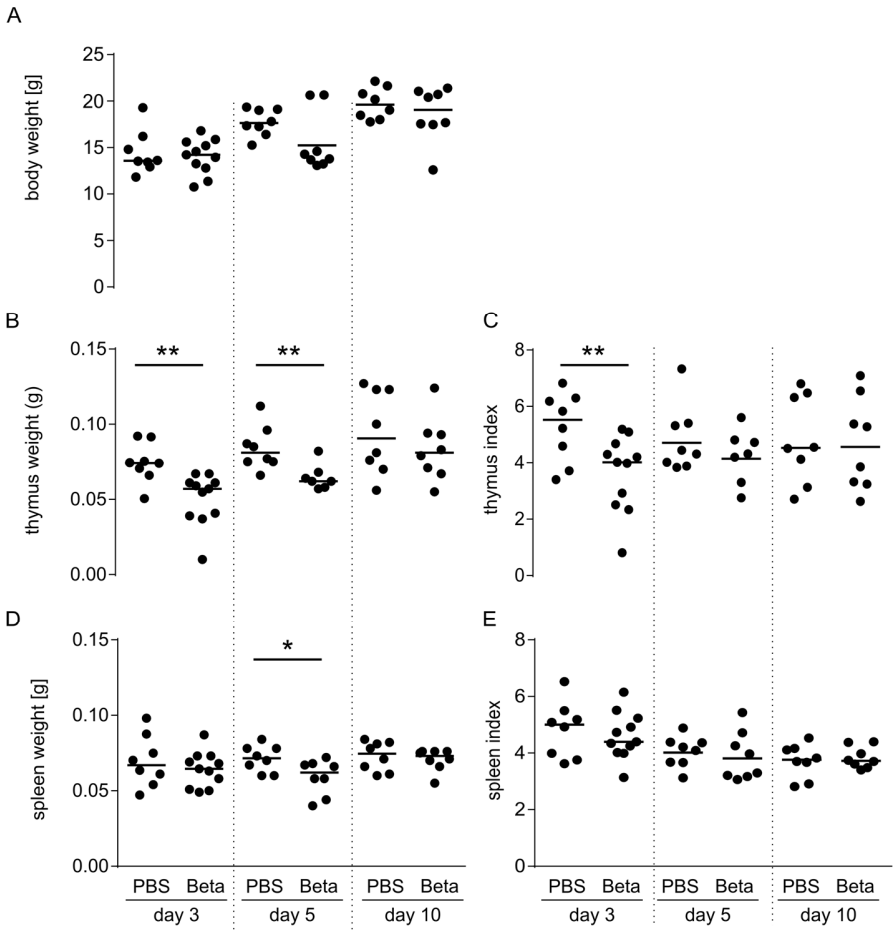

Figure 21. Transient reduction in thymic weight after prenatal steroid treatment. $GR^{flox}Lck^{Cre}$ mice were injected i.p. with 10µg Betamethasone or PBS (control) and body weight (A), thymus weight (B) and spleen weight (D) were evaluated on day three, five and ten after treatment. Weight index for thymus (C) and spleen (D) was calculated as ratio of organ to body weight. *p<0.05, **p<0.01

TRA expression in mTECs is required for proper negative selection of developing thymocytes. The transcription factors autoimmune regulator (Aire) and Forebrain Embryonic Zinc Finger-Like Protein 2 (Fezf2) promote the expression of a large number of TRAs. AIRE and Fezf2 each drive the expression of a specific set of TRAs with only low overlap.

To investigate the effect of steroid treatment on TEC, we had to consider the massive cell death of thymocytes after incubation with steroids. To circumvent this problem, we used the $GR^{fl/fl}$Lck-Cre mice, which should have a thymus with comparable cellularity, even after steroid treatment. We performed qPCR analysis on mRNA from thymi of betamethasone or PBS treated $GR^{fl/fl}$Lck-Cre mice. After an initial (day 3) increase in the gene expression of *Krt5*, a cytokeratin exclusively expressed in mTECs, and *Krt8,* a

cytokeratin exclusively expressed in cTEC, we found a subsequent decline in gene expression that reached basal levels on day five for *Krt5* and day ten for *Krt8* (Figure 22 A + B).

To assess if changes in the mTEC compartment affect the expression of the major transcription factors driving TRA expression, we compared *Aire* and *Fezf2* expression in $GR^{fl/fl}$Lck-Cre mice after steroid treatment. We found a slight transient 1.5 fold increase in *Aire* and *Fezf2* expression on day three after betamethasone treatment, but not in PBS treated control animals (Figure 22 C + D).

An alteration in the gene expression of *Aire* and *Fezf2* - even if very mild - might result in subsequent changes of TRA expression. To explore the effect on TRA expression, we analyzed the mRNA levels of two TRAs which expression is exclusively induced by Aire (Insulin2) or Fezf2 (Klk1b16) (Takaba et al., 2015). The relative gene expression levels of the two TRAs Insulin2 and Klk1b16 were equally altered after steroid treatment (Figure 22 E + F). In brief, gene expression of *Insulin2* and *Klk1b16* in thymi from $GR^{fl/fl}$Lck-Cre mice treated with steroids increased three days after treatment, but was reduced on days five and ten compared to PBS treated animals. The expression of *Klk1b16* was affected twice as much as *Insulin2,* which is accompanied by a slightly higher increase in the gene expression of *Fezf2* than of *Aire*. We found the same ratio of the percentage change in gene expression of Ins2:Aire (2.8) and Klk1b16:Fezf2 (2.9).

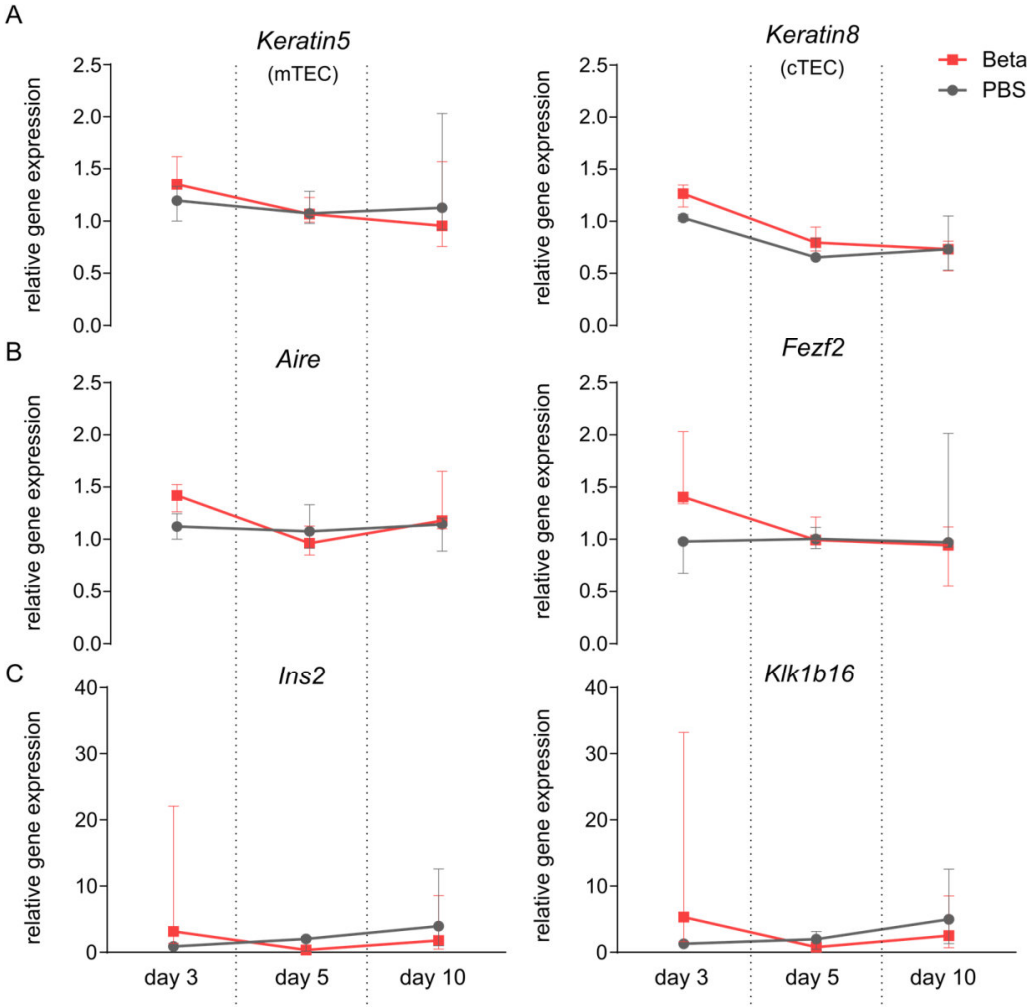

Figure 22. Transiently increased gene expression of transcription factors that drive the expression of TRAs. Expression levels of *Krt5* (mTEC) and *Krt8* (cTEC) (A), the major transcription factors driving TRA expression in mTECs *Aire* and *Fezf2* (B) and of the TRAs *Ins2* (Aire-dependent) and *Klk1b16* (Fezf2-dependent) three, five and ten days after betamethasone treatment. Gene expression was normalized to the gene expression of the house keeping gene Polr2A. One PBS control on day three was used as calibrator for all samples.

3.2.3 Increased Treg frequency and a bias towards Vδ2 cells in cord blood cells of neonates after prenatal steroid treatment

The transient reduction in thymus size, the subsequent rapid thymic growth and the alterations in self-antigen expression in the murine thymus after steroid treatment, raised the question of possible effects of prenatal steroid treatment on the peripheral T cell compartment. A cooperation with the Department of Pediatrics at the UKSH Lübeck (Prof. C. Härtel & Dr. J. Pagel) provided us access to a collection of 30 cord blood samples from children born late-preterm (GW30-35) (PP Lübeck cohort), which were either untreated (n=10) or treated with prenatal steroids (n=20) (Table 9). We subdivided the group of treated individuals into children who were recently (two weeks prior to birth) treated with steroids (n=10), or treated longer than two weeks before birth (n=10). Samples consisted of frozen CBMCs, precluding the analysis of neutrophils. In addition, due to the small amount of material available, we had to restrict the analysis to a single staining panel, and we decided to focus on T cells. We analyzed 31 populations with one multicolor flow cytometry panel, using CBMCs isolated from 100µL cord blood. Five samples had to be excluded due to a poor cell recovery rate (< 1500 T cells) upon thawing, and the remaining samples were distributed as follows: untreated (n=8), recently treated (n=9), and neonates treated with steroids longer than two weeks before birth (n=8). Gestational age range was carefully matched for all three groups (Table 9).

Table 9. Population characteristics of PP Lübeck cohort

		PRETERM BIRTH		
SAMPLE SIZE		**8**	**9**	**8**
TREATMENT		No ACS	ACS recently *< 14 days before birth*	ACS long-term *> 14 days before birth*
GESTATIONAL AGE AT BIRTH	Median	33.2	32.1	31.1
	Mean	33.8	31.6	30.7
	min	30.5	30.4	29.6
	max	34.0	34.4	33.1
SEX	% male	50 %	44 %	50 %

Flow cytometric analysis showed a two-fold higher frequency of Tregs in cord blood of neonates that had received prenatal steroids shortly before birth (Figure 23 A). In contrast, if the treatment had taken place longer than two weeks before birth, the frequency of Tregs was only slightly higher than in untreated neonates, indicating normalization. CD4 and CD8 cell frequencies were not significantly altered, but we found a slight trend towards lower frequencies of CD8 cells after prenatal steroid treatment (Figure 23 A). In addition, we found a significant increase in Vδ2 cells in cord blood of recently treated neonates compared to untreated controls (Figure 23 B). More than two weeks between prenatal steroid treatment and birth resulted in a less pronounced increase in the frequency of Vδ2 cells, again closer to normal values. We also found that the increase in the frequency of Vδ2 cells lasts longer than the transient increase for Treg cells. The frequency of total TCRγδ cells was slightly diminished in treated newborns, but differences did not reach statistically significant values.

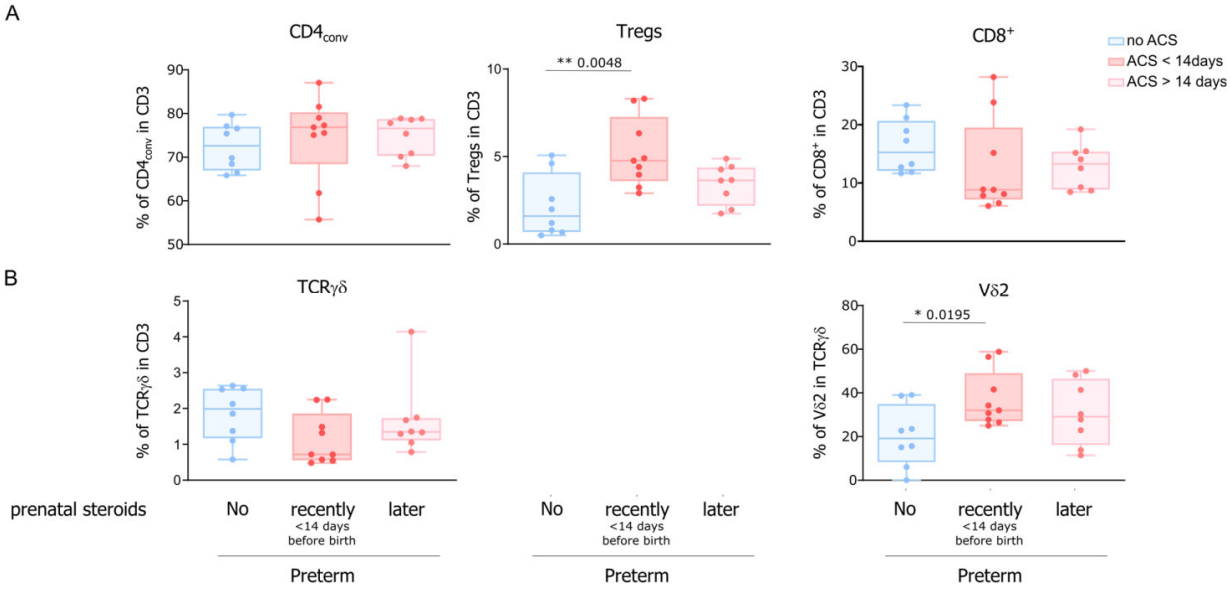

Figure 23. Transient increase in the frequency of Tregs and Vδ2 cells in cord blood after prenatal steroid treatment. A. Relative frequency of CD4conv, Tregs and CD8 cells in CD3 cells (A) and total TCRγδ cells and Vδ2 cells (B) in cord blood samples from untreated (blue) or neonates treated with prenatal steroids less (darker red) or more (lighter red) than two weeks before birth. Data was generated on samples from the PP Lübeck cohort. Boxes represent 5-95 percentile. *p<0.05, **p<0.01

In addition, we analyzed an independent cohort of children born late-premature (PP UKE cohort), provided by an add-on cohort from the PRINCE study of the Department of Gynecology and Obstetrics at the UKE Hamburg (Prof. P. Arck, PD Dr. A. Diemert, Prof. K. Hecher). In this cohort, we analyzed 26 cord blood samples with the complete set of panels of our deep immunophenotyping approach (Table 10). Samples were classified again in untreated neonates (n=10) and neonates treated recently (n=9) or longer than two weeks before birth (n=7) with prenatal steroids. Gestational age at birth ranged from 30 to 36 weeks in treated individuals and 34 to 37 weeks in the untreated ones. Normality ranges were obtained from the analysis of 39 term birth (GW 38+) samples without prior steroid treatment using the same staining panels

Table 10. Population characteristics of PP UKE cohort.

		PRETERM BIRTH			TERM BIRTH
SAMPLE SIZE		**10**	**9**	**7**	**39**
TREATMENT		No ACS	ACS recently *< 14 days before birth*	ACS long-term *> 14 days before birth*	No ACS
GESTATIONAL AGE AT BIRTH	Median	35.1	34.1	34.5	40.0
	Mean	35.0	33.1	34.4	39.9
	min	34.0	30.4	30.4	38.2
	max	36.4	34.2	36.0	41.4
SEX	(% male)	60 %	66 %	71 %	36 %

In this cohort, we found a slight positive trend in the Treg frequency of cord blood from neonates recently treated with steroids (Figure 24 A). Of note, the differences were not significant, but a great variance (±5%) of the overall Treg frequency was observed in all groups. In this cohort, we found a higher proportion of CD8

and a correspondingly lower proportion of CD4 in neonates recently treated with steroids, in a ratio that is commonly found in term-born children. Cord blood from children who received steroids more than two weeks before birth showed almost normal frequencies of CD4 conventional, Treg and CD8 cytotoxic T cells. A tendency to higher percentages of Vδ2 cells – not significant in this cohort – could also be observed (Figure 24 B).

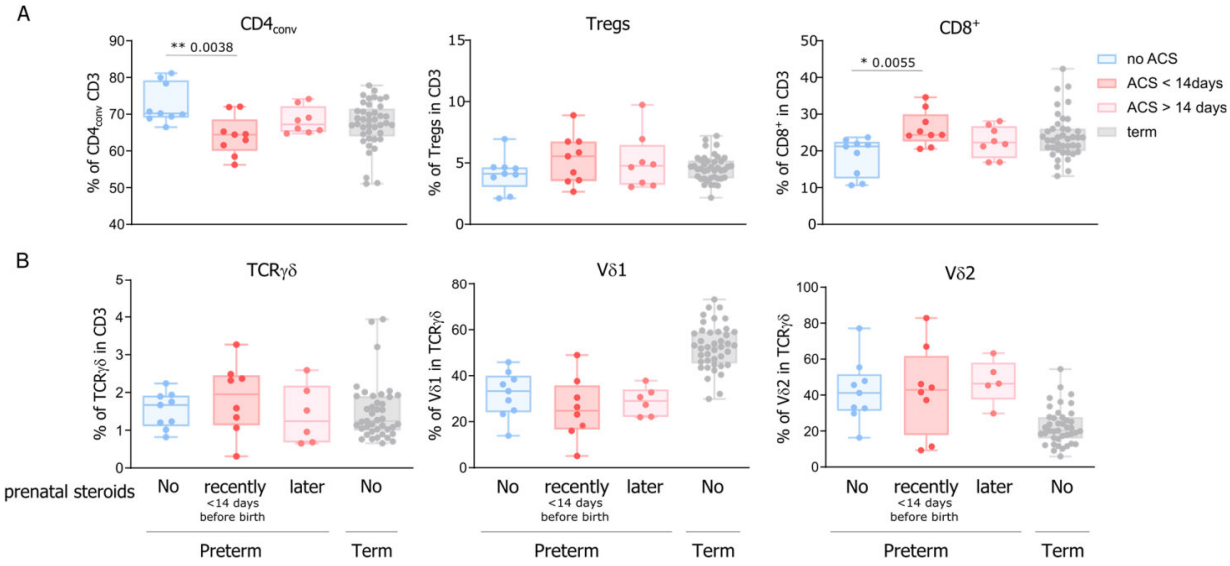

Figure 24. Cord blood analysis of the frequency of Tregs and TCRγδ cells in a second cohort of steroid-treated newborns. A Relative frequencies of CD4conv, Tregs and CD8 cells in CD3 cells (A) and total TCRγδ cells and Vδ2 cells (B) in cord blood samples from untreated (blue) or neonates treated with prenatal steroids less (darker red) or more (lighter red) than two weeks before birth. Data were generated on PP UKE cohort. Boxes represent 5-95 percentile. *p<0.05, **p<0.01

3.2.4 Prenatal steroid treatment results in short-term increase of cytokine production by CD4 cells

We investigated the cytokine production after stimulation of CBMC in term and preterm births (Figure 25). As expected, CD4 cells from preterm neonates showed limited cytokine production. We detected IL-2 in one third, IL-8 in 10%, and TNFα in two thirds of CD4 cells, while IL-4, IL-10, IL-17 and IFN-γ responses were negligible in untreated preterm children. We found significantly higher production of IL-2, IL-8 and IL-10 in samples from neonates recently treated with prenatal steroids compared to samples from untreated neonates, or neonates treated more than two weeks before birth. CD8 cells from cord blood of untreated preterm neonates produced IL-2 (9 ± 6 %), IL-8 (6 ± 5 %), IFNγ (5 ± 2 %) and TNFα (19 ± 14 %), but no differences were found in newborns prenatally treated with steroids. In contrast to CD4 cells, IFNγ production by CD8 cells was clearly detectable in all preterm neonates, independently of the treatment.

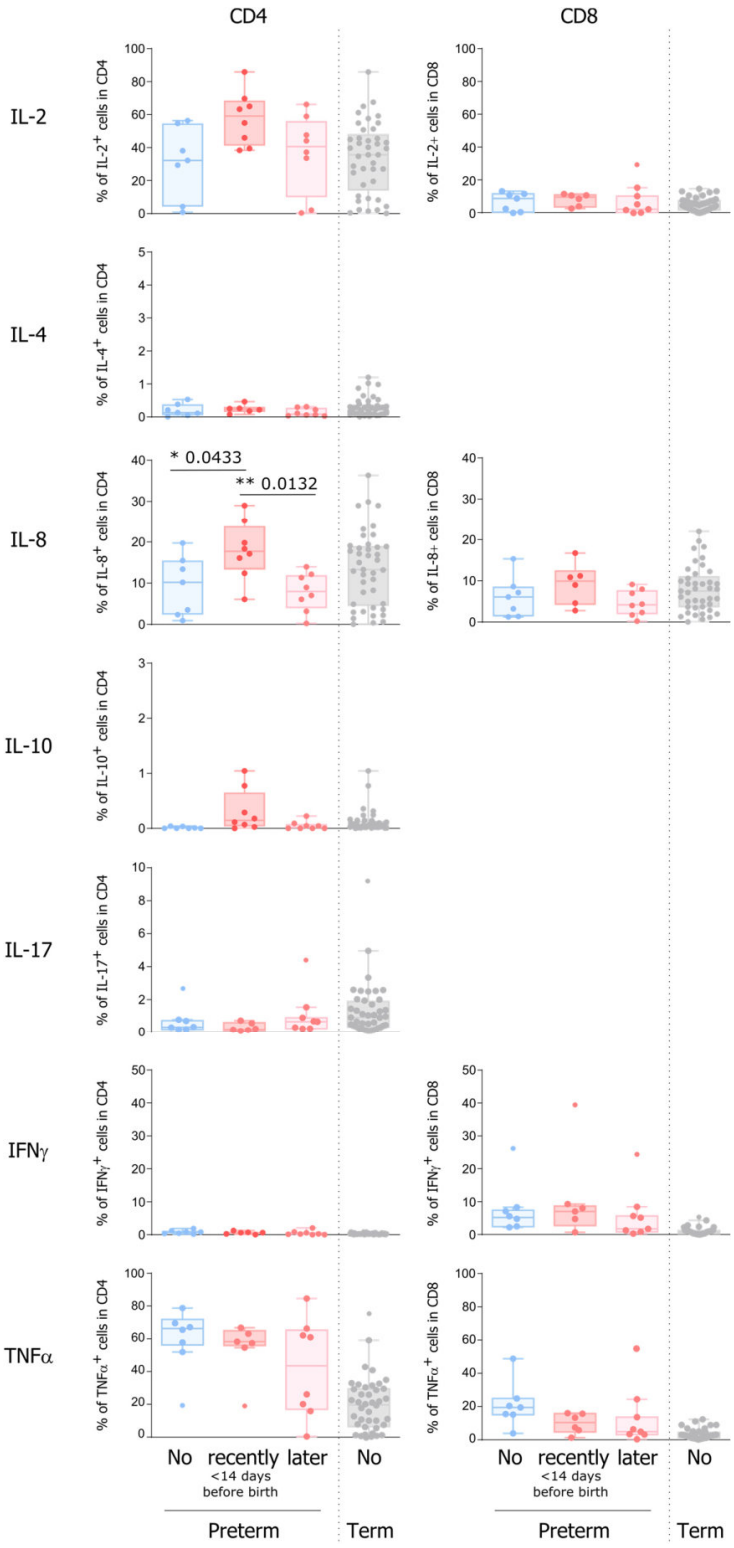

Figure 25. Cytokine production by cord blood T cells from prenatally treated and untreated preterm and term births. Cytokine production (IL-2, IL-4, IL-8, IL-10, IL-17, TNFα, IFNγ) after *ex vivo* PMA/Iono stimulation in CD4 (top) and CD8 (bottom) T cells from cord blood cells of untreated (blue) or neonates treated with prenatal steroids less (darker red) or more (lighter red) than two weeks before birth. Data was generated on samples from the PP UKE cohort. Boxes represent 5-95 percentiles. *p<0.05, **p<0.01.

