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The effects of maternal type 1 diabetes on autoreactive $CD4^+$ T cells in neonates

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Abbreviations

AHR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
BCL	B-cell lymphoma
c/mTEC	Cortical/medullary thymic epithelial cell
CBMC	Cord blood mononuclear cell
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTLA4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DN / DP	Double negative / positive
ELISPOT	Enzyme-linked immunosorbent spot
FACS	Fluorescence activated cell sorting
FoxP3	Forkhead box P3
FSC	Forward scatter detector
GAD65	65-kDa isoform of glutamate decarboxylase
GDM	Gestational diabetes
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome wide association studies
HLA	Human leucocyte antigen
HSC	Hematopoietic stem cell
IA-2	Islet antigen-2
IAA	Insulin autoantibody
ICOS	Inducible costimulator
IFN	Interferon
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL	Interleukin

KLH	Keyhole lympet hemocyanin
LGA	Large for gestational age
MACS	Magnetic activated cell sorting
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MO-DCs	Monocyte-derived dendritic cells
MPP	Multipotent progenitor
MS	Multiples sclerosis
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa light chain enhancer of activated B-cells
NK	Natural killer
NOD	Non-obese diabetic
OGTT	Oral glucose tolerance test
PBMC	Peripheral mononuclear cell
PMA	Phorbol myristate acetate
PMT	Photomultiplier
PPI	Pre-proinsulin
RAG	Recombination-activating gene
RUNX1	Runt-related transcription factor 1
SCF	Stem cell factor
SD	Standard deviation
SNP	Single nucleotide polymorphism
SP	Single positive
SSC	Side scatter detector
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th	T helper
TNFα	Tumor necrosis factor alpha
T_{reg}	Regulatory T cell
tSNE	t-distributed Stochastic Neighbor Embedding
VNTR	Variable number of tandem repeats
ZnT8	Zinc transporter 8

Summary

Type 1 diabetes is a T cell-mediated autoimmune disease resulting in the destruction of insulin-producing beta cells in the pancreas, orchestrated by autoantigen-specific $CD4^+$ T cells. Type 1 diabetes has an initiation phase identified by seroconversion to islet autoantibody positivity, and a progression phase with persistence and often discontinuous spreading of islet autoantibodies, resulting in decline of beta cell function and onset of clinical diabetes. There is a peak period of initiation of autoimmunity that is found at around 1 year of age. Thus, factors that occur prior to this are likely to influence the development of type 1 diabetes. Of particular interest, it is known that the risk of developing type 1 diabetes is decreased in children born to mothers who have type 1 diabetes as compared to children born to fathers with type 1 diabetes or with a type 1 diabetes sibling. The same reduction of risk was also observed for the development of islet autoantibodies and in particular insulin autoantibodies in the first year of life. In addition, the risk of