3.2.5 No lasting changes in the immune signature, but risk for the development of immune diseases in children prenatally treated with steroids

We wondered whether the observed effects of prenatal steroid treatment observed at birth may lead to long-lasting changes in the immune compartment of children born preterm. Analysis of the T cell signature of young children (≤5 years) from the PRINCE cohort who were born preterm showed no differences to term-born controls (Figure 26). Furthermore, we found no changes in the immune signature of children prenatally treated with steroids compared to children born preterm who did not receive prenatal steroids.

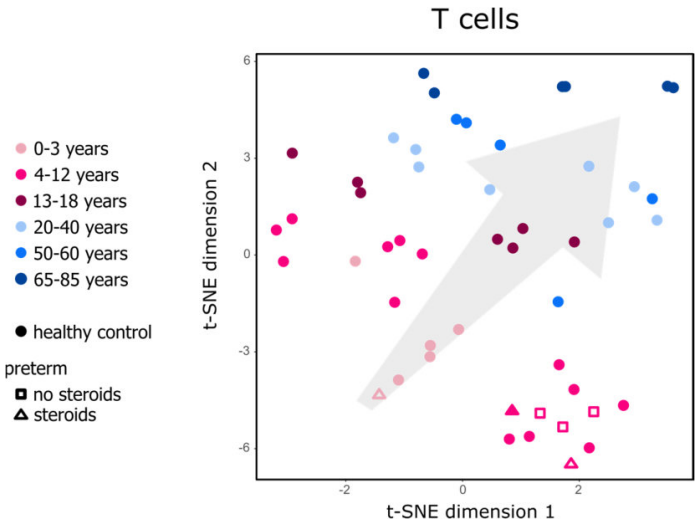

Figure 26. The peripheral T cell signature of steroid-treated and untreated late preterm born children is indistinguishable from untreated controls. tSNE analysis for 104 T cell subpopulations in healthy controls (filled circles) and children born preterm and prenatally treated with steroids (empty triangle) or untreated (empty square). Each symbol represents one donor. Age-classes are indicated by colors. Manually drawn grey arrow reflects progression by age.

When looking at the populations that specifically showed changes related to prenatal steroid treatment, namely Tregs and the major TCR$\gamma\delta$ subsets Vδ1 and Vδ2Vγ9, we found no differences at the age of five years (Figure 27)

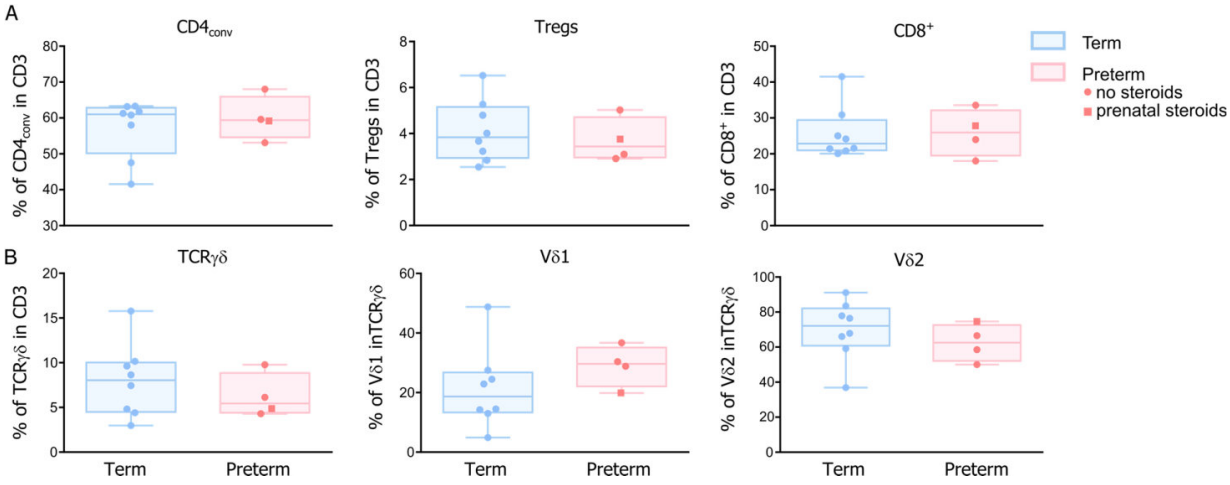

Figure 27. Term-born and preterm children show comparable frequencies of Tregs and TCRγδ subsets at the age of 5 years. Analysis of the frequencies of T cell subsets (A) and TCRγδ subsets (B) in children born term (blue) or preterm (red), at the age of 5 years. One preterm born child received prenatal steroid treatment (■).

To further investigate the effect of prenatal steroid treatment on disease susceptibility, even in the absence of obvious alterations in the immune signature, I used data arising from exhaustive questionnaires collected for the PRINCE study. The questionnaires were given to the parents at six month of age and yearly from one to five years. The health-related parameters as analyzed include the number of medical consultations, the prevalence of diseases such as mumps or measles, diseases of the respiratory and gastro-intestinal tract, allergy, reactive immune diseases and the intake of medication for allergy or immune reactive diseases. The variables generated summarizing the collected parameters are depicted in Table 11.

Table 11. Overview of the analyzed parameters and their underlying variables

	PARAMETER	INCLUDED VARIABLES
NUMBER OF	GENERAL MEDICAL CONSULTATIONS	Number of medical consultations excluding regular "U"-examinations
	INFLUENZA INFECTIONS	Influenza infections
	LOWER RESPIRATORY TRACT INFECTIONS	Pneumonia, bronchitis, angina, croup (diphtheric/non-diphtheric)
	UPPER RESPIRATORY TRACT INFECTIONS	Conjunctivitis, inflammation of the middle ear, thrush
	URINARY / GASTRO-INTESTINAL TRACT INFECTIONS	Gastrointestinalinfections, colic, bladder/urinary tract infections
	OTHER VIRAL OR BACTERIAL INFECTIONS	mumps, measles, rubella, pertussis, chicken pox, scarlet fever, mononucleosis, fifth disease, salmonella, herpetic infections

PREVALENCE OF	ALLERGIES	cow milk, soy products, eggs, peanuts, pollen, house dust mites, dye-/preservative agent, dogs, cats, antibiotics, medication, soap, insect bites
	IMMUNE REACTIVE DISEASES	hay fever, psoriasis, asthma, contact dermatitis
	MEDICATION FOR ALLERGY OR REACTIVE IMMUNE DISEASES	sprays for asthma treatment (such as Budecort, Aarane, Aerodur), steroidal therapy, or anti-histaminic

The collection of questionnaire data for the PRINCE cohort is still ongoing. Currently, data are available for sample sizes varying between four and 21 individuals, and are decreasing with age mainly due to the fact that study participants have not yet reached the 5 year age mark (Table 12), but also drop-out of participants or missing datasets. Gestational age at birth of preterm born children ranged from 31 to 37 weeks of gestation in prenatally-treated children, and from 32 to 36 weeks in untreated children born preterm. We found a slightly higher number of medical consultations during the first two years of life in preterm born children prenatally treated with steroids compared to untreated children. This tendency, however, disappears between two and five years, and presumably reflects an enhanced medical follow-up during the first months of life. Finally, we did not find any clear difference in the prevalence of influenza infections, lower- and upper respiratory tract infections, urinary-gastro-intestinal tract infections, and other viral or bacterial infections between preterm children receiving prenatal steroids and controls.

Table 12. Prevalence of medical consultations and infections in children born preterm during first five years of life.

	AGE AT ASSESSMENT	6 MONTHS		1 YEAR		2 YEARS		3 YEARS		4 YEARS		5 YEARS	
	SAMPLE SIZE	21	12	14	10	10	5	11	4	10	5	6	4
	PRENATAL STEROIDS	-	+	-	+	-	+	-	+	-	+	-	+
Prevalence [events/individual/year]	MEDICAL CONSULTATIONS	4.85	7.5	4.93	5	2.6	7.8	2.5	2.3	1.75	2	1.2	1.8
	INFLUENZA INFECTIONS	2.9	2.3	4.53	5	4.1	6	3.75	2.2	3.6	2.2	2	2.2
	LOWER RESPIRATORY TRACT INFECTIONS	-	-	2.13	1.4	1.4	2.3	1.9	1.5	2.6	0.6	0.7	0.4
	UPPER RESPIRATORY TRACT INFECTIONS	-	-	1.73	0.6	1.2	2.4	0.9	0.7	1.1	0	0.4	1
	URINARY / GASTRO-INTESTINAL TRACT INFECTIONS	-	-	1.06	1.2	0.7	1	1	0.5	1.6	0.2	1	0.4
	CONTAGIOUS CHILDHOOD DISEASE	-	-	0.13	0.2	0.3	0.2	0.5	0.2	0.6	0.2	0.3	0.2

We also analyzed the incidence of immune-mediated diseases, allergy or medication reported by the parents of children born preterm who received prenatal steroid treatment or not (Figure 28). Prenatally-treated children showed a higher probability of developing reactive immune diseases, allergy, or require more medication than untreated preterm children. Here, the group sizes are small and re-assessment of the data after completion of data collection will be needed to validate these results.

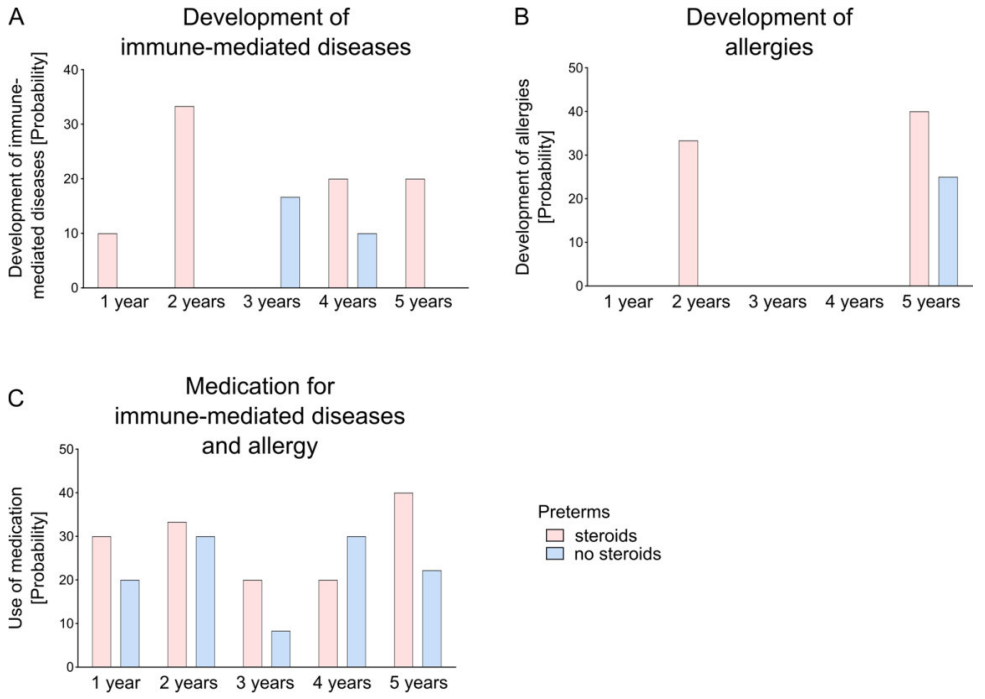

Figure 28. Increased probability of developing immune-mediated diseases or allergy in children prenatally treated with steroids. Parent-reported prevalence of reactive immune diseases (A), allergy (B) or medication for either of these conditions (C) in children born preterm and prenatally treated with steroid (red) or untreated (blue) during their first five years of life. The sample size for each time point corresponds to the cases specified in Table 12.

In summary, we show that prenatal steroid treatment results in short-term effects on the thymus size and in the cord blood composition. We observed a reduced fetal thymus size and a decrease in the growth rate lasting up to six weeks after prenatal steroid treatment. In the murine model, we found a transient slight increase in the gene expression of structural genes (*Keratin 5*, *Keratin 8*), the transcription factors Aire and Fezf2 and in two TRAs, Insulin2 (Aire-dependent) and Klk1b16 (Fezf2-dependent), in thymus cells after *in vivo* steroid treatment. Moreover, we also report transient alterations of the cord blood composition, namely an increase in the frequency of Treg and Vδ2 cells and significantly higher amounts of the cytokines IL-2, IL-8 and IL-10 in CD4 cells after *ex vivo* stimulation. We did not observe any long-term changes of the peripheral immune compartment in children previously receiving prenatal steroids, however, parent-reported prevalence of allergy and reactive immune diseases indicates a higher risk to development disease for preterm children receiving prenatal steroids compared to untreated preterm born children.

3.3 Effects of early life thymectomy on the developing immune system

3.3.1 Prematurely aged immune signature in children who underwent thymectomy early in life

We have shown that transient thymic ablation due to prenatal steroid treatment results in alterations in the immune compartment at birth. A more drastic form of thymic ablation is thymectomy or surgical removal of the thymus upon cardiac surgery. Because cardiac surgery is often necessary soon after birth, these children are irreversibly deprived of the main source of naive T cells very early in life. Here, we aimed to investigate the effects on the immune compartment of children undergoing early life thymectomy by using our deep immunophenotyping approach. Thanks to a cooperation with the Department of Pediatrics (PD Dr. R. Kobbe, Dr. U. Schulze-Sturm), we obtained blood samples from four children that had undergone complete thymectomy early in life (age range: 1.5-17 years). We found no distinct differences in the surveyed populations of the innate compartment (DCs, monocytes, basophils, neutrophils, NK, eosinophils) in children after thymectomy compared to age-matched controls. In the adaptive immune compartment, we found a decrease in the relative frequency of T cells ($CD3^+$) and an increase in B cells ($CD19^+$) in all thymectomized individuals. Analysis of the B cell subpopulations revealed a slightly reduced frequency of class-switched memory B cells ($CD27^+sIgD^-$) and higher frequencies of non-class-switched memory B cells. Tfh (CXCR5+) cells provide B cell help by inducing immunoglobulin class switch and high-affinity antibody production in memory B cells, and we thus expected a reduction in their frequency. Surprisingly, we detected higher frequencies of Tfh cells in peripheral blood from early thymectomized children than in healthy controls. The T cell compartment was overall biased towards an activated and memory phenotype, in detriment of naïve cells and recent thymic emigrants, both cell types reflecting a healthy thymic output. Repeatedly stimulated effector CD4 cells, defined by the lack of CD25 and CD127 expression, were higher in children after early life thymectomy. Moreover, we observed elevated frequencies of $CD25^+$ conventional $CD4^+$ T cells. All four patients showed increased Th2 cell frequencies and three of them featured elevated frequencies of Th1 cells. In the TCR$\gamma\delta$ cell compartment, TCR$V\delta1$ cells were slightly reduced compared to age-matched controls, leading to a premature predominance of TCR$V\delta2V\gamma9$ cells - similar to what we have seen in cord blood upon prenatal steroid treatment. Table 13 summarizes the immune phenotype of the analyzed thymectomized children.

Table 13. Common changes in subpopulation frequencies in lymphocytes of four children who underwent early life thymectomy

		Donor 1	Donor 2	Donor 3	Donor 4	Feature in 100%	Feature in ≥ 75%
	Age [years]	1.5	4.5	8	17		
	Sex	F	F	M	?		
Conventional T cells	↓ Total T cells	x	x	x	x	■	■
	↓ CD4conv RTE (CD31$^+$CD45RA$^+$)	x	x	x	x	■	■
	↓ CD4conv naive	x	x	x	-		■
	↙ CD8	x	x	x	x	■	■
	↙ CD8 naïve	x	x	x	x	■	■
	↑ Repeatedly activated CD4 cells (CD25$^-$CD127$^-$)	x	x	x	x	■	■
	↑ IL-17 prone CD4 (CCR6$^+$CD161$^+$)	x	x	x	x	■	■
	↑ Activated Tregs (CCR4$^+$)	x	x	x	-		■
	↑ CD4conv CD25+	x	x	x	x	■	■
	↑ CD4conv memory	x	x	x	x	■	■
	↑ CD8 memory	-	x	x	x		■
	↑ CD4conv CD25+ memory	x	x	x	-		■
	↑ Th1	-	x	x	x		■
	↑ Th2	x	x	x	x	■	■
	↑ Th17	x	x	-	-		■
	↑ T follicular helper cells	-	x	x	x		■
Tregs	↓ Tregs RTE (CD31$^+$CD45RA$^+$)	x	x	x	-		■
	↙ Tregs naive	x	x	x	x	■	■
	↑ Tregs memory	x	x	x	x	■	■
TCR$\gamma\delta$& MAIT	↙ TCRVδ1	x	x	x	x	■	■
	↖ TCR Vδ2Vγ9	x	x	x	x	■	■
	↙ MAIT	x	x	x	x	■	■
B cells	↑ Total B cells	x	x	x	x	■	■
	↑ Unswitched memory B cells (CD27+sIgD+)	-	x	x	x		■
	↙ Switched memory B cells (CD27+sIgD-)	x	x	x	x	■	■

We used the tSNE dimensionality reduction approach to visualize the effect of early thymectomy on the T cell signature. Two cases showed a marked shift in the T cell signature towards older age-classes (Figure 29), indicating premature aging of the T cell compartment

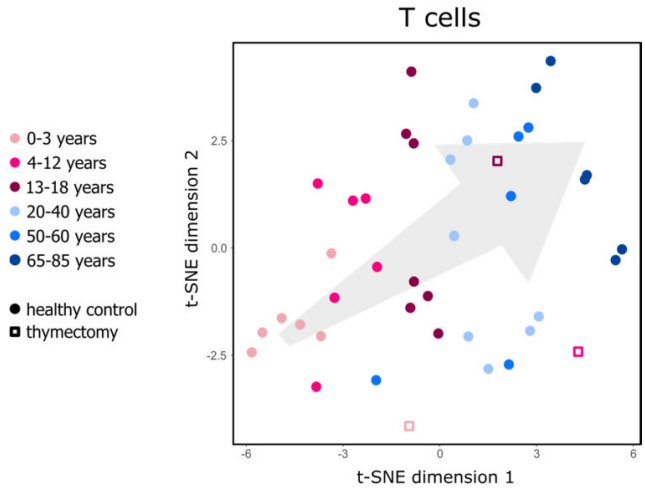

Figure 29. Children with early life thymectomy exhibit a prematurely aged T cell signature. Multidimensional reduction algorithm tSNE analysis for T cell populations in healthy controls (filled circles) and children with early life thymectomy early in life (empty squares). Each symbol represents one donor. Age-classes are indicated by colors. The grey arrow reflects progression of the T cell signature with age.

3.3.2 Minor alterations of the adaptive immune compartment are already present at the time of heart surgery

We next inquired if the stated perturbations are definitively resulting from thymus removal upon heart surgery, or if the immune compartment could be already altered before the operation. To answer this question, we analyzed peripheral blood of young children (<1 year) taken at the time of corrective heart surgery (n=13). The samples were provided by the Department of Pediatric Cardiology (Dr. S. Sachweh). In these children with heart disease, but not yet thymectomized, we found lower absolute cell counts of lymphocytes and monocytes, but increased values for granulocytes (data not shown). In the lymphocyte compartment, we detected lower frequencies of B and T cells, but normal ranges for the main T cell subsets, namely CD4conv, Tregs and CD8 cells (Figure 30 A + B). The frequency of RTE and naïve cells in CD4conv and Treg cells was strongly reduced in very young children (< 0.5 years) undergoing surgery compared to age-matched controls (Figure 30 C, D), suggesting poor thymic output This difference, however, was not observed in children undergoing heart surgery after 6 months of age. (Figure 30 D). We also observed a concomitant increase in the memory T cell compartments of Tconv, Tregs and CD8 T cells in very young children undergoing surgery compared to healthy controls (Figure 30 E).

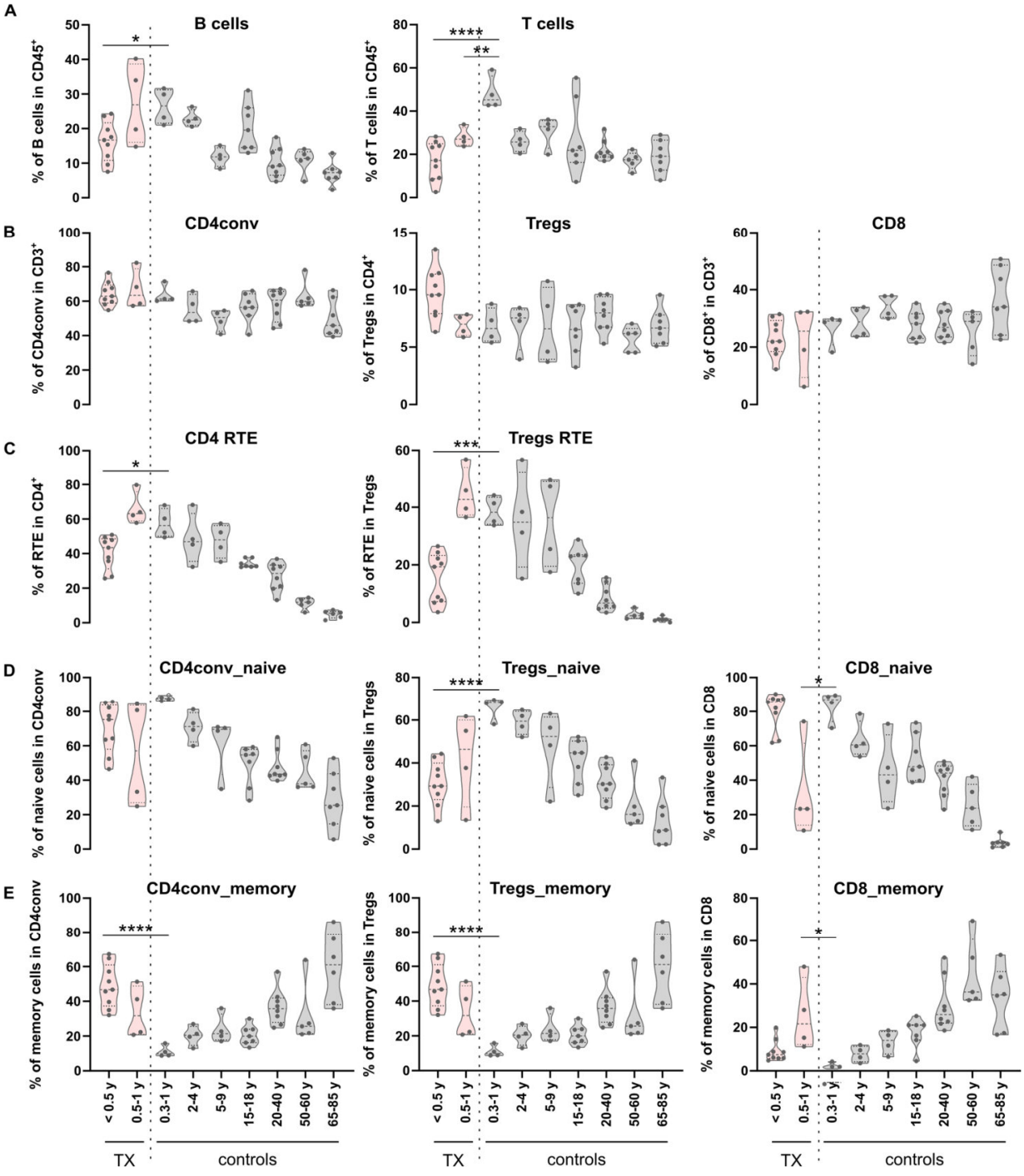

Figure 30. Changes in adaptive immune populations occur already before thymectomy. Flow cytometric analysis of immune populations in children undergoing thymectomy. Relative frequencies of B- and T cells (A), major T cell subsets (B), recent thymic emigrants in CD4 conventional and Treg cells, naïve (D) and memory (E) T cells. Children undergoing surgery (TX, red) are subdivided in children younger than 6 months (< 0.5y) and between 6 months and one year (0.5-1y) and compared to healthy controls (grey) of indicated age-classes. Statistical differences between children undergoing thymectomy and age-matched controls (0.3-1y) are indicated. *p<0.05, **p<0.01, ***p>0.005, ****p<0.0001

In summary, we show that children undergoing early life thymectomy feature a skewed adaptive immune compartment, with an increase in B cells and a reduction in T cells. A memory and activated phenotype (data not shown) is predominant in the T cell pool, and is accompanied by elevated frequencies in chronically activated CD4 T cells ($CD25^{-}CD127^{-}$). Altogether, this indicates a prematurely aged immune signature. Analysis of thymectomized children at the time of surgery indicates that features observed in thymectomized children are already acquired during the period before surgery, and become more prominent after thymectomy.

3.4 Immune signature of patients with intrinsic ablation of the thymic microenvironment: DiGeorge syndrome

3.4.1 Reduced frequency of T cells is concomitant to an increase in NK and B cells

Children with DGS present a congenital aplasia or hypoplasia of the thymus. In these children, the microenvironment for T cell development is already altered during fetal development. The underlying genetic microdeletion results in heterogeneous consequences for immune function in affected individuals. However, a highly detailed characterization of the peripheral immune compartment in young children with DGS has not been published yet. Blood samples from children with genetically proven 22q11.2 microdeletion (DGS) were provided by the Department of Pediatrics (PD Dr. R. Kobbe, Dr. U. Schulze-Sturm). Our cohort (n=16) spans an age-range from one month to seventeen years. Nine out of 16 DiGeorge patients showed elevated frequencies of neutrophils (Figure 31 A), and seven featured increased NK frequencies (Figure 31 B). Similar to thymectomized children, we found elevated frequencies of B cells (Figure 31 C) and reduced frequencies of T cells (Figure 31 D) in 44% and 63% of individuals, respectively.

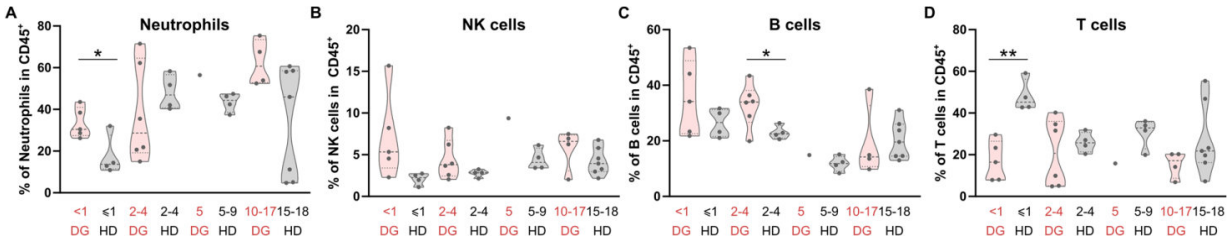

Figure 31. Altered frequencies of NKs, Neutrophils, B and T cells in children with DiGeorge syndrome. Flow cytometric analysis of Neutrophils (A), NK cells (B), B cells (C) and T cells (D) in peripheral blood of children with DGS (red) and healthy controls (grey). Samples are subdivided by age-classes. Statistical analysis was performed for each age-class, except in the age-class of 5-9 years due to only one sample for children with DGS. *p<0.05, **p>0.01.

3.4.2 Strongly activated and prematurely aged T cell compartment in a subset of DiGeorge patients

The overall frequencies of the major T cell subsets – CD4conv, Tregs and CD8 – were comparable between DiGeorge patients and controls, except for a lower CD8 frequency in very young children (< 1year) (Figure 32 A-C). CD4conv cells expressing CD25 were increased, particularly in very young children (<1 year) in DGS patients (Figure 32 D).

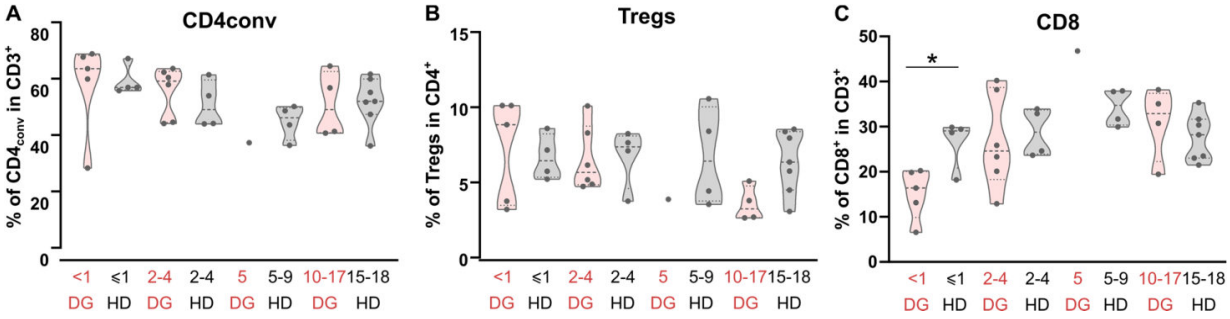

Figure 32. CD4, Tregs and CD8 frequencies are unaffected in children with DiGeorge syndrome. Flow cytometric analysis of CD4conv (A), Tregs (B), and CD8 T cells (C) in peripheral blood of children with DGS (red) and healthy controls (grey). Samples are subdivided by age-classes. Statistical analysis was performed for each age-class, except in the age-class of 5-9 years due to only one sample for children with DGS. *p<0.05, **p>0.01.

We analyzed the frequency of recent thymic emigrants, the naïve/memory phenotype and the activation status of T cells. We found a heterogeneous phenotype in children with DGS, with changes in only a fraction (less than half) of patients. This subgroup of patients showed similar features in the common T cell subsets (CD4conv, Tregs, CD8), including reduced frequency of RTEs, more T cell activation and a bias towards a memory phenotype (Figure 33). Remarkably, a reduced naïve Treg pool was observed in 14 of 16 cases.

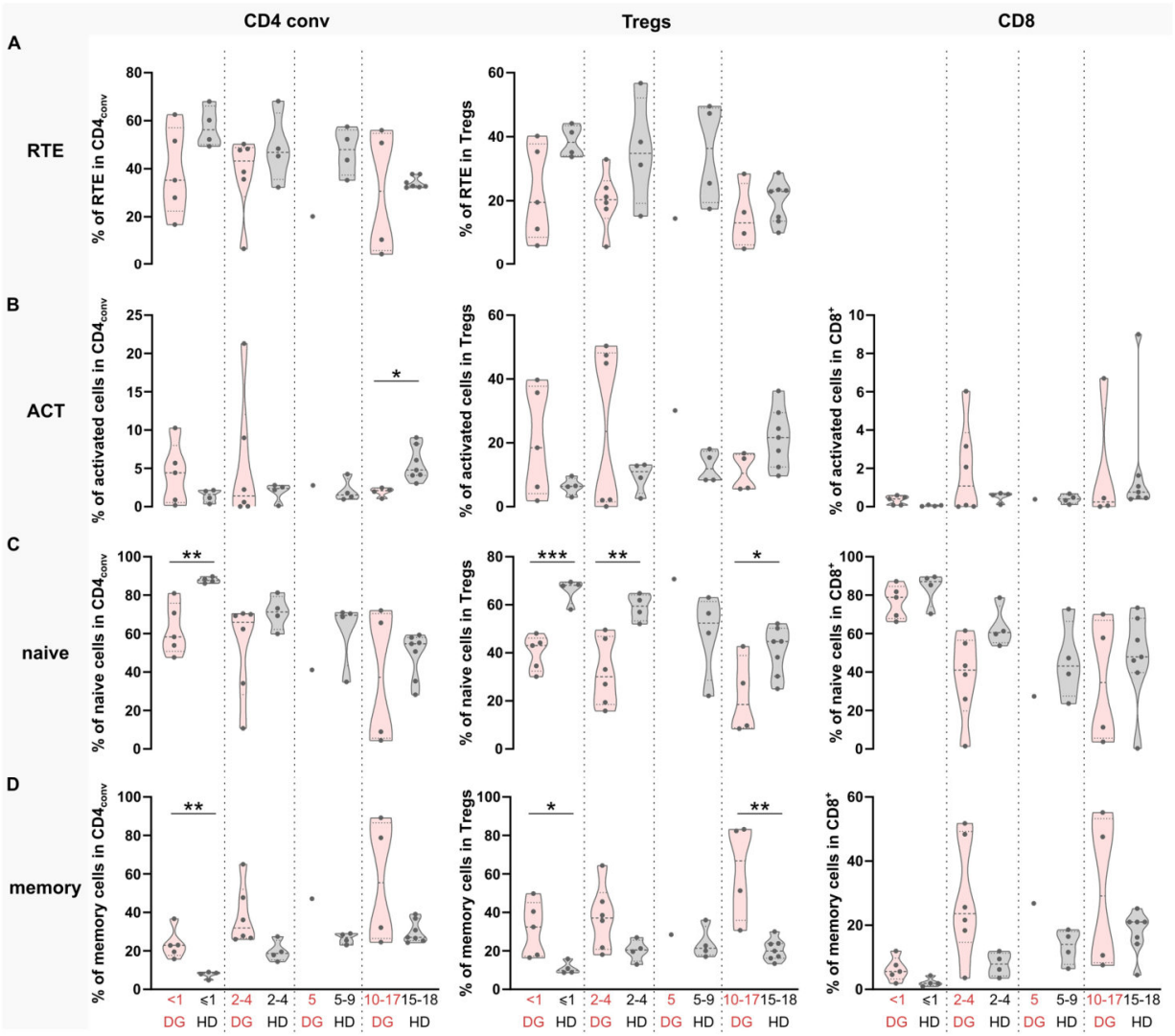

Figure 33. High frequencies of activated and memory T cells concomitant to low frequencies of recent thymic emigrants and naïve T cells in DiGeorge patients. Flow cytometric analysis of recent thymic emigrants (A), activation status(B), naïve (C) and memory (D) T cells in peripheral blood of children with DGS (red) and healthy controls (grey). Samples are subdivided by age-classes. Statistical analysis was performed for each age-class, except in the age-class of 5-9 years due to only one sample for children with DGS. *p<0.05, **p>0.01.

3.4.3 Trend towards increased frequency of CD39 expression in T cells of children with DiGeorge syndrome

In addition to the marked changes in the naïve and memory compartments, we found a conspicuously high expression of CD39, especially on Tregs, in 69% of DiGeorge patients (Figure 34 B). CD39 is an exonucleotidase involved in extracellular ATP degradation and generation of adenosine, and constitutes one of the mechanisms of suppression used by Treg cells. The expression of CD39 is upregulated in T cells upon activation. In line with our findings of an increase in the frequency of activated T cells, we found higher frequencies of CD4, CD8 and Treg cells expressing CD39 (Figure 26 A+C). Children with DGS featured a

twofold increase in the frequency of CD39 in Tregs compared to healthy controls. Statistical analysis of the differences between children with DGS and controls did not result in statistically significant values.

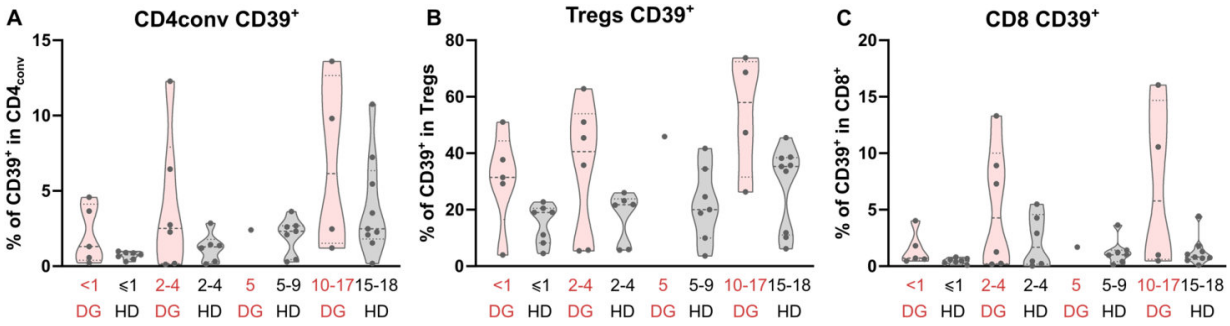

Figure 34. Increased CD39 expression in children with DiGeorge syndrome. Flow cytometric analysis of CD39 expression in CD4conv (A), Tregs (B), and CD8 (C) T cells in peripheral blood of children with DGS (red) and healthy controls (grey). Samples are subdivided by age-classes. Statistical analysis was performed for each age-class, except in the age-class of 5-9 years due to only one sample for children with DGS. Changes between children with DGS and controls did not result in statistically significant values.

3.4.4 Premature increase of Vδ2Vγ9 cells in very young children with DiGeorge syndrome

The two major TCRγδ subsets in peripheral blood are TCRVδ1 and TCRVδ2γ9. Dynamic changes happen in the distribution of Vδ1 and Vδ2γ9 during early life. While Vδ1 are the predominant TCR subtype in cord blood, Vδ2γ9 cells increase rapidly after birth and represent the major TCRγδ subset in peripheral blood throughout adulthood (Vermijlen and Prinz, 2014). Very young healthy controls have intermediate frequencies of Vδ1 and Vδ2Vγ9 cells, reflecting the transition from a predominance of Vδ1 to Vδ2Vγ9 cells (Figure 35). We found that very young children with DGS show a premature increase in the Vδ2Vγ9 subset, with frequencies comparable to older controls (Figure 35 B+ C). Of note, the frequency of T cells with a γδ TCR was comparable between controls and children with DGS (Figure 35 A).

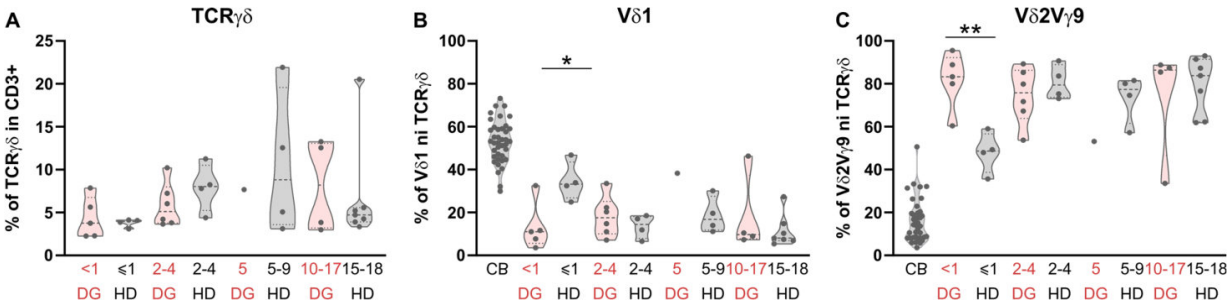

Figure 35. Premature increase of Vδ2Vγ9 T cells in very young children with DGS. Flow cytometric analysis of total TCRγδ (A) and Vδ1 (B) or Vδ2Vγ9 (C) chain usage in peripheral blood of children with DGS (red) and healthy controls (grey). Samples are subdivided by age-classes. CB - cord blood; HD – healthy donor; DG – DiGeorge syndrome. Statistical analysis was performed for each age-class, except in the age-class of 5-9 years due to only one sample for children with DGS. *p<0.05, **p>0.01.

3.4.5 Reduced IL-4 production in T cells and decreased frequency of class-switched B cells in children with DiGeorge syndrome

To test the functional capacity of T cells to produce cytokines, we analyzed cytokine production upon stimulation in PBMCs from DiGeorge patients and healthy controls. While IFNγ production by CD8 cells was increased, IL-4 production by CD4 T cells was reduced in children with DGS compared to controls throughout all age-classes (Figure 36 A). Secretion of IL-4 by T cells induces class switch of B cells from IgG and IgM to IgE production. Loss of IL-4 results in a restricted IgE/IgG1 ratio and a bias in IgG subtypes (Kopf et al., 1995, Thorbecke et al., 1994). At the same time, IFNγ levels in CD8 cells were increased (Figure 36 B). IFNγ supports class-switch of IgM to IgG, but represses IgM to IgE conversion. Therefore, we wondered if the frequency of class-switched B cells is reduced. Indeed, we found slightly reduced frequencies of class-switched B cells in children with DGS compared to controls (Figure 36 C). The major producer of IL-4 for class-switch of B cells in lymphoid tissues are Tfh cells (Reinhardt et al., 2009, Belanger and Crotty, 2016, Varricchi et al., 2016). In line with the reduced frequency of IL-4, we also expected reduced levels of Tfh cells. In contrast, we found slightly increased frequencies of Tfh cells in children with DGS compared to controls (Figure 36 D).

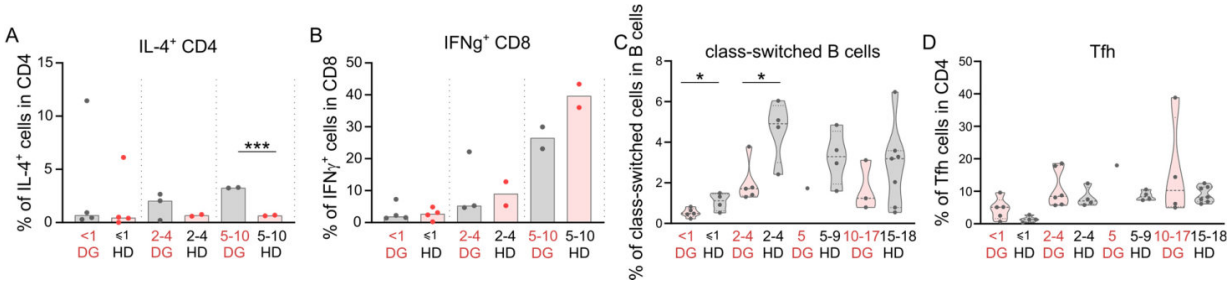

Figure 36. Reduced IL-4 production in CD4 cells, increased IFNγ levels in CD8 cells and diminished frequencies of class-switched B cells in children with DGS. Flow cytometric analysis of IL-4 (A) and IFNg (B) production in T cells upon *ex vivo* stimulation with PMA/Iono. Cytokine production is shown in relation to age [years] (left) or age-groups (right). Relative frequencies of class-switched B cells (C) and Tfh cells (D). Children with DGS are represented in red, controls in black. Statistical analysis was performed for each age-class. *p<0.05, **p>0.01, ***p>0.005.

3.4.6 Increased frequency of virtual memory CD8 cells as potential marker for immune aging in the CD8 compartment

We found a reduced frequency of RTEs, a bias towards a memory phenotype in CD4 cells, CD39 expression in Tregs, and a premature increase in Vδ2Vγ9 cells to serve as useful markers to identify children with immune deficiencies. However, we are still lacking a potential marker on the CD8 compartment. The reduction of RTEs leads to increased homeostatic proliferation to maintain the peripheral T cell pool, which might favor the increase in CD8 virtual memory cells. Virtual memory cells exhibit a memory phenotype, but are antigen-inexperienced, and are increased in individuals with lymphopenia (White et al., 2017), as a result of homeostatic proliferation. We used antibodies against CD49d, NKG2A, pan-KIR and CD45RA to identify human virtual memory cells as described by White and colleagues (White et al., 2016). We analyzed the frequency of CD8 virtual memory cells in five children with DGS and found a strong increase in three of

the children, which also presented a prematurely aged T cell compartment and increased activation of T cells, compared to age-matched controls (Figure 37).

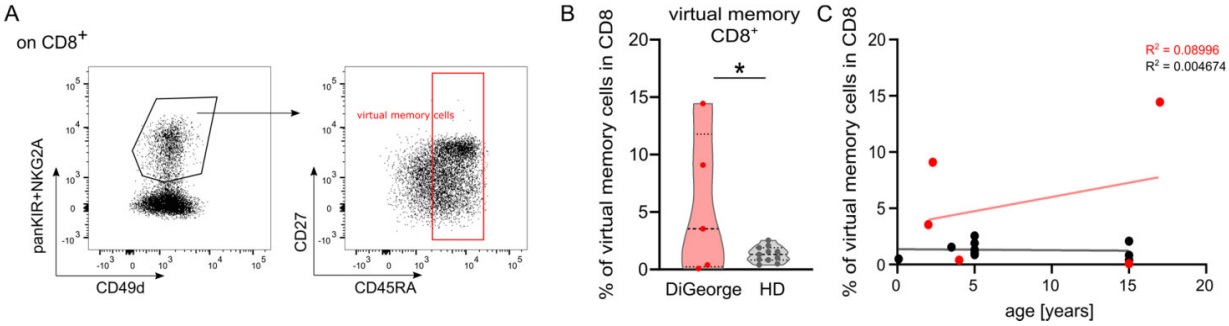

Figure 37. Virtual memory CD8 cells are increased in a subset of children with DiGeorge syndrome. A) Gating strategy for virtual memory cells (CD45RA$^+$panKIR$^+$NKG2A$^+$CD49d^{low}) within CD8 cells. B) Relative frequency of CD8 virtual memory cells in DiGeorge patients (red) and healthy controls (black). C) Correlation of virtual memory frequency with age [years] in patients (red) and healthy controls (black). The Regression line is based on data from untreated healthy controls (black) or children with DGS (red).Statistically significant differences are indicated. *p<0.05.

In conclusion, we found that children with DGS show a marked reduction in RTEs and naïve cells, concordant with an increase in activated and memory T cells, in half of the cohort, and a premature increase of the frequency of the Vδ2Vγ9 subset in TCRγδ cells, and CD8$^+$ virtual memory cells. These features were observed in less than half of the DGS children, confirming the inter-individual heterogeneity of DGS on the immune system. In addition, we propose that the frequency of virtual memory CD8 cells can be a useful marker for perturbations of the CD8 compartment in DGS patients.

3.5 Combinations of thymic reduction-causing conditions - “Multiple hit theory”

3.5.1 Multiple hits on the thymus integrity intensify the premature aging effect

Preterm birth and DGS are often associated with cardiac anomalies requiring early life surgery and subsequent thymectomy. We hypothesized that children undergoing a combination of thymic reduction-causing conditions (“multiple hits”) exhibit a distinct phenotype and their T cell compartment is more affected than children with a single condition. After immune phenotyping analysis, we used the tSNE algorithm to visualize the immune signature of children affected by a thymus-related condition (prenatal steroids, early life thymectomy or DGS) in comparison to healthy controls (Figure 38A). As described before, the immune signature of children born preterm is indistinguishable from controls at all ages, except at birth, while the immune signature of children after early life thymectomy and a portion of children with DGS is reminiscent of premature aging of the immune system. To quantify the differences between controls and affected children, we calculated the median center for the controls of each age-class. Next, we computed the distance between the immune signature of each donor and its particular age-class center, which reflects the variation of the immune signature (Figure 38A). Since tSNE representations differ slightly with repeated algorithm runs, the algorithm was run three times with different seeds to calculate the mean distance from three two-dimensional embeddings. Because tSNE dimensions are calculated on the entire dataset, the

representation of the immune signature of healthy donors could be compromised by the inclusion of a high number of affected children (n=30). We verified the conserved spatial orientation of the immune signature of controls by comparing the mean distance of each sample to the center of the respective age-class when plotted only for healthy donors, or for healthy donors and cases (Figure 38B). No significant difference between both plots were found. Next, we measured the distances of each immune signature mark to the age-class median to assess how each individual control and each patient diverged from the median. Confirming our hypothesis, we found greater distances in samples from children with some kind of thymic reduction than in controls (Figure 38C). For two children exposed to a combination (double hits) of preterm birth and early life thymectomy, and one case with DGS, premature birth and early life thymectomy (multiple hits), we observed markedly increased distances to the healthy donor age-class median exceeding the median distance of healthy controls in all three cases. Of note, six out of 30 donors featuring a single condition showed a greater distance to the age-class median than children with double or multiple hits. This suggests that multiple hits pose a higher risk for accelerated T cell perturbation and premature aging, although other factors such as pathogen encounter, lifestyle and environment also influence the individual pace of premature aging.

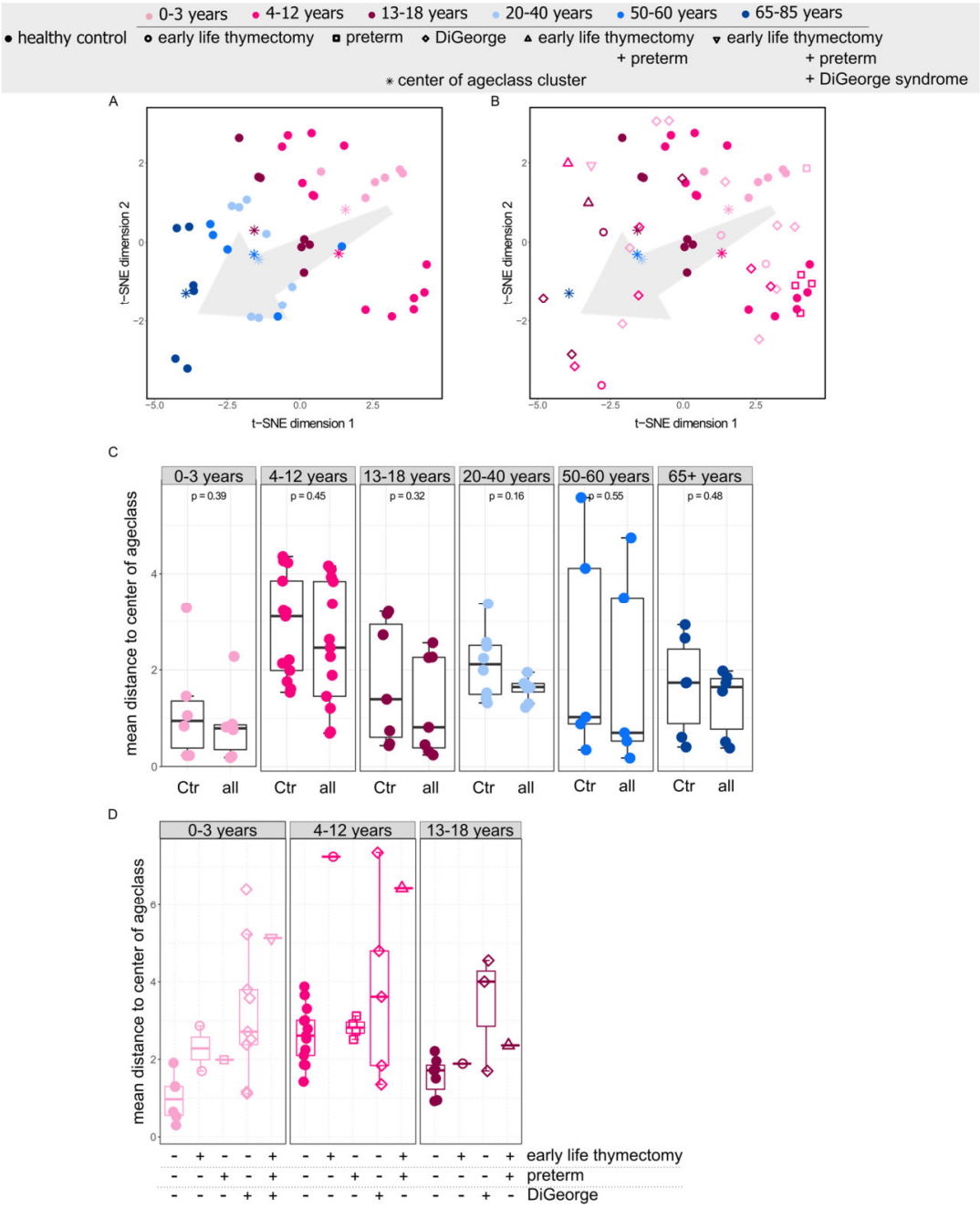

Figure 38. Combinations of thymic reduction- causing conditions increased the risk for premature aging of the T cell compartment. A) tSNE analysis for T cell populations in healthy controls (filled circles). The median center of each age-class is represented by a star. B) tSNE analysis for healthy children (≤ 18 years) and children with early life thymectomy (empty circles), born preterm (empty squares), DGS (empty diamond) or with multiple conditions (empty triangles). The median center of each age-class is represented by a star. Age-classes are indicated by colors. The manually drawn grey arrow shows the dynamic progression of the immune system with age. C) Comparison of the median distance to the center of the respective age-class of healthy donors calculated by plotting exclusively healthy donors (controls, left columns) or healthy donors and affected children (total, right columns) for each age-class. Wilcoxon rank-sum test was used for comparison of the two groups. C) Distances to the normal median center for children affected by either one or multiple conditions (empty symbols) and healthy controls (filled circles). Conditions are indicated at the bottom with "+" (applicable) or "–" (not applicable).

3.5.2 Two hits – One case report: preterm birth and early life thymectomy

3.5.2.1 Clinical history and scope of analysis

We had the chance to comprehensively analyze one case with multiple hits during early infancy (Figure 39). The male patient received prenatal steroids, was born prematurely at 31 weeks of gestation, and underwent cardiac correction surgery after being diagnosed with Tetralogy of Fallot. This procedure involved complete thymectomy at the age of seven weeks. During infancy, he suffered from recurrent viral infections and a mild atopic eczema. The presence of anti-nuclear antibodies (ANA), cyclic citrullinated peptides (CCP), and slightly increased levels for Scl-70 (directed against DNA-Topoisomerase I) autoantibodies in serum indicated rheumatoid arthritis (RA), an autoimmune disease causing destruction of the synovial membranes and chronic infection of the joints, at the age of 13 years. Morphological characteristics, cardiac defect and frequent lymphopenia suggested a DGS, but FISH-analysis was negative and additional whole exome sequencing analysis (Prof. B. Grimbacher, University of Freiburg) did not show any mutations in any gene associated with T cell deficiency or defective T cell development.

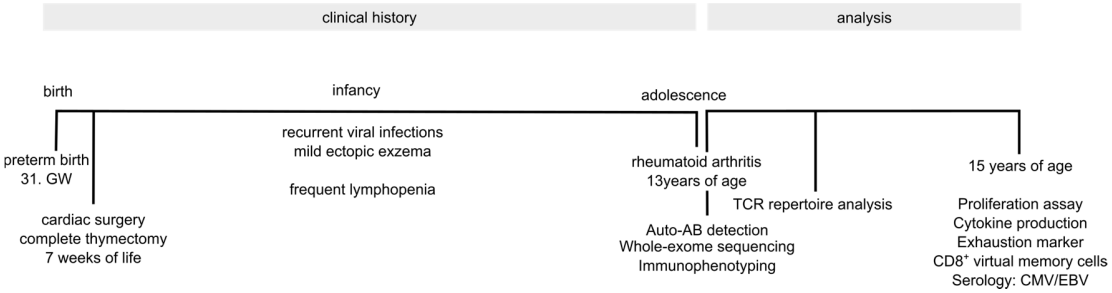

Figure 39. Simplified clinical history and immune tests performed on patient with multiple hits. This patient was born preterm (GW 31) and underwent thymectomy early in life.

3.5.2.2 Strong bias towards a prematurely aged immune signature with highly reduced total T cells, Tregs and recent thymic emigrants

One month before our first analysis, the patient started methotrexate medication for RA treatment at the age of 13 years. Immunophenotyping analysis showed a prematurely aged phenotype for the T cell signature (Figure 40 A), since the patient clustered together with the samples corresponding to adults over 50 years of age. We detected low T cell numbers and frequencies, but normal amounts of B cells (Figure 40 B+C) and conserved CD4 to CD8 ratio (Figure 40 D+E). The frequency of Tregs was decreased to one third of the values found in controls (Figure 40 F). Within the T cell compartment, the most remarkable features were very low frequencies of Treg cells (3.2% of CD4 compared to the normal range of 5 to 10%), and of RTE in the CD4 compartment, with values comparable to those found in controls aged forty years and older (Figure 40 G).

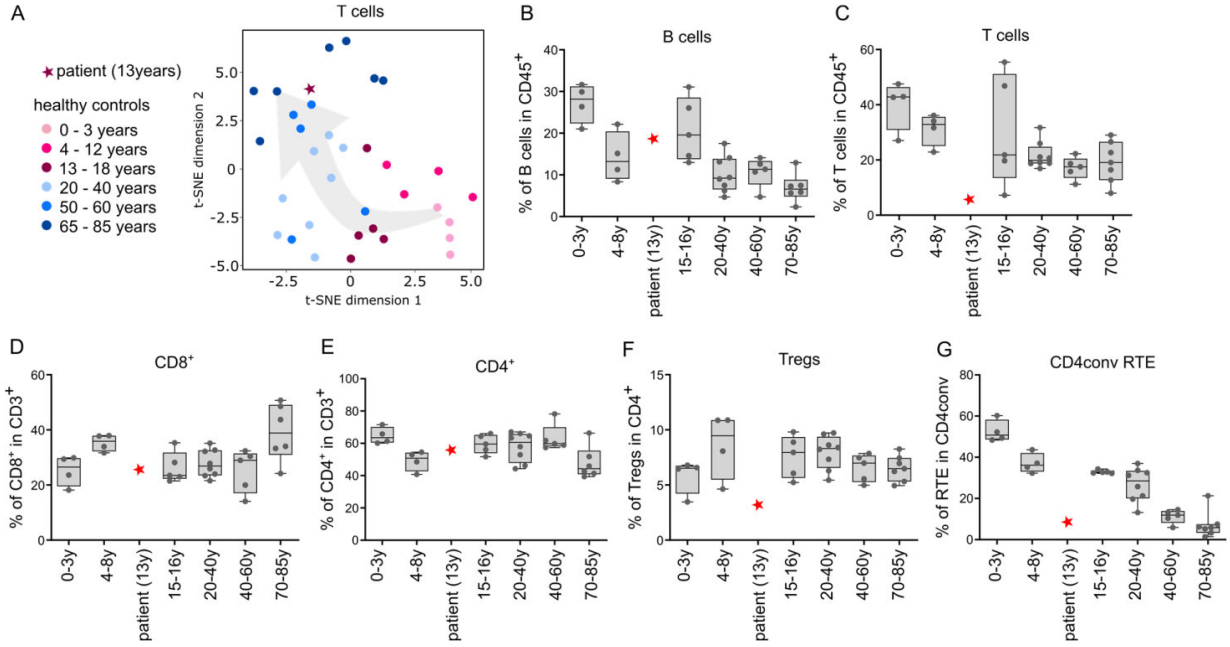

Figure 40. Prematurely aged T cells signature with highly reduced T cells, Tregs and RTE. A) Multidimensional reduction algorithm tSNE analysis for T cell populations in healthy controls (filled circles) and patient (star). Each symbol represents one donor. Age-classes are indicated by colors. Grey arrow reflects progression by age. B-G) Flow cytometric analysis of B cells (B), total T cells (C), CD8+ (D), CD4+ (E), Tregs (F) and CD4conv RTE (G). Boxplots represent median with range. Healthy donors are represented by grey dots, patient is depicted by the star.

3.5.2.3 Increased frequencies of activated and memory T cells and bias to Vδ2Vγ9 cells

Similar to findings in other patients undergoing thymectomy or DGS, we found increased frequencies of activated T cells and memory T cell subsets, Tfh cells and TCRγδ cells in this patient (Figure 41 A-E). Vδ1 and Vδ2Vγ9 subset distribution in TCRγδ cells was highly skewed towards Vδ2 cells. The frequency of Vδ1 cells corresponds to a quarter of the frequencies observed in healthy children at the age of 8-18 years (median: 10.7% of TCRγδ; min: 5.3%; max: 30.1%) (Figure 41 F).

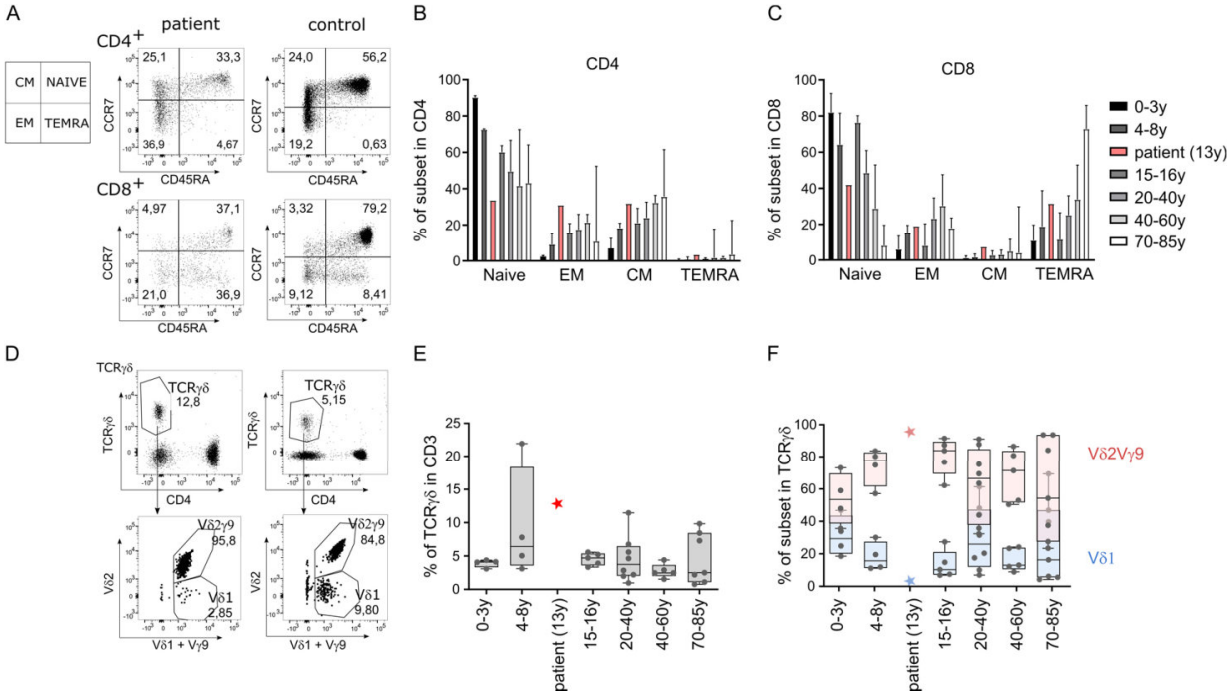

Figure 41. Increased frequency of memory T cells and strong bias towards the Vδ2Vγ9 chain usage in TCRγδ cells. A) Gating strategy for naïve to TEMRA differentiation of CD4 and CD8 T cells in the patient (left) and a representative control (right). B+C) Relative frequencies of naïve, effector memory (EM), central memory (CM) and terminally differentiated effector memory cells re-expressing CD45RA (TEMRA) in CD4 (B) and CD8 (C) T cells. D) Gating strategy for TCRγδ in CD3+ cells followed by gating of Vδ sub chains in the patient (left) and a representative control (right). E+F) Relative frequencies of TCRγδ cells in T cells (E) and distribution of Vδ1 (blue) and Vδ2 (red) within TCRγδ cells (F).

TCR repertoire analysis of the patients' PBMCs was performed at the Medical School in Hannover (Prof. I. Prinz & L. Kuhlmann). Contrary to what we expected, we found a higher frequency of rare clones in CD4 and CD8 cells of the patient compared to age-matched controls, representing a highly diverse TCR repertoire (Figure 42 A+B). The distribution of rare and polyclonal TCRs was comparable to one of the three age-matched controls (Figure 42 C).The amount of Treg cells for TCR sequencing was very low due to lymphopenia and low frequency, thus data obtained for Tregs requires careful interpretation.

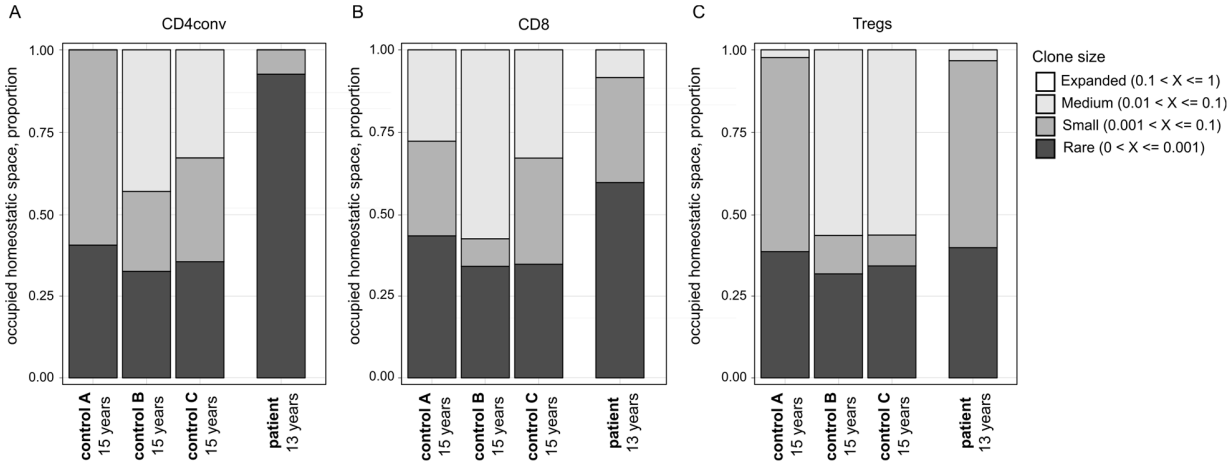

Figure 42. The TCR repertoire of CD4 and CD8 cells from the patient with "multiple hits" is highly diverse. TCR repertoire analysis of CD4conv (A), CD8 (B) and Treg (C) cells from three controls (control A-C) and patient (right). Shading indicates clonal size from rare (dark) to expanded (white) clones.

3.5.2.4 High proliferative capacity and cytokine production upon TCR stimulation

We next analyzed functional properties of the patients' T cells, such as their proliferation capacity and cytokine production after *in vitro* stimulation. T cells from the patient strongly exceeded the proliferative potential of cells from age-matched controls after stimulation of the TCR (anti-CD3) (Figure 43 A+E). We found the strong proliferation to be independent of exogenous CD28 (Figure 43 B+F). On day three after stimulation, more than 75% of the patient's CD8 cells had undergone division, while this was the case for less than 30% of CD8 cells in healthy age-matched controls (Figure 43 E+F+I). A similar relation was found for CD4 cells (Figure 43 A+B+I). Five days after TCR stimulation, CD8 and CD4 cells of the patient showed five times higher percentages of divided cells than controls, independently of exogenous co-stimulation with CD28. Similarly high rates were obtained for division and proliferation index. Proliferation in response to IL-2 and IL-7, cytokines controlling survival and homeostatic proliferation, was measured at five and seven days in the absence of TCR stimulation. We found no difference in the proliferation capacity in response to IL-2 or IL-7 for CD4 and CD8 cells between patient and age-matched controls, indicating a normal potential for homeostatic proliferation (Figure 43 C+D+G+H).

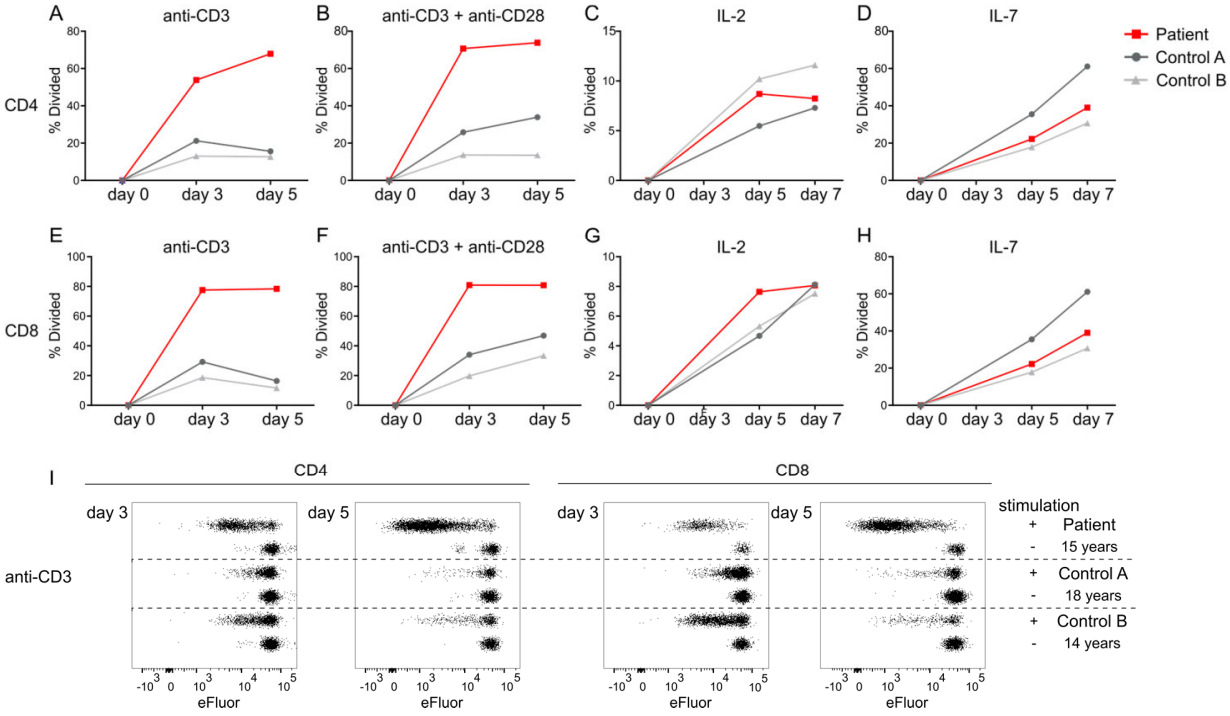

Figure 43. High proliferative capacity of CD4 and CD8 cells after TCR stimulation. Analysis of cell division after *ex vivo* stimulation with anti CD3 (A+E), anti-CD3 and anti-CD28 (B+F), IL-2 (C+G) or IL-7 (D+H) in CD4 (A-D) and CD8 (E-H) cells using cells from patient (red) or age-matched controls (grey). I) Flow cytometric detection of proliferation by eFluor dilution three and five days after anti-CD3 stimulation in CD4 (left) and CD8 (right) T cells.

The expression of CD25 and HLA-DR, markers of T cell activation, was very high in all CD4 and CD8 cells of the patient, but not in cells of the controls after TCR stimulation (Figure 44). We found CD25 and HLA-DR expression levels to remain high throughout day five after anti-CD3 stimulation, while marker expression was substantially reduced in controls. Stimulation with IL-2 and IL-7 resulted in comparable frequencies of CD25 and HLA-DR in patient and controls. These findings indicate lasting activated T cells, which is in line with the previously detected high proliferative capacity upon TCR stimulation (Figure 43).

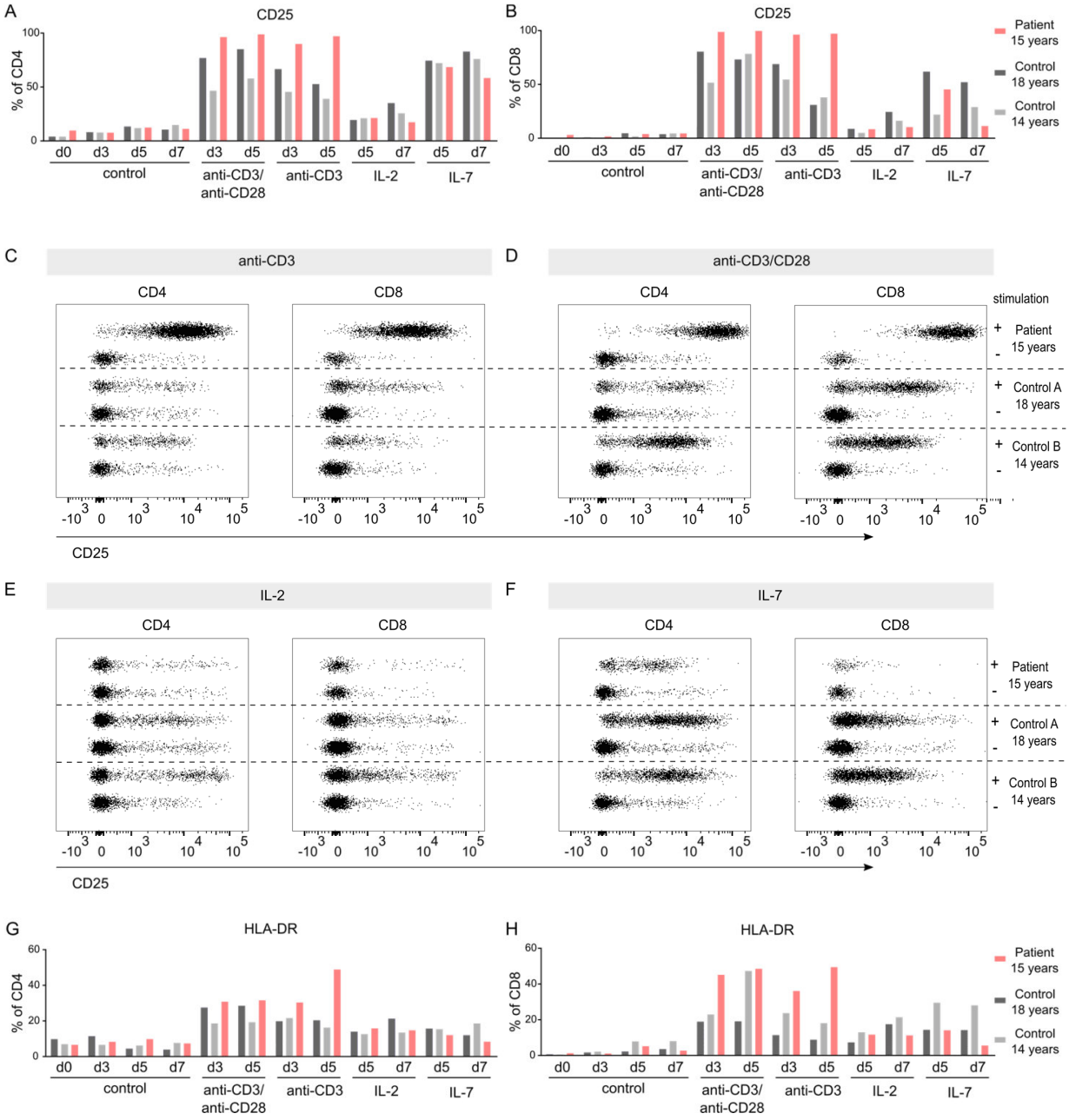

Figure 44. Strong and persistent expression of CD25 and HLA-DR after TCR stimulation. A-B) The relative frequency of CD25 in CD4 (A) and CD8 (B) cells after stimulation with anti-3, anti-CD3/CD28, IL-2 or IL-7. C-F) Flow cytometric detection of CD25 expression upon anti-CD3 (C) or anti-CD3/CD28 (D) five days after stimulation or upon IL-2 (E) or IL-7 (F) seven days after stimulation in CD4 (left) and CD8 (right) T cells. G-H) The relative frequency of HLA-DR expression after stimulation in CD4 (G) and CD8 (H) T cells.

The cytokines released upon T cell stimulation were measured using the bead-based immunoassay LEGENDplex™ XX (BioLegend), which allows the simultaneous detection of thirteen cytokines (IL-2, IL-4, IL-5 IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17f, IL-21, IL-22, IFNγ, and TNFα). We found increased amounts of cytokines in samples stimulated with anti-CD3 or anti-CD3/CD28 from the patient (Figure 45 A+B).

Increased concentrations were found for all measured cytokines, except IFNγ, IL-4 and IL-21. The stimulation with IL-2 or IL-7 resulted in a comparable cytokine production in the patient and the controls (Figure 45 C+D).

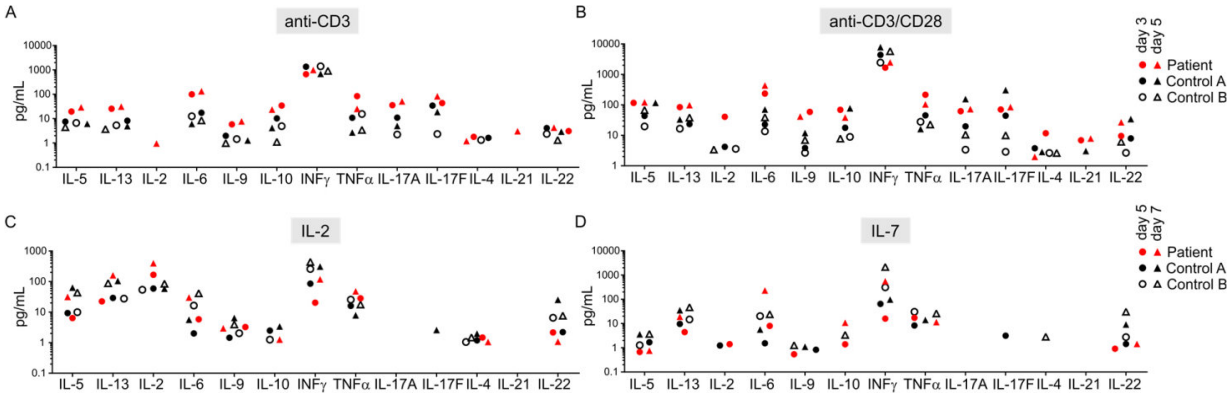

Figure 45. Prominent cytokine production in cell culture supernatants from the patient. Concentration (pg/mL) of the indicated cytokines upon stimulation with anti-CD3 (A), anti-CD3/CD28 (B), IL2 (C) and IL-7 (D) for patient (red) and controls (black). Culture supernatants were harvested on days 3 (circle) and 5 (triangle) for anti-CD3 and anti-CD3/CD28, and on days 5 (circle) and 7 (triangle) for IL-2 or IL-7 stimulation.

3.5.2.5 Increased cytokines levels in plasma

In addition to cytokine production after *in vitro* stimulation, we investigated steady-state cytokine levels in plasma of the patient using the same assay as above (LEGENDplex™, BioLegend). Consistent with the *in vitro* results, we detected higher concentrations of IL-2, IL-4, IL-5, IL-17f, IL-21, IL-22, IFNγ and TNFα in the plasma of the patient compared to controls (Figure 46). IL-17A was undetectable in all samples. The concentration of IL-2, IL-5 and IFNγ was fivefold increased, while the concentrations of TNFα, IL-17F and IL-21 were 8 to 9-fold higher in the patient. IL-4 (56 pg/mL) and IL-22 (19 pg/mL) were detectable in the plasma from the patient, but not in the controls. The sensitivity limit of the assay was 12 pg/mL.

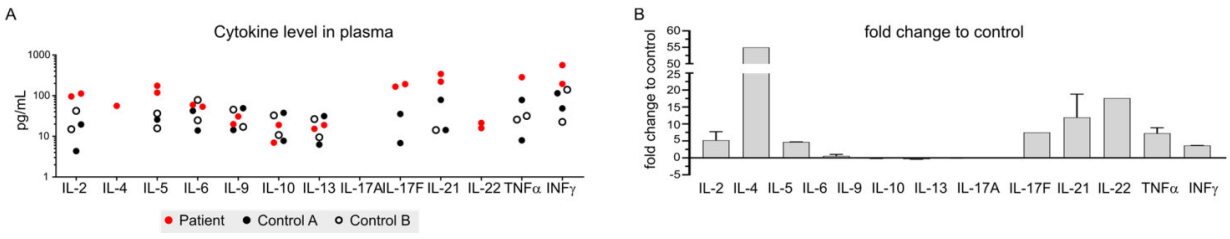

Figure 46. Increased cytokine concentration in plasma *ex vivo*. Cytokine detection using LEGENDplex™ assay (BioLegend) was performed on plasma samples from patient and two age-matched controls. A) Concentration (pg/mL) of indicated cytokines in patient (red) and controls (black). Measurement was performed with two dilutions of plasma samples. B) Differences in cytokine concentrations are represented by fold change compared to the healthy controls.

3.5.2.6 Reduced KLRG-1 expression in T cells and increase in PD-1 and CD57

Our findings of a marked prematurely aged T cell compartment (section 4.5.2.2 and 4.5.2.3) and a potent response to T cell stimulation (section 4.5.2.4), prompted the question whether T cells in the patient had an increased expression of exhaustion and senescence markers (KLRG1, PD-1, Tim-3, 2B4 and CD57). We found a strongly reduced KLRG-1 expression in CD8 cells and lower levels in CD4 (Figure 47 A+B). PD-1 expression was increased in CD4 cells, while CD8 cells showed increased frequency of CD57 compared to controls. We found no differences between the patient and controls for the expression of 2B4 and Tim-3. In addition, we analyzed the frequency of $CD8^+$ virtual memory cells, which we have shown to be increased in some patients with DGS (section 4.4.6, Figure 37). In contrast to DGS patients, the frequency of virtual memory CD8 cells was fairly normal in the patient. (Figure 47 C). Serology testing revealed no infections with EBV or CMV.

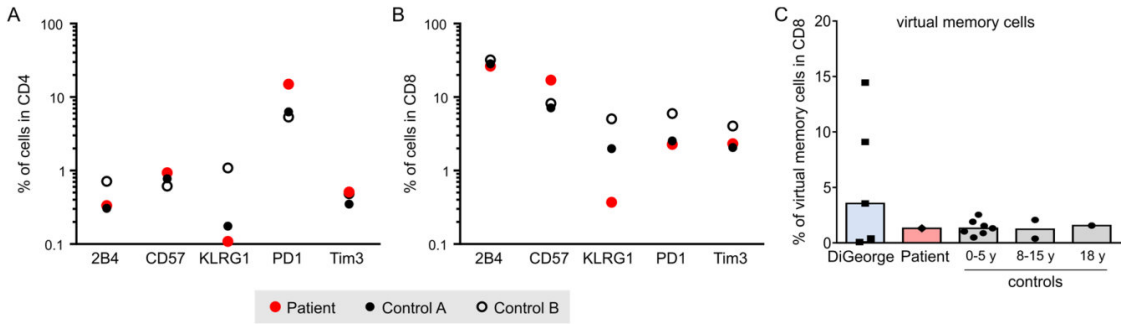

Figure 47. Diminished KLRG-1 expression, increase in PD-1 and CD57, but no difference in virtual memory T cells. Flow cytometric analysis of exhaustion markers (2B4, CD57, KLRG1, PD-1, Tim-3) in CD4 (A) and CD8 (B) cells and CD8 virtual memory cells (C) in the patient (red) and controls (black). For comparison reasons, frequency of virtual memory cells (C) are also indicated for children with DGS (blue)

In summary, we found that a patient who was born prematurely, underwent early life thymectomy and developed early onset RA, showed a strong, prematurely aged immune signature. Surprisingly, the TCR repertoire was more diverse than that detected in age-matched controls. Proliferation capacity after *in vitro* stimulation of the TCR was greater than in controls, and was accompanied by a high expression of activation markers and cytokine production. Analysis of exhaustion markers did not reveal a consistent upregulation, but showed reduced levels for KLRG1 in CD4 and CD8 cells and increased frequencies of CD57 in $CD8^+$ cells and PD-1 in $CD4^+$ cells. We suggest that the combination of preterm delivery and early life thymectomy, promoted the premature aging of the T cell compartment – as we have shown before for other cases featuring only one thymic reduction-causing condition (sections 4.3 and 4.4).

4. Discussion

The thymus provides the microenvironment for T cell development by precisely orchestrating the maturation and selection of T lymphocytes. Impairment of the thymic function, particularly in very young children when thymic output is greatest, may result in long-lasting effects on the adaptive immune system. Premature reduction of thymic tissue is associated with a higher risk for autoimmunity, cancer and opportunistic infections (van Baarlen et al., 1988, Gelfand et al., 1972, Savino, 2006, Cromi et al., 2009, Abe et al., 2015, Taub and Longo, 2005, Chinn et al., 2012, Gudmundsdottir et al., 2018). During my thesis, I have established a comprehensive flow cytometry-based immune phenotyping approach to analyze 141 immune cell subpopulations in less than 1mL blood. With this multiparametric flow cytometry approach, and the use of bioinformatics tools to analyze and visualize the data, we overcame the limitations of most studies in immune aging, which considered only the number of the total T cells, or the frequencies of the main T cell subsets. We used this method on cord blood or peripheral blood from children with thymic reduction caused by i) prenatal steroid treatment, ii) early life thymectomy or iii) with DiGeorge syndrome to analyze in detail the immune composition, with a focus on T cells. We have observed a transient reduction in thymus size upon prenatal steroid treatment, accompanied by an increase in the frequency of circulating Tregs and in the production of cytokines in cord blood. Analysis of children that were prenatally treated with steroids revealed normal numbers and frequencies of all immune cell subsets, however, our data also hints towards a higher risk for the development of allergy and immune-related diseases. Children with early life thymectomy show a phenotype similar to DiGeorge syndrome, namely reduced frequency of recent thymic emigrants and a premature shift to a memory phenotype. Unexpectedly, we found that children undergoing thymectomy present an altered adaptive immune compartment already at the time of surgery, possibly a result of hypoxia-induced stress, medication or increased exposure to pathogens during hospitalization. PBMCs from children with DiGeorge syndrome show a decreased frequency of IL-4 in CD4, accompanied by a reduction in class-switched memory B cells. In all three conditions, we found elevated levels of T cells bearing the Vδ2Vγ9 TCR chain combination, which may result from the accumulation of phosphoantigens due to increased homeostatic proliferation, an increased rate of infections, or from impaired development of Tab cells during T cell development. Finally, we show that a combination of thymectomy, preterm birth and/or DiGeorge syndrome results in premature aging of the immune system, and we describe a case report where preterm birth and early childhood thymectomy are accompanied by immune deficiency and early onset rheumatoid arthritis.

4.1 Age-related progression of the adaptive immune compartment

During aging the immune system undergoes profound changes, particularly in the adaptive compartment (Miller, 1996, Cambier, 2005). As expected, using our comprehensive immune phenotyping approach, we found that the major age-related changes occur within the T cell compartment. The strong age-related effect on T cells is mainly caused by the decline in thymic output – assessed by the frequency of RTE -, together with an increase in memory T cells, diversification of the Th lineages, a drastic reduction in IL-8 production, and an increase in IL-4 and IFNγ production by T cells. Decreasing frequencies of RTE with age are the consequence of a physiological age-related involution of the thymus, which is accompanied by a diminished T cell egress. The estimated daily rate of T cells emigrating from the thymus reaches the highest values at one year of age with 2 x 10^9 cells per day and then decreases in an age-dependent manner to 2 - 4 x 10^8 cells per day in young adults (20 – 25 years) (Vrisekoop et al., 2008, Bains et al., 2009). Of note, the loss in thymic output by age is slightly stronger for CD8 cells than for CD4 cells (Mold, 2019). The maintenance of the peripheral T cell pool is ensured by IL-7 dependent homeostatic proliferation, whose contribution to the peripheral T cell pool rises from 60% at the age of 20 to over 95% at age 65 (Mold, 2019). Homeostatic proliferation and memory generation upon antigen encounter drive the increase of the memory compartment by age.

Th2 is the predominant T-helper subset until birth. The perinatal period features a tolerance-prone immune system, which prevents an excessive inflammatory response upon encounter with environmental pathogens, and enables peripheral seeding of antigen rich tissues, namely the gut and the lung, by T cells. Mechanisms such as T cell-derived production of IL-8, which recruits and activates neutrophils and induces IFNγ production by TCR$\gamma\delta$ cells, may have evolved to help innate and innate-like cells to distinguish between commensal and pathogenic bacteria (Gibbons et al., 2014). In the B cell compartment, the immune signature of very young donors clustered clearly apart from older donors, in whom we did not detect any further age-dependent progression. The high frequency of immature (transitional) B cells early in life, which decline rapidly with age, accounted for the separation of very young donors, while the general inter-individual variation observed in the B cell compartment is likely due to individual pathogen encounter independent of the age. In contrast to adaptive lymphocytes, innate-like and innate lymphoid cells do not follow an age-related progression. Age-dependent changes in innate and innate-like cells are rather associated with functional impairment than with phenotypic alterations (Weiskopf et al., 2009, Patin et al., 2018). Interindividual variability of the innate compartment is driven by environmental exposure. Classical innate cells show a separation into young donors (< 18 years) and adults, which is mostly driven by the distribution of NK subsets and maturation of Neutrophils. The age-related increase in the frequency of NK^{dim}, concomitant to the decrease of NK^{bright} cells, as well as the loss of immature $CD16^-$ neutrophils, have been described before (Hazeldine and Lord, 2013, Prabhu et al., 2016). The NK^{dim} subset is primarily responsible for elimination of virus-infected, stressed and malignant cells, and their increase in the elderly is accompanied by a loss of cytotoxicity, and is associated with an increased susceptibility to infections (Jacobs et al., 2001, Ogata et al., 2001). NK^{bright} cells are a source of cytokines (e.g. IFNg,

TNFa) and chemokines, but present impaired function in the elderly (Hazeldine and Lord, 2013, Cooper et al., 2001). High frequency of immature neutrophils in cord blood is paralleled by abundant production of IL-8 in T cells upon stimulation, responsible for the recruitment and activation of neutrophils (Gibbons et al., 2014).

Approximately 2% of the total T cell pool is localized in peripheral blood, while most memory T cells reside in tissues (Farber et al., 2014, Mueller et al., 2013). We did not attempt to analyze the immune cell composition in tissue, as it has been done by other studies (Kumar et al., 2018, Szabo et al., 2019). Our study investigated circulating immune cells in blood, which provides valuable findings for diagnostics, as peripheral blood is a common and readily accessible source.

4.2 Effects of prenatal steroid treatment on the immune system

The dramatic effect of steroids on thymocytes is known for almost 40 years (Craven et al., 1981, Wyllie, 1980). Further studies in birds showed a drastic decrease in thymic output of naïve cells upon high-dose steroid treatment (Kong et al., 2002). Even though prenatal glucocorticoids are standard treatment for RDS, surprisingly little is known on their effects on fetal thymus. We report here that the fetal human thymus undergoes a transient reduction of size upon prenatal steroid treatment. This finding is consistent with our previous studies in a murine model for prenatal steroids showing significant decrease in thymus volume (Diepenbruck et al., 2013). Similarly, radiographs of the human thymus taken within the first 36 hours of life had revealed an absence of thymic shadow that lasted for several months in preterm children that had received prenatal steroids (Michie et al., 1998).

In addition to thymocytes, the thymus is populated by several types of stromal cells, among them thymic epithelial cells. A single study reported a significant reduction of thymic epithelial cells, which lasted for two weeks, using a high dose of steroids (20 mg/kg) in a murine *in vivo* setting (Fletcher et al., 2009). These findings underline the profound effects of this treatment also on the thymic stroma. Since the proportion of stromal cells in the murine thymus is minimal (less than 0.5%), particularly early in life, it is most likely that the decrease in thymic size results from the loss of the highly abundant thymocytes rather than from changes in the thymic epithelium (Gray et al., 2006). TEC, however, play a crucial role in the selection processes during thymocyte development ensuring a functional, non-autoreactive T cell repertoire. The expression of tissue-restricted antigens by mTEC is a key mechanism of negative selection for the elimination of potentially autoreactive T cells and alterations – even if transient - might result in an altered T cell repertoire.

The massive apoptosis of thymocytes upon steroid treatment prevented a meaningful analysis of the gene expression in TECs due to accumulation of DNA and cell debris, and an imbalance in the cellularity of treated and untreated thymi. We circumvented this issue using mice with a knockout of the GR, by excision of the essential exon 3 of the GR locus under the control of the *lck* promoter, specifically expressed in thymocytes and T cells (Tronche et al., 1999, Orban et al., 1992, Baumann et al., 2005). Therefore, the

thymocyte compartment in these mice should be preserved after steroid treatment. Surprisingly, we detected a significant 25% reduction of the thymic weight three days after steroid treatment in GR^{flox} mice with knocked-down GR (Cre^{+}) signaling, which was comparable to the effects in mice with functional GR (Cre^{-}). Stromal cells comprise less than 1% of the total thymic cellularity, so that it is unlikely that the effects are due to a reduction in TEC (Gray et al., 2006). We analyzed the effects of steroid treatment on thymocytes *in vitro* to exclude residual GR signaling in thymocytes, which would led to their apoptosis, and caused the decrease in thymic size. Thymocytes of GR^{flox}Lck-Cre mice were resistant to steroids, confirming the lack of the GR. We hypothesize that the reduction in thymic weight might thus be an exclusive feature of *in vivo* steroid treatment. GR is ubiquitously expressed by almost all cell types and plays a role in the control of metabolic, cardiovascular or homeostatic functions. The decrease in thymic weight might thus result from changes induced by GC on a variety of cells. In addition, we found that gene expression of keratins (*Keratin 5* und *Keratin 8*), the transcription factors *Aire* and *Fezf2*, driving the expression of TRAs, and the TRAs *Insulin2* and *Klk1b16* are transiently increased shortly after steroid treatment, followed by reduced transcriptional activity. Of note, changes did not result in statistical significant differences. Previously reported effects of high dose steroid treatment *in vivo* showed a strong reduction of the absolute number of TECs, and an almost complete ablation of $mTEC^{high}$ cells three days after treatment and lasting for 2 weeks (Fletcher et al., 2009). The overall increased gene expression in TECs three days after low dose steroid treatment, detected by our analysis, may indicate enhanced proliferation during the recovery of mTECs. We have used a five times lower concentration of steroids, thus, the effects on TECs were expected to be less dramatic, and the recovery of TECs occurred within one week , as indicated by the stronger differences between day three and later time points after steroid treatment, compared to the published two weeks recovery after high dose steroid treatment (Fujikado et al., 2016). The drop of *Aire* expression five days after treatment might thus mirror the period of $mTEC^{high}$ cells, which express *Aire* but not *Fezf2*, returning from the transient peak to normal values. The increased frequencies of $mTEC^{low}$ after high dose steroid treatment implies that $mTEC^{low}$ cells are less sensitive to steroid treatment than $mTEC^{high}$ cells (Fletcher et al., 2009). The expression of *Fezf2,* which is expressed in $mTECl^{ow}$ and $mTEC^{high}$, was indistinguishable between steroid-treated and control animals at days five and ten after treatment, underlining the less severe effect on $mTEC^{low}$ compared to $mTEC^{high}$. The early changes in the gene expression of *Insulin2* (Aire-dependent) and *Klk1b16* (Fezf2-dependent) after steroid treatment are proportional to the alterations found in the expression of *Aire* or *Fezf2*, thus implicating down-stream effects on TRA expression in mTECs.

In human fetuses, we calculated that the reduction in thymic size after prenatal steroid treatment persisted up to six weeks. Maturation of thymocytes takes approximately three weeks. Many women receiving prenatal steroids eventually give birth within six weeks of treatment, and their offspring are born with a thymus that is insufficiently equipped at a time of confronting the multitude of environmental challenges associated with birth. This might lead to inefficient immune responses to pathogens and facilitate colonization with unfavorable microbiota. In addition, we observed a rapid growth of the thymus following the initial six weeks delay period. Thymic growth rates of the treated fetuses in this accelerated phase lay

within the upper 50% of healthy controls. The accelerated growth of thymic tissue, most likely due to restoration pressure of the thymocyte pool, might lead to error-prone selection processes and subsequent egress of potentially autoreactive cells to the periphery.

Thymus-produced endogenous GCs are relevant for proper selection processes in the thymus (Vacchio and Ashwell, 1997, King et al., 1995). Thymocytes bearing a TCR with low or no avidity for MHC:peptide complexes die due to GC-induced apoptosis, while thymocytes with high avidity for MHC:peptide complexes undergo TCR-activation induced apoptosis. TCR-signaling with intermediate avidity to MHC:peptide complexes is antagonized by GCs signaling, resulting in survival of thymocytes (Vacchio et al., 1999, Stephens et al., 2003, Zacharchuk et al., 1990). Low production of thymic glucocorticoids shifts the threshold for positive and negative selection towards the survival of thymocytes with low avidity, rescuing cells which would normally die by neglect, and leads to the death of cells with intermediate to high TCR avidity for self-peptides, resulting in less autoreactive cells (Mittelstadt et al., 2012, Tolosa et al., 1998). We hypothesized that increased GC levels have a contrary effect, inducing positive selection of cells that would normally be eliminated by negative selection. Recently, GC-signaling was shown to be predominantly present in DP thymocytes that have been stimulated before through their TCR (Taves et al., 2019). These findings support the role of GC during the selection processes and explain the high rates of apoptosis detected particularly in DP cells after steroid treatment (Diepenbruck et al., 2013). Betamethasone, routinely used for prenatal steroid therapy, features a biological half-life of 36-54 hours, and cannot be inactivated by 11ß-HSD-2, a placental enzyme protecting the fetus from excessive maternal steroid levels (Cottrell et al., 2012, AlSaad, 2019). The long-lasting and sufficient availability of exogenously administered steroids, together with the high pressure to refill the thymocyte pool, may thus lead to error-prone and altered selection processes, which result in increased survival of autoreactive cells.

The thymus plays an essential role in the generation of Treg cells (Sakaguchi et al., 2008). Interestingly, we found increased frequencies of Tregs in cord blood of neonates born shortly after prenatal steroid treatment (<14 days before birth). This finding is in agreement with a previous report on the inverse correlation of thymic volume und the Treg frequency in cord blood (Diemert et al., 2016). The generation of murine tTreg cells during the perinatal time window is crucial for the establishment of self-tolerance and protection against autoimmunity, and is generated in an AIRE-dependent manner (Yang et al., 2015). Depletion of murine perinatal Treg cells cause notably similar autoimmune diseases as ablation of AIRE expression, or thymectomy during the first days of life, stressing the importance of an intact thymic environment for perinatal Treg generation (Yang et al., 2015, Betterle et al., 1998, Samy et al., 2008). Neonatal Treg cells feature a specific gene expression profile, and possess greater tolerogenic potential in the prevention of autoimmune diseases than their adult counterparts (Mold et al., 2010, Kim et al., 2007, Hori et al., 2003, Fontenot et al., 2003). Already thirty years ago, differences between neonatal and adult T cells were proposed to result from sequential waves of distinct hematopoietic stem populations giving rise to cells with different functional properties adapted to the respective developmental requirements (Herzenberg and

Herzenberg, 1989). Tregs are abundant during mid-gestation and decrease towards birth to levels comparable to those detected in adult blood, indicating that the first wave of cells appears early during pregnancy (Kim et al., 2012, Pagel et al., 2016). The generally high Treg frequency in cord blood of preterm children compared to term-born children - independent of prenatal steroid treatment - might thus rather reflect an earlier developmental stage than a consequence of preterm birth. We have found that prenatal steroid treatment results in an additional increase in Treg frequency in cord blood of preterm neonates. We excluded a gestational age bias by comparing steroid treated and control preterm neonates of similar age-ranges. In addition to the possible effect of the altered thymic environment and AIRE expression in the generation of Treg cells, we have reported that murine tTregs are less sensitive to steroid treatment *in vitro* and *in vivo* in adult and prenatally treated animals (Gieras et al., 2017). Unpublished data from our lab confirms the same effect on human thymocytes, with a relative increase of the Treg proportion after *in vitro* culture in the presence of glucocorticoids. In addition, *in vivo* steroid treatment of mice resulted in increased frequency of Treg cells in peripheral organs, such as spleen and lymph nodes, and *in vitro* culture of murine spleenocytes with GCs showed the same increase in the frequencies of Tregs as seen in thymocytes (Chen et al., 2004, Engler et al., 2017). A study using high-dose steroid injection in immunocompetent mice and in humans, however, showed no differences in relative Treg frequency in spleen (mice) and blood (man, mouse) after treatment (Sbiera et al., 2011). The greater survival of Tregs may be explained by different mechanisms. First, we found lower mRNA expression of the GR in Treg thymocyte precursors than in conventional T cells in untreated mice, possibly resulting in better resistance to steroids (Pekar, 2014). It could also be that Tregs are preferentially generated in the thymus under glucocorticoids. In this line, TGFβ is known to induce pTreg transformation (Zhang et al., 2017), and TGFβ is released upon cell death. Therefore, abundant TGFβ released upon glucocorticoid-induced thymocyte death could favor the generation of Treg. We also found increased production of IL-2 by CD4 cells in preterm children that were recently treated with prenatal steroids. IL-2 controls Treg homeostasis and was shown to selectively protect murine $CD4^+CD25^+$ Tregs from steroid-induced cell death (Laurence et al., 2007, Shevach et al., 2006, Tai et al., 2013, Lio and Hsieh, 2008, Chen et al., 2004). Of note, increased frequencies of Tregs are associated to an increased risk for preterm neonates to develop early onset sepsis, possibly due to excessive immune suppression (Pagel et al., 2016).

The increased frequency of Treg cells could explain the elevated production of IL-8 in CD4 cells found upon prenatal steroid treatment. IL-8 (CXCL8), one of the few cytokines released by neonatal T cells, is produced in great amounts by CD4conv in neonates, but virtually absent in adult T cells (Himmel et al., 2011, Gibbons et al., 2014). Similar to neonatal T cells, Tregs do not secrete the classical pro-inflammatory cytokines, but were reported to produce IL-8 to direct the recruitment of immune cells, particularly neutrophils, into inflamed tissue (Himmel et al., 2011). IL-8 also enhances IFNγ production by TCRVδ1 and Vδ2 cells (Gibbons et al., 2014). The increased frequency of Tregs upon steroid treatment might impair a proper response to pathogens or the control of autoreactive cells. Moreover, we find elevated levels of IL-10 in CD4 cells upon

in vitro activation with PMA. CXCL8 signaling results in activation of IL-10 producing TCRVδ2 cells in neonates (Gibbons et al., 2009).

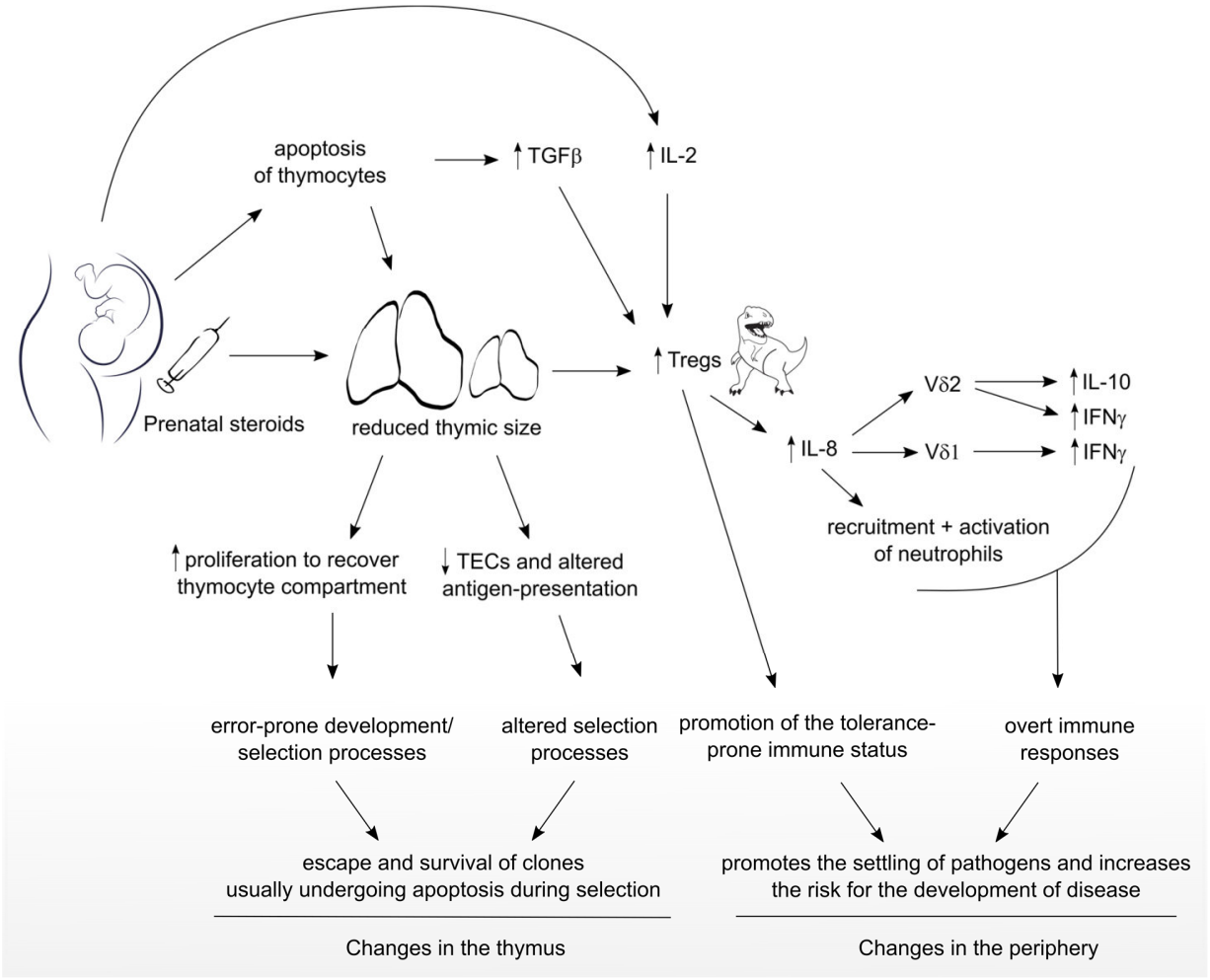

Figure 48. A possible link of prenatal steroid treatment, reduction of thymic size and increase in Treg frequency.

4.3 Prematurely aged T cell compartment in children with DiGeorge syndrome and in children thymectomized early in life.

Thymectomy in the first months of life and DiGeorge syndrome are associated with thymic reduction and reduced thymic output, both persisting lifelong. Children affected by any of these two conditions exhibit a prematurely aged T cell compartment, characterized by low numbers of T cells, an oligoclonal repertoire and diminished frequency of recent thymic emigrants and naïve T cells (Eysteinsdottir et al., 2004, Giardino et al., 2019, Halnon et al., 2005, Kobrynski and Sullivan, 2007, Madhok et al., 2005, Mancebo et al., 2008, Piliero et al., 2004, Prelog et al., 2009, Sauce et al., 2009, Gudmundsdottir et al., 2017). We confirmed these studies showing a reduced T cell compartment, a bias towards the memory phenotype, and a drastic

reduction of RTEs in CD4conv and Treg cells in peripheral blood of children with early life thymectomy or DiGeorge syndrome. CD4conv cells expressing $CD25^+$ cells are enriched in children with DiGeorge syndrome and after early life thymectomy, especially in young infants (less than five years of age). The lack of CD31 and PTK7 expression in $CD4convCD25^+$ cells, suggests their extrathymic origin (Zhang et al., 2014). The increased proportion of these cells in children with hypoplasia may be due to their thymus-independent generation. We found changes in nearly two thirds of the individuals with DiGeorge syndrome, in agreement with the broad heterogeneity of the syndrome and with previous studies showing low T cell numbers in 70-85% of infants with 22q11.2 microdeletion syndrome (Gennery, 2012). Compared to our findings, this study shows a slightly increased frequency of children with an affected T cell compartment, which might result from the inclusion of children with very mild T cell reduction by Gennery and colleagues.

In accordance with an early activation of the adaptive immune system, we found a strikingly high expression of CD39 on T cells, particularly on Treg cells, in children with DiGeorge syndrome. The ectonucleotidase CD39 degrades ATP and ADP to AMP, which in turn is converted to adenosine by another ectonucleotidase, CD73. In contrast to the immune suppressive function of adenosine, ATP mediates pro-inflammatory signals (Zarek et al., 2008, Huang et al., 1997, Lokshin et al., 2006). Therefore, high expression of CD39 attenuates inflammatory signaling and supports immune suppression. CD39 on T cells is upregulated upon activation, enriched in T cells present at sites of acute inflammation, and is a hallmark of Treg cells (Rissiek et al., 2015). Children with DGS showed increased frequency of activated T cells, determined by the expression of CD25, HLA-DR, and CD39, *ex vivo,* even in the absence of apparent infections, suggesting latent chronic immune activation. The increased frequencies of activation and memory phenotypes in children with DiGeorge syndrome are well reflected by the elevated frequency of CD39. CD39 expression increases with age, most likely due to pathogen encounters over the years (Rissiek et al., 2015). We excluded an age bias by comparing children with DGS to age-matched controls. Importantly, the frequency of T cells expressing CD39 is genetically determined by a single nucleotide polymorphism in the *ENTPD1* locus: Tregs from individuals with GG genotype at rs10748643 show a higher frequency of CD39 on Tregs and possess higher suppression capacity than Tregs from individuals with AA genotype (Rissiek et al., 2015). The strong suppressive capacity of $CD39^+$ Treg cells and their high frequency in children with DGS suggest a bias towards an immune suppressive status, possibly favoring tolerance to pathogens that would normally provoke an immune reaction, and thus facilitate the development of disease. Due to the small cohort and the lack of genotyping, we cannot exclude the influence of the SNP on the described differences in CD39 expression. A direct effect of the chromosome 22 microdeletion underlying the DiGeorge syndrome on the increased frequency of CD39 is highly unlikely, since CD39 is encoded on chromosome 10. In addition, CD39 on CD8 cells identifies terminally exhausted T cells, thus, the high expression of CD39 in children with DGS provides another sign of a prematurely aged T cell compartment (Gupta et al., 2015).

In addition to the prematurely aged T cell phenotype, we observed low IL-4 production by T cells from children with DGS upon *in vitro* stimulation with PMA. Production of IL-4, and particularly IL-21, which was not analyzed in our study, by T cells contribute to class-switch of B cells, which leads to the conversion of

IgG and IgM to IgE (Crotty, 2015, Thorbecke et al., 1994). Loss of IL-4 results in a restricted IgE/IgG1 ratio and biased IgG subtypes (Kopf et al., 1995, Thorbecke et al., 1994). We found reduced frequencies of class-switched B cells in children with DGS, particularly in early childhood, which was associated with reduced availability of IL-4 in children with DGS compared to age-matched controls. Alterations of Ig levels in children with DGS range between normal and reduced levels. Low immunoglobulin levels were associated to impaired vaccine response and a more severe course of disease in infections in most of the patients with DGS (Patel et al., 2012, Finocchi et al., 2006, Junker and Driscoll, 1995, Chinen et al., 2003, Giardino et al., 2019). Routine screening of newborns for low T cell numbers and T-cell receptor excision circles (TRECs) started in 2018 in the US and several European countries, including Germany, providing a precious chance for early diagnostic of DiGeorge syndrome. In patients with common variable immunodeficiency (CVID), characterized by low or absent immunoglobulins, the reduction in switched memory B cells correlates to higher rates of autoimmunity, susceptibility to infections and requirement of intensive care (Vodjgani et al., 2007). The primary producers of IL-4 in lymphoid tissue are $CXCR5^+$ Tfh cells, thus we expected this compartment to be reduced in children with DGS, but this was not the case (Yusuf et al., 2010, Reinhardt et al., 2009) The frequency of circulating Tfh cells is low, since they mainly reside in tissue. This might result in normal frequencies of Tfh cells in blood, but show diminished IL-4 in lymphoid tissue resulting in decreased frequencies of B cells. Klocperk and colleagues also reported elevated frequencies of Tfh cells in DiGeorge patients, but showed no correlation with serum IgG levels or switched-memory B cells (Klocperk et al., 2018). Taken together, diminished frequencies of class-switched memory B cell might contribute to insufficient immunoglobulin generation in DiGeorge patients. We report slightly elevated B cell levels in children with DiGeorge syndrome, suggesting that this impairment appears to be more of an indirect effect by disabled B cell help by T cells than a direct effect of the syndrome on the B cell compartment. In addition to the impaired T cell response, a reduction in antibody titers by reduced frequencies of class-switched B cells increases the susceptibility to infectious diseases, autoimmunity and cancer in children with DiGeorge syndrome.

We found an increased frequency of virtual memory cells (T_{VM}) in children with DGS. T_{VM} express a memory phenotype, but are antigen-inexperienced cells that are generated from naïve $CD8^+$ cells in the periphery by IL-15 and possibly also IL-4 (White et al., 2017). Similar to antigen-experienced $CD8^+$ memory cells, T_{VM} cells produce IFNγ in response to cognate antigens, however, they can also produce IFNγ and show lytic activity in response to an inflammatory cytokine milieu (IL-12, IL-15, IL-18) in the absence of any antigen encounter (White et al., 2017). An increase in the frequency of T_{VM} cells was found during aging and lymphopenia (White et al., 2016). The antigen-unspecific production of IFNγ by T_{VM} cells could therefore be a beneficial mechanism to elicit protective CD8 responses in a compromised T cell compartment, as we have observed for children with DGS. Considering its use as diagnostic marker, T_{VM} cells might therefore serve as CD8 counterparts to RTEs in CD4 cells.

4.4 Alterations of the T cell compartment in children affected by congenital heart disease

A decline in T cells or in T cell diversity may prevent an appropriate immune response to newly encountered antigens, as it happens in elderly individuals (Cicin-Sain et al., 2010). Moreover, the number of naïve T cell inversely correlates to the success of vaccination and the tolerance to opportunistic infections (Lewin et al., 2002, Roux et al., 2000). Studies in HIV individuals have shown that patients with high numbers of RTE control disease better than those with low numbers. This conclusion is, however, controversial, since disease itself might influence the frequency of RTE (Fink, 2013). A decline in total T cells, reduced frequencies of naïve cells and RTEs are common features of patients with DiGeorge syndrome or early life thymectomy, and multiplies their risk for developing severe opportunistic infections, autoimmunity and allergy. Indeed, the majority of children with complete DiGeorge syndrome, characterized by a complete absence of the thymic tissue, show immune features reminiscent of severe combined immunodeficiency (SCID) (Davies et al., 2017). In children with the more common partial DiGeorge syndrome, the effects on the immune system vary greatly between life-threatening immune deficiency and normal immunity. A recent study on 467 patients with partial DGS showed that more than half of the cohort suffered from upper respiratory tract infections, possibly a consequence of insufficient immune protection and anatomical anomalies (Giardino et al., 2019). Allergy was registered in 24% of the individuals, and autoimmunity was diagnosed in 8% of the children, in line with other reports on DGS patients (Jawad et al., 2001, Oskarsdottir et al., 2005, Tison et al., 2011).

The underlying cause for autoimmunity due to immunodeficiency can be traced to defective or altered thymic selection leading to the escape of autoreactive clones and their subsequent peripheral expansion due to increased homeostatic proliferation (Maggadottir and Sullivan, 2014). Until recently, there were no clinical reports of immunodeficiency or autoimmunity in humans after thymectomy early in life, and infection frequencies were similar to controls (Eysteinsdottir et al., 2004). A functional thymic T cell development during the fetal and neonatal window was accounted for the lower susceptibility to infections and autoimmunity in children thymectomized early in life compared to DiGeorge patients (Sauce and Appay, 2011). A recent study investigating 5664 individuals thymectomized before the age of five years revealed an increased risk for the development of autoimmune disease, cancer and infectious diseases (Gudmundsdottir et al., 2018). We found a reduced frequency of T cells and RTEs, and an increased memory T cell phenotype in children undergoing early life thymectomy. Normal thymic function prior to surgery should ensure a functional and diverse T cell repertoire and limit the effects of the unavoidable upcoming thymic reduction. Unexpectedly, we found that the T cell compartment of children with congenital heart disease showed moderate signs of premature aging prior to surgery. To my knowledge, we are the first to describe premature aging in children before surgery. One study reported no difference in the total lymphocyte count at the time of early life heart surgery, but a reduction in the lymphocyte count after surgery that was independent of thymectomy (Michie and Tulloh, 1998). The lack of healthy controls without any congenital cardiac pathology, and the marked age differences in the different study groups does not allow comparison with our data. Another study reported that children undergoing early life thymectomy show

similar frequencies in the main T cell subsets than age-matched controls, but no study investigated the effect on the T cell compartment beyond the main subsets (Wells et al., 1998). The alterations observed in the T cell pool might be a consequence of factors associated to the cardiac condition, namely insufficient oxygen-supply provoking stress-induced thymic reduction, or steroids prescribed to stabilize the child during the perioperative period that may temporarily reduce thymic output (Craven et al., 1981). The effects of steroids on the thymus in adult myasthenia gravis patients, as well as the effect of prenatal steroids on the fetal thymus, are discussed in detail above. Lastly, severe congenital heart defects require frequent hospitalization, thus increasing the risk for infections and the accelerated generation of the memory compartment.

4.5 Changes in the TCRγδ compartment in relation to thymic reduction or impaired T cell development

The major subpopulations of TCRγδ cells in human peripheral blood use the Vδ1 and Vδ2 receptor chains. TCRγδ cells expressing the Vδ2 chain primarily pair with the Vγ9 receptor chain to build the Vδ2Vγ9 TCR in adults. The frequency of TCRVδ1 and TCRVδ2 in blood varies greatly during development. In mid-gestation, Vδ2 cells are the dominant TCRγδ subset, and their frequency decreases towards birth. In contrast, Vδ1 cells are rare in mid-gestation, but constitute the predominant TCRγδ subset in cord blood of term-born babies. Shortly after birth, the frequency of TCRVδ2 cells increases dramatically, and remains stable throughout adulthood (Vermijlen and Prinz, 2014, Willcox et al., 2018, Dimova et al., 2015). Vδ2 cells react to small molecular weight, non-peptide molecules (phosphoantigens), which are metabolites of the mammalian mevalonate pathway involved in the biosynthesis of cholesterol and steroids or the bacterial desoxyxylulose-phosphate pathway. Phosphoantigens accumulate in the course of inflammation, infection or cell transformation due to dysregulated metabolic processes, thus signaling cellular distress and activating Vγ9δ2 cells (Vantourout and Hayday, 2013, Kronenberg and Kinjo, 2005, Hayday, 2009). Viral infections, such as CMV infection, or tumors may shift the ratio in favor of the Vδ1 compartment. Postnatal expansion of TCRVδ2 cells upon birth was suggested to result from microbial encounter (Carding and Egan, 2002, Parker et al., 1990). In line with this hypothesis, TCRVδ2 cells in cord blood are predominantly naïve, but almost all of them have developed a memory phenotype after the first year of life (De Rosa et al., 2004). This “massive expansion hypothesis” was challenged by the finding of abundant Vδ2 cells in mid-pregnancy, generated in the sterile *in utero* setting (Dimova et al., 2015). In addition, the discovery of a differential J-segment usage in cord blood (Jδ3, Jδ2) and adult (Jδ1) cells casted doubts on this theory (Davodeau et al., 1993, Davey et al., 2018, Dimova et al., 2015). Together with the absence of Vδ2 in the pediatric thymus, the described findings suggest two independent waves of Vδ2 cells in the fetus and during life, or extrathymic selection of specific TCRγδ clones (Willcox et al., 2018). This hypothesis is supported by a recent study that reported disparate CDR3 repertoires in fetal and adult Vγ9Vδ2 cells in periphery and thymus, implying different progenitor cells for fetal and adult cells (Papadopoulou et al., 2019). Another explanation might be

a transiently increased migration of cells into tissues, in preparation or in response to the transition from the sterile *in utero* into the outer environment (Dimova et al., 2015). We found increased frequencies of Vδ2 cells in all three studied conditions (prenatal steroid treatment, early life thymectomy and DiGeorge syndrome), which can be explained in different ways. First, increased homeostatic proliferation during the recovery of the thymic niche after prenatal steroid treatment, or to maintain the peripheral T cell pool after thymic reduction, might lead to metabolic stress, thus accumulating phosphoantigens that promote TCRVδ2 proliferation. Next, lack of AIRE expression in the thymus was associated with increased frequencies of IL-17-producing Vδ2Vγ9 cells (Fujikado et al., 2016). These IL-17 producing γδ T cells are thought to develop, particularly during the perinatal period, from immature γδ T cells that encountered cognate antigens in the thymus, and are involved in immune, autoimmune and anti-tumor responses (Fujikado et al., 2016). We could show that prenatal steroid treatment results in temporarily altered expression of AIRE in mice that might cause increased frequencies of Vδ2Vγ9 cells. Lastly, higher frequencies in Vδ2 cells might also be the result of early (prenatal) encounters with pathogens. Intrauterine infections, even if undiagnosed, might result in preterm birth or congenital heart disease (Ye et al., 2019, Helmo et al., 2018).

Figure 49 summarizes possible mechanisms underlying the increase in the frequency of TCRVδ2 cells in children with prenatal steroid treatment, DiGeorge syndrome, and early life thymectomy. Given the association of an increase in TCRγδ cells and the development of autoimmunity, inflammation and cancer, it is plausible that children with DiGeorge syndrome or early life thymectomy show a higher risk for the development of disease (Sutton et al., 2009, Markle et al., 2013, Cai et al., 2011, Wu et al., 2014, Van Hede et al., 2017, Coffelt et al., 2015). Thus, the enrichment of TCRVδ2 cells may synergize with a prematurely aged immune system to increase the susceptibility to autoimmunity and worsen the long-term outcome for these children.

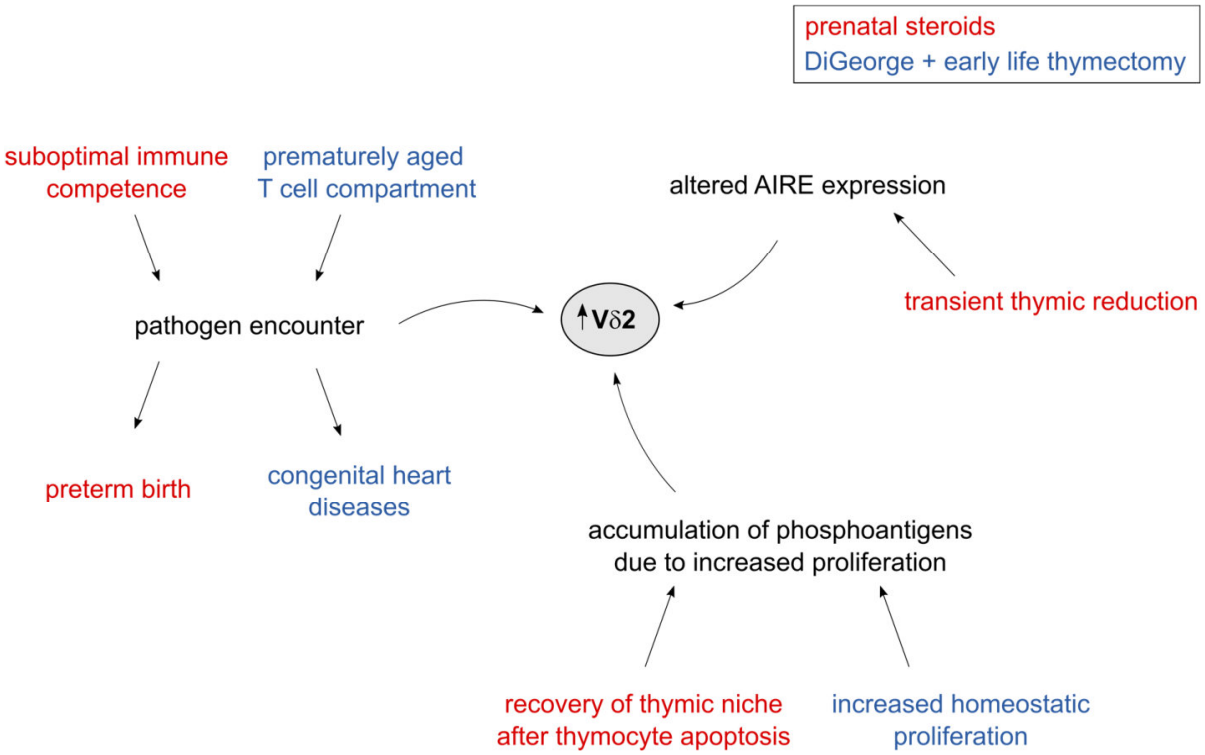

Figure 49. Proposed mechanisms underlying the increase in TCR Vδ2 cells in children affected by preterm birth, DiGeorge syndrome and early life thymectomy. Prenatal steroid treatment (red), or persistent thymic reduction, caused by DiGeorge syndrome and early life thymectomy (blue), increase the frequency of TCRVδ2 cells by increased pathogen encounter, reduced thymic expression of AIRE, and an accumulation of phosphoantigens.

4.6 Factors influencing the severity of the prematurely aged T cell phenotype

We have reported severe short-term effects after prenatal steroid treatment on the immune compartment, and prematurely aged T cells in children with DiGeorge syndrome or early life thymectomy. Severe congenital heart defects can lead to premature birth, and often belong to the array of features characteristic of DiGeorge syndrome. It is therefore important to consider situations where children are not only exposed to one of the conditions studied, but experience several of them. Children born preterm and treated with prenatal steroids experience a period of transient thymic reduction and alterations of the peripheral T cell compartment. In the case of a subsequent early life thymectomy, these initial changes might lead to fare worse changes of the adaptive immune compartment compared to children who had a stress-free prenatal period and birth, and underwent thymectomy a few weeks after birth. Even though it may be difficult to identify such cases, we found that donors with multiple (prenatal and postnatal) hits exhibited a tremendously aged T cell compartment. Therefore, our data indicates that the combination of prenatal steroids and early life thymectomy (two hits) or in combination with DiGeorge syndrome (three hits) worsen the effect of premature T cell aging. Yet, the individual's exposure to pathogens is a critical factor for the extent of skewing. In addition, we have shown that preterm born children prenatally treated with steroids may have an increased risk to develop allergy or reactive immune diseases compared to untreated preterm

born children. We believe that a second hit during the thymic recovery phase, such as early life thymectomy, multiplies the risk of long-term effects on the immune system. It should be noted that our analysis of combined conditions and children with thymectomy early in life is limited to a small number of donors, but the results obtained demand further studies on these special cases.

As an example, we had the chance to study the case of a child born preterm who had received prenatal steroids and underwent early life thymectomy. This child presented characteristics of a prematurely aged T cell compartment (low frequencies of total T cells, naïve T cells and RTEs, increased memory compartment and skewing of the TCR$\gamma\delta$ compartment towards Vδ2 cells), as described above for children with thymectomy or DiGeorge syndrome. Of note, this patient developed arthritis in his teenage years. We expected a less diverse TCR repertoire due to increased homeostatic proliferation of T cell clones, but unexpectedly found a TCR repertoire for CD4, CD8 and Treg cells in this patient which was comparable to age-matched controls. This might indicate either residual thymic tissue or regeneration of thymic tissue as shown before for children with early life thymectomy (van den Broek et al., 2016, van Gent et al., 2011). A second thymus, found in the neck of mice (cervical thymus), was shown to generate a diverse repertoire of T cells, although in lower numbers than in the ordinary thymus found in the chest (Terszowski et al., 2006). In human fetuses, a cervical thymus is found frequently, however, its presence after birth is rare and considered to be a result of a defective translocation to its final location and is associated with malignant transformation in some cases (Cornu et al., 2001, Pai et al., 2005, Wu et al., 2001, Terszowski et al., 2006). The restoration of the thymus function results in an increase in the proportion of newly generated T cells, contributing to the diversification of the peripheral T cell pool. In contrast, we observed a significant decrease in the frequencies of RTE and naïve T cells, concomitant to an increase in memory cells, which was indicative of a strongly reduced thymic output.

T cells in the elderly down-regulate the co-stimulatory molecule CD28, leading to impaired T cell stimulation (Czesnikiewicz-Guzik et al., 2008, Goronzy et al., 2007). With regard to the prematurely aged T cell compartment, we assumed that T cells might exhibit this age-related down-regulation of CD28. We found that *in vitro* stimulation of the TCR (anti-CD3) led to strong T cell proliferation independent of exogenous anti-CD28, suggesting that T cells in these patients are perfectly capable of responding to antigen. The high proliferation capacity of the T cells corresponds to our observation of the low expression of KLRG-1 (killer cell lectin-like receptor subfamily G), a common marker for senescent cells, which are terminally differentiated cells, and possess poor proliferative capacity, while remaining their potential to produce cytokines. Considering the high proliferative capacity of T cells from the patient, we were surprised to measure an increased frequency of $CD57^+$, another common marker for senescent cells, on $CD8^+$ cells (Kared et al., 2016). The frequency of senescent cells increase with age and with persistent infections such as CMV or HIV (Kared et al., 2014, Papagno et al., 2004). Serology testing showed negative results for CMV or EBV infections in the patient, excluding any involvement in the increase in senescent cells. In contrast to senescent cells, exhausted cells feature impaired proliferation and function. We found increased expression of PD-1, a well-known marker for exhausted cells, on $CD4^+$ cells concomitant with increased

levels of cytokines detected in plasma *ex vivo* that suggests persistent infections. In summary, we propose three explanations for our results: i) the diverse T cell repertoire results from remaining or recovered thymic function, ii) the high proliferation capacity of T cells and cytokine production after TCR stimulation, as well as the predominant memory phenotype results from hyperresponsiveness of naive and memory cells, and iii) the senescence and exhaustion phenotype is most likely expressed by terminal memory cells and caused by high proliferation capacity of T cells and persistent infections. The hyperresponsiveness observed after TCR stimulation, which resulted in the accumulation of senescent cells, might thus be involved in the development and progression of rheumatoid arthritis. In summary, this clinical case presents features of poor thymic output with concomitant premature aging of the T cell compartment, and alterations in TCR stimulation, altogether favoring development of autoimmunity. Prenatal steroid treatment followed by early life thymectomy result in one short perinatal period and an additional long-lasting period of thymic reduction that lead to changes in the thymus microenvironment and the T cell compartment, thereby promoting the appearance of autoreactive cells and the development of early onset rheumatoid arthritis.

4.7 Conclusion and Perspective

In this study, I showed that thymic reduction as a consequence of prenatal steroid treatment, early life thymectomy or DiGeorge syndrome is accompanied by alterations in the immune cell compartment, particularly affecting T cells, and may contribute to the development of autoimmunity later in life.

Prenatal steroid treatment causes an interruption of the human fetal thymic growth for up to six weeks and results in slightly altered expression of murine genes in the thymus that are important for the selection of functional, mature thymocytes. Transient reduction of the thymic size upon prenatal steroid treatment is accompanied by short-term effects on the relative frequency of several immune cell subsets, including Tregs, TCRVδ2 cells and cytokine-producing cells in cord blood. Functional perinatally cells proved to be important for the prevention and attenuation of autoimmune diseases (Kim et al., 2007, Hori et al., 2003, Fontenot et al., 2003, Yang et al., 2015). Prenatal steroid treatment might not only cause changes in T cell development and selection, by reduction of the thymic size, but also interfere with the generation of important perinatal immune cell populations promoting tolerance and long-lasting protection from autoimmunity. Prenatal steroids are routinely used in the prophylactic treatment of women at risk of premature birth. The incidence of premature delivery in high-income countries remains high and increasing (Vogel et al., 2018). The preterm birth rate in Germany is 8.6% which is one of the highest in Europe (Bäurle, 2017, Chawanpaiboon et al., 2019, WHO, 2014). A large number of women deliver beyond the effective period of the drug (>1 week after treatment), resulting in exposure of the offspring to steroids without getting any benefit on lung maturation. Considering our findings of altered proportions of immune cells during the fragile perinatal window, we suggest a re-evaluation and more restricted guidelines for the use of prenatal steroid treatment instead of its current prophylactic use. The routine application of prenatal steroid treatment began in the mid-1990s, therefore, possible long-term consequences on the immune system can only be expected in the next several decades (ACOG, 1995, NIH, 1995, Leviton et al., 1999). In addition to the use

of prenatal steroids for lung maturation in preterm births, its application has also been proposed before planned term caesarian section for prevention of respiratory complications (Sotiriadis et al., 2018). Our data hints toward an increase in immune-mediated diseases and allergy in young children prenatally treated with steroids. The extended prenatal application beyond preterm birth enlarges the pool of affected individuals and stresses the demand for research on long-term effects on the immune system.

Early life thymectomy and DiGeorge syndrome feature long-term thymic reduction that leads to reduced T cell output. Increased homeostatic proliferation to maintain the T cell pool, and the diminished thymic egress of naïve cells lead to a prematurely aged T cell compartment. I have found that children undergoing early life thymectomy already show signs of premature aging of T cells at time of surgery. These changes might be a consequence of insufficient oxygen supply leading to stress-induced thymic reduction, or the prescription of steroids to stabilize the child during the perioperative period. The removal of the thymus leads to an enhanced premature aging of the T cell compartment later in life. In children with DiGeorge syndrome, we additionally detected low IL-4 production in CD4 cells upon *in vitro* stimulation. This increase is accompanied by reduced frequencies of class-switched B cells, supporting previous findings of an impaired humoral immunity. Impaired antibody responses together with an aged immune T cell compartment strongly hamper adaptive immune responses and constitute an increased risk for the development of infections and other diseases. Congenital heart defects requiring early life surgery accompanied by thymectomy occur in 0.5% live births, and DiGeorge syndrome in 0.03% (Hoffman and Kaplan, 2002, Reller et al., 2008, Bauer, CDC, 2018, Devriendt et al., 1998, McDonald-McGinn et al., 2015). The constant technical and medical progress has led to a decrease in death rates of congenital malformations and chromosomal abnormalities in Germany by approximately 50% during the last three decades (WHO, 2018b). The improved chances of survival of children with congenital malformations or chromosomal abnormalities increases the number of children requiring early life thymectomy or are affected by DiGeorge syndrome. For children undergoing early life thymectomy, we recommend to preserve as much thymus tissue as possible to keep a certain degree of thymic output, and slow down aging of the T cell compartment. Taken together, our results suggest that the immune system is already altered at surgery, highlighting the necessity of a broad investigation of children with severe congenital heart defects. Strict monitoring of progressive immune status after early childhood surgery is therefore recommended and may allow early diagnosis of immune pathology.

Due to the heterogeneity of symptoms and a wide spectrum of effects, the diagnosis of DiGeorge children is challenging. In addition to the reduced total number of T cells, and the bias towards a memory phenotype of the T cell compartment, we identified increased frequencies of TCR$\gamma\delta$ cells bearing the$V\delta2V\gamma9$ receptor, and virtual memory CD8 cells, as well as reduced IL-4 production by CD4 cells as useful markers for DiGeorge syndrome. The inclusion of TREC analysis in the newborn screening program will allow the early identification of cases with DGS, providing further insights into the characteristics and heterogeneity of this condition.

Application of prenatal steroids and early life thymectomy are routinely performed for approximately thirty years now. This might be a major health burden for those children. Our study is limited to a small number of children, most of them under the age of ten. Further studies with increased cohort sizes are required to confirm our findings. Autoimmunity or immune-mediated diseases triggered by environmental or interventional factors tend to appear beyond childhood. The inclusion of older individuals in the study could improve the significance of the analysis with regard to the risk to develop autoimmunity and immune-mediated disease.

5. Summary

The prevalence of immune-mediated diseases in industrial countries is rapidly increasing. Our genome has not fundamentally changed in the last decades, but our lifestyle, diet and exposure to environmental factors has changed dramatically. Therefore, it is conceivable that the increase in autoimmune diseases and allergies is most likely triggered by a combination of interventional, environmental and genomic factors. T cells play a central role in adaptive immune responses. They develop in the thymus, where precisely orchestrated processes generate an extensive repertoire of functional T cells and eliminate potential autoreactive cells. We hypothesize that thymic reduction in early life may be involved in the development of immune disorders. A premature reduction in thymus function reduces the diversity of the T cell pool. In addition, altered selection processes might lead to thymic egress of autoreactive cells. Both aspects increase the risk of immune-mediated diseases and autoimmunity. In this context, I investigated the effects of extrinsic (prenatal steroid treatment or early life thymectomy) and intrinsic (DiGeorge syndrome) factors related to thymic reduction in early childhood on the development of the immune system, particularly T cells.

To study the role of the thymus on the immune composition in blood, I have established a comprehensive multicolor flow cytometry approach covering the innate and adaptive immune system. Using *the t-distributed stochastic neighbor embedding* algorithm, we generated a unique “immune signature” for each donor. This immune signature reflects 141 immune subpopulations, including 104 subpopulations of T cells, but also B cells, cells of the innate immune system, and recently described immune cell populations, such as innate lymphoid cells. We explored age-related changes in the innate and adaptive immune compartments of healthy donors and showed the progression of the T cell compartment with age. Using this detailed analysis of blood immune cells, particularly of T cells, we compared the immune signature of children with thymic reduction to healthy controls. We found that prenatal steroid treatment – routinely given to women at risk of preterm delivery to promote fetal lung maturation – caused an interruption of the human fetal thymic growth for six weeks, and transiently altered the thymic expression of genes important for thymus structure and negative selection of thymocytes in a murine model. Furthermore, we observed a short-term increase in the frequency of regulatory T cells and TCRVδ2 cells in in cord blood of preterm neonates prenatally treated with steroids. In addition, we found an increased production of IL-2 and IL-8 by T cells after *in vitro* stimulation. We found no long lasting changes in prenatal steroid treatment on the immune composition in blood, however, children who had received prenatal steroids showed a trend towards a higher risk of developing immunological diseases. Permanent thymic reduction caused by early life thymectomy – as a side effect of corrective heart surgery - or DiGeorge syndrome – caused by a chromosomal microdeletion – resulted in a premature aging of the T cell compartment. This was characterized by low frequencies of recent thymic emigrants and a predominance of the memory phenotype. Interestingly, in children who underwent early life thymectomy, we found mild alterations of the T cell compartment already at time of surgery. In addition to the prematurely aged T cell compartment, our data indicated that high CD39 expression, particularly on Tregs, and an increased frequency of virtual memory cells might be promising diagnostic markers for early detection of immune deficiency in infancy. Finally, I could show that a combination of

prenatal steroid treatment, early thymectomy and/or DiGeorge syndrome accelerated changes in the T cell compartment.

In conclusion, in this thesis I showed that early life disturbances of the thymus function such as prenatal steroids and early life thymectomy, as well as thymic hypoplasia associated with DiGeorge syndrome, result in changes in the progression and composition of the immune system that might promote the development of immune-related diseases in later life. We found promising diagnostic markers for the identification of children with immune deficiency. The routine practice of prenatal steroid treatment and early life thymectomy during the last thirty years may represent a burden for future generations. The results of this thesis and future studies call for further studies to develop awareness of the possible long-term consequences of prenatal steroid treatment and early life thymectomy and suggest the revision of clinical guidelines.

6. Zusammenfassung

Die Prävalenz von immunologischen Erkrankungen in den Industrieländern hat rasant zugenommen. Unser Genom hat sich in den letzten Jahrzehnten nicht grundlegend verändert, aber unser Lebensstil, unsere Ernährung und Umweltfaktoren haben sich dramatisch gewandelt. Daher ist es denkbar, dass die Zunahme der Autoimmunerkrankungen und Allergien durch eine Kombination aus medizinischen Maßnahmen, Umwelteinflüssen und genomischen Faktoren ausgelöst wird. T-Zellen sind ein essentieller Teil der adaptiven Immunabwehr. Sie entwickeln sich im Thymus, wo präzise abgestimmte Prozesse ein umfangreiches Repertoire an funktionellen T-Zellen erzeugen und potenzielle autoreaktive Zellen eliminieren. Wir gehen davon aus, dass die frühkindliche Verkleinerung des Thymus (Atrophie oder Hypoplasie) an der Entwicklung von Immunstörungen beteiligt ist. Eine vorzeitige Verringerung der Thymusfunktion reduziert die Vielfalt des T-Zell Repertoires und veränderte Selektionsprozesse könnten zum Austritt von autoreaktiven Zellen aus dem Thymus führen. Diese beiden Aspekte erhöhen das Risiko von immunologischen Erkrankungen und Autoimmunerkrankungen. In diesem Zusammenhang untersuchte ich die Auswirkungen von extrinsischen (pränatale Steroidbehandlung oder frühkindliche Thymektomie) und intrinsischen (DiGeorge-Syndrom) Faktoren im Zusammenhang mit frühkindlicher Atrophie oder Hypoplasie des Thymus auf die Entwicklung des Immunsystems, insbesondere der T-Zellen.

Um die Rolle des Thymus auf die Zusammensetzung der Immunzellpopulationen des peripheren Blutes zu untersuchen, habe ich eine umfassende Analyse der Immunzellen mittels Durchflusszytometrie entwickelt, die sowohl Zellen des adaptiven als auch des angeborenen Immunsystems umfasst. Mit Hilfe des *t-distributed stochastic neighbor embedding* (tSNE) Algorithmus, erzeugten wir für jeden Spender eine einzigartige "Immunsignatur". Diese Immunsignatur spiegelt 141 Immunsubpopulationen wider, darunter 104 Subpopulationen von T-Zellen, aber auch B-Zellen, Zellen des angeborenen Immunsystems und kürzlich beschriebenen Immunzell-Populationen, wie z.B. *innate lymphoide Zellen* (ILC). Wir untersuchten Veränderungen in den angeborenen und adaptiven Immunzellen gesunder Spender unterschiedlichen Alters und zeigten die altersbedingte Entwicklung des T-Zell-Kompartiments. Mittels dieser detaillierten Analyse von Immunzellen, insbesondere von T-Zellen, verglichen wir die Immunsignatur von Kindern mit frühkindlicher Atrophie oder Hypoplasie des Thymus mit gesunden Kontrollen. Wir fanden heraus, dass die pränatale Steroidbehandlung - die routinemäßig bei Frauen durchgeführt wird, welche von einer Frühgeburt bedroht sind, um die fetale Lungenreifung zu fördern - eine Unterbrechung von sechs Wochen im Wachstums des fetalen Thymus verursachte. Darüber hinaus führte die Applikation von Steroiden im Mausmodel zu einer vorübergehenden Veränderung der thymischen Expression von Genen, die für die Thymusstruktur oder die negative Selektion von Thymozyten von Bedeutung sind. Wir beobachteten einen kurzfristigen Anstieg in der Häufigkeit von regulatorischen T-Zellen und TCRVδ2 Zellen im Nabelschnurblut von pränatal steroidbehandelten Frühgeborenen. Darüber hinaus zeigten wir, dass die Produktion von IL-2 und IL-8 in T-Zellen nach *in vitro* Stimulation erhöht war. Wir fanden keine lang anhaltenden Veränderungen der pränatalen Steroidbehandlung auf die Zusammensetzung der Immunzellen im Blut, zeigten aber, dass Kinder, die pränatale Steroide erhalten hatten, möglicherweise ein höheres Risiko für die Entwicklung immunologischer Erkrankungen aufweisen.

Eine permanente Reduktion des Thymus, wie dies durch eine frühkindliche Thymektomie - im Rahmen einer Herzoperation - oder durch das DiGeorge Syndrom - verursacht durch eine chromosomale Mikrodeletion - auftritt, führte zu einer vorzeitigen Alterung des T-Zell Kompartiments. Diese war gekennzeichnet durch einen geringeren Anteil an neu gebildeten T-Zellen aus dem Thymus (*recent thymic emigrants*), und einem überwiegenden Anteil an Gedächniszellen. Interessanterweise fanden wir bei Kindern mit frühkindlicher Thymektomie, bereits zum Zeitpunkt der Operation leichte Veränderungen im T-Zell-Kompartiment. Neben dem vorzeitig gealterten T-Zell Kompartiment zeigten unsere Daten, dass eine hohe Expression von CD39, insbesondere bei Tregs, und ein erhöhter Anteil an antigen-unerfahrenen CD8 Gedächniszellen (*virtual memory cells*) vielversprechende Diagnosemarker für die Erkennung von Patienten mit Immunschwäche sind. Schließlich konnte ich zeigen, dass eine Kombination aus pränataler Steroidbehandlung, frühkindlicher Thymektomie und/oder DiGeorge Syndrom die Veränderungen im T-Zell-Kompartiment beschleunigt.

Zusammenfassend zeigte ich in dieser Arbeit, dass Störungen der Thymusfunktion während der frühen Kindheit, wie dies bei pränatalen Steroiden, frühkindlicher Thymektomie oder dem DiGeorge Syndrom der Fall ist, zu Veränderungen in der Entwicklung und der Zusammensetzung der Immunzellen führen und somit die Entwicklung immunologischer Erkrankungen im späteren Leben begünstigen können. Wir haben vielversprechende diagnostische Marker für die Identifizierung von Patienten mit Immunschwäche ermittelt. Die routinemäßige Praxis der pränatalen Steroidbehandlung und der frühkindlichen Thymektomie in den letzten dreißig Jahren stellt möglicherweise eine Belastung für zukünftige Generationen dar. Die Ergebnisse dieser Arbeit verlangen weitere Studien um ein Bewusstsein für die möglichen langfristigen Folgen der pränatalen Steroidbehandlung und der frühkindlichen Thymektomie zu schaffen und empfehlen eine Neubewertung der klinischen Richtlinien.

7. Abbreviations

11ß-HSD-2	11β-hydroxysteroid dehydrogenase type 2
ADP	adenosine diphosphate
AIRE	auto immune regulator
AMP	adenosine monophosphate
APC	antigen-presenting cell
APS	autoimmune polyglandular syndrome type 1
ATP	adenosine triphosphate
BCR	B cell receptor
Breg	regulatory B cell
CBG	corticosteroid-binding globulin
cDC	conventional dendritic cell
CD	cluster of differentiation
$CD4_{conv}$	conventional T cells
CMV	Cytomegalovirus
cTEC	cortical thymic epithelial cell
DC	dendritic cell
DGS	DiGeorge syndrome
DP	double positive
CVID	common variable immunodeficiency
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
ETP	early thymic progenitor cells
FEZF2	forebrain embryonic zinc finger-like 2
FOXP3	forkhead box P3
GA	gestational age
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid response elements
HIV	human immunodeficiency viruses
HPA	hypothalamic-pituary-adrenal
HSC	hematopoietic stem cells

11β-HSD2	11ß-Hydroxysteroid dehydrogenase type 2
IDO	indolamin-2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IL	interleukin
ILC	innate lymphoid cells
Iono	Ionomycin
IPP	Isopentenyl pyrophosphate
ISP	intermediate single positive
KIR	killer cell Ig-like receptor
KLF2	Krüppel-like factor 2
KLRG-1	killer cell lectin-like receptor subfamily G-1
MAIT	mucosal-associated invariant T cells
MHC	major histocompatibility complexes
MIF	macrophages migration inhibitory factor
mTEC	medullary thymic epithelial cell
NET	neutrophil extracellular trap
NKG2A	NK receptor 2G family
NKT	natural killer T cells
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell
pDGS	partial DiGeorge syndrome
PLP	proteolipid protein
PMA	Phorbol 12-myristate 13-acetate
PPR	pattern recognition receptors
pTreg	peripherally-induced regulatory T cell
RDS	respiratory distress syndrome
RNA	ribonucleic acid
ROS	radical oxygen species
RTE	recent thymic emigrants

S1P	sphyngosin-1-phospat receptor
SCID	severe combined immunodeficiency
SNP	single-nucleotide polymorphism
TEM	T effector memory cells
TRA	tissue-restricted antigens
TRB	TCRβ chain
TCR	T cell receptor
TEC	thymic epithelial cell
TEP	thymic early progenitor
Tfh	T follicular helper
TGF	transforming growth factor
Th	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
TRECs	T-cell receptor excision circles
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
tSNE	t-stochastic neighbor embedding
TSSP	thymus-specific serine protease
tTreg	thymic regulatory T cell

8. References

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11. Curriculum Vitae

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

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12. Appendix

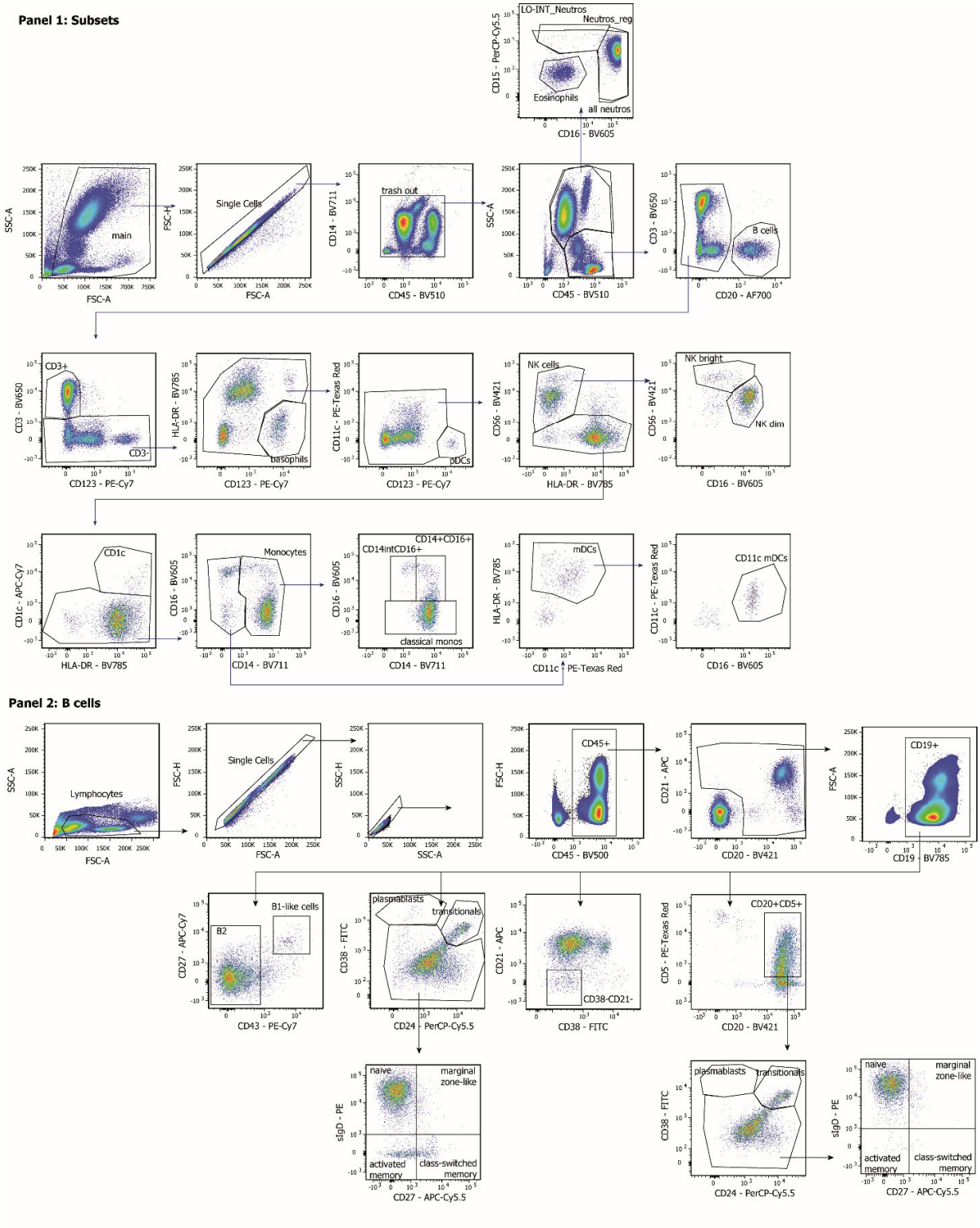
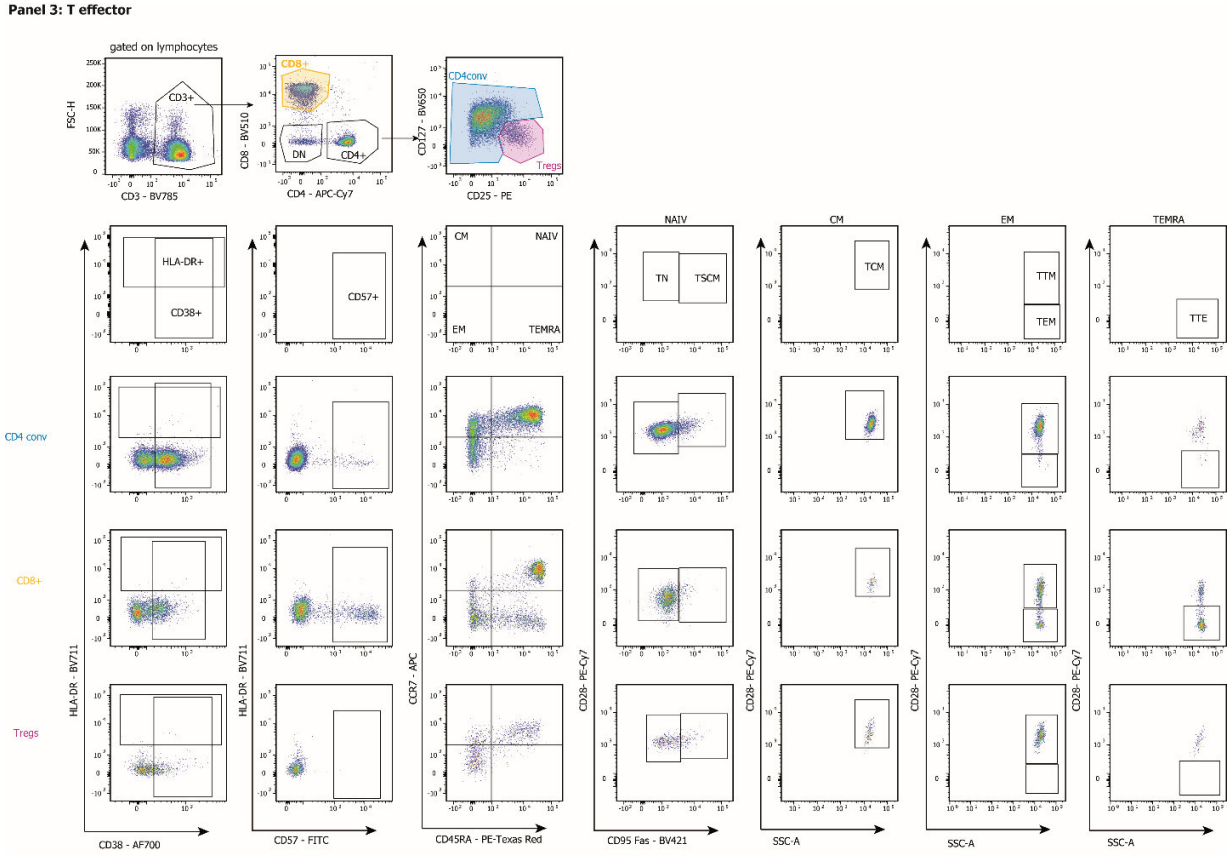


Figure S1 Gating strategy for panel 1 (subsets) and panel 2 (B cells).

Panel 3: T effector



Panel 4: T helper

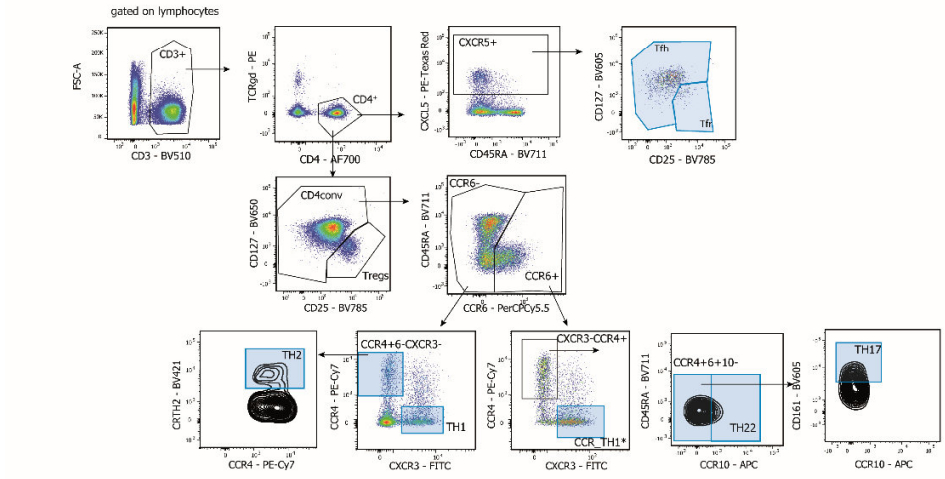


Figure S2. Gating strategy for panel 3 (T effector) and panel 4 (T helper).

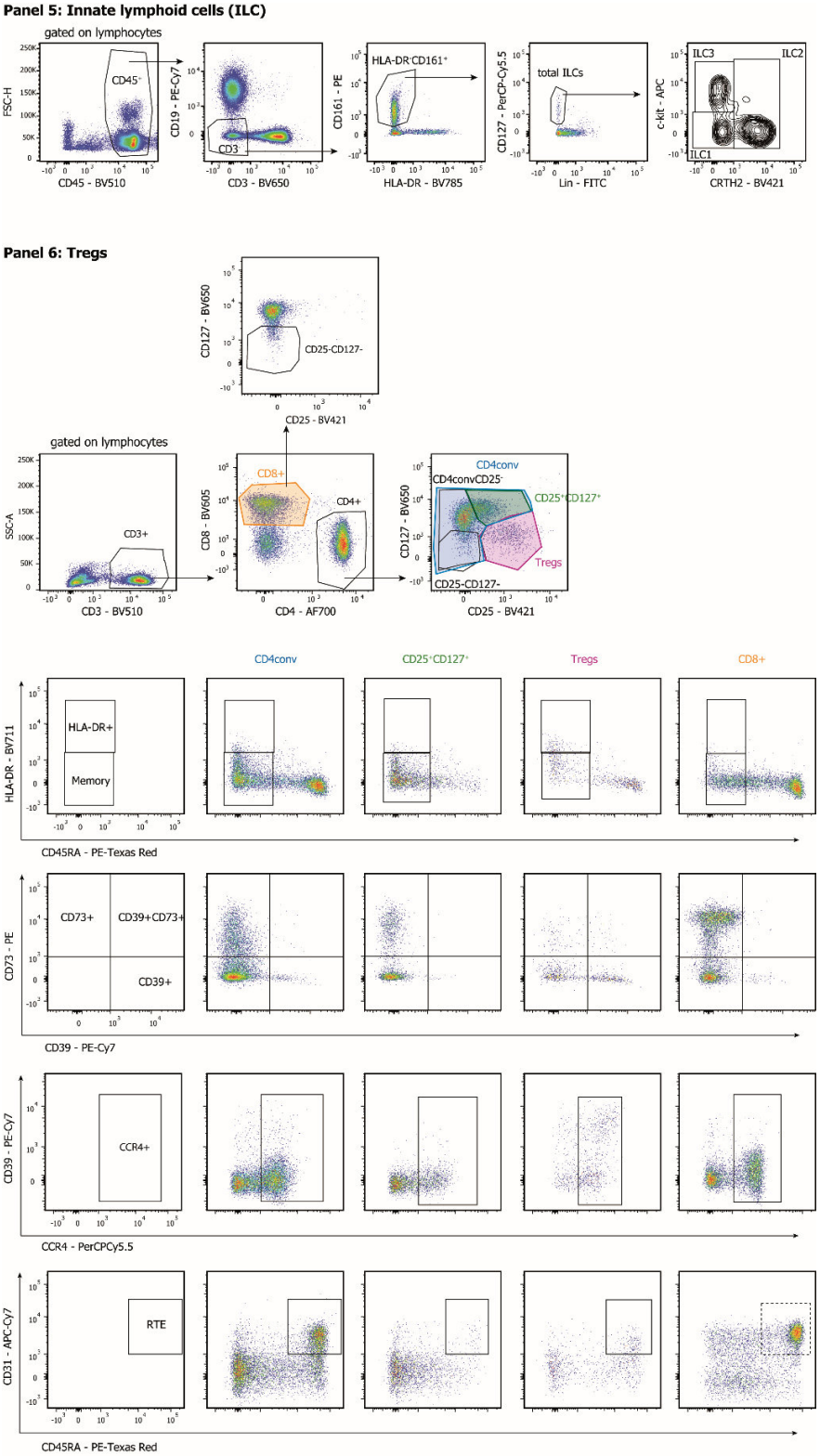
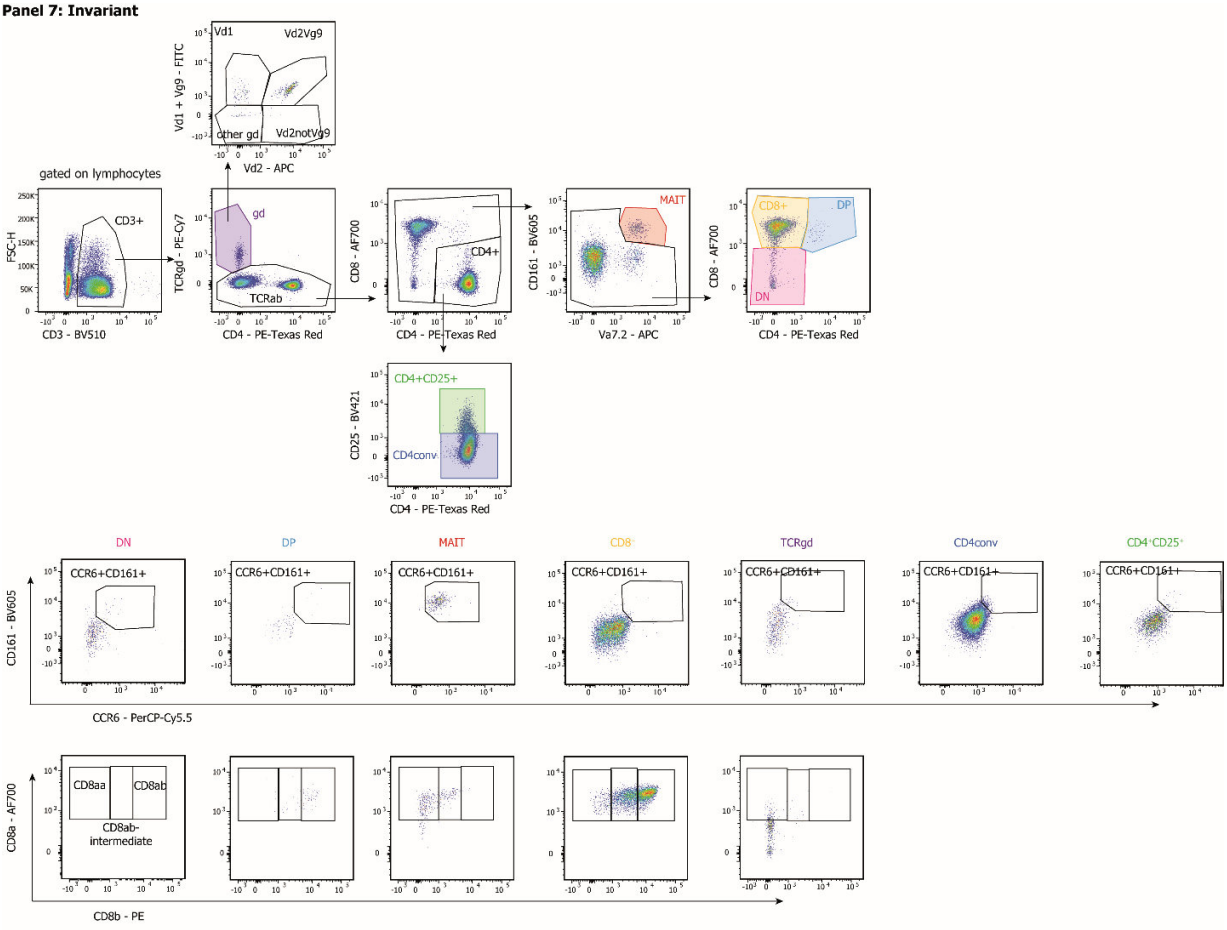


Figure S3. Gating strategy for panel 5 (Tregs) and panel 6 (Innate lymphoid cells).

Panel 7: Invariant



Panel 8: Cytokines (ICC)

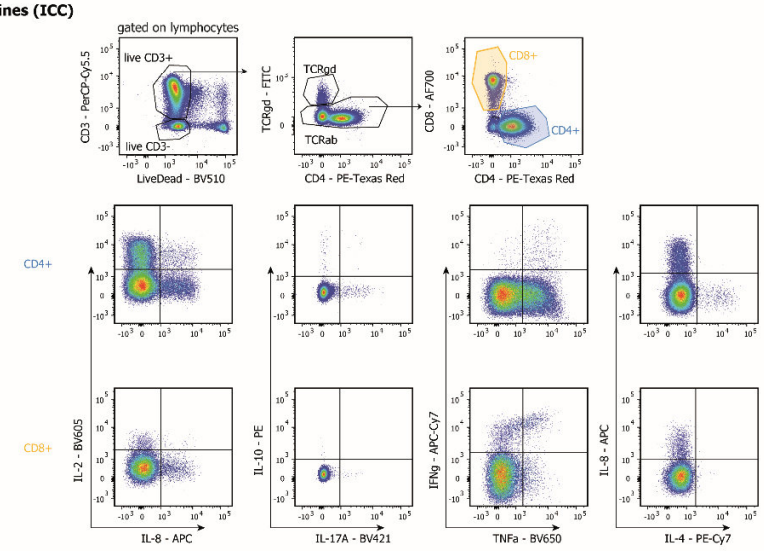


Figure S4. Gating strategy for panel 7 (invariant) and panel 8 (cytokines).

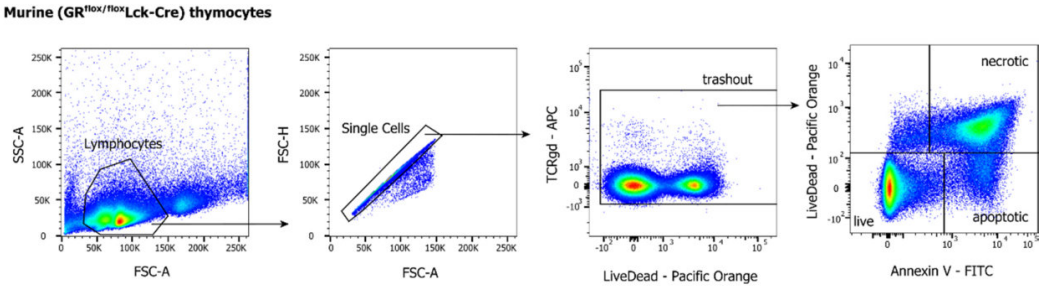


Figure S5. Gating strategy for murine (GRflox/floxLck-Cre) thymocytes from *in vitro*experiments.

13. Eidesstattliche Versicherung

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.

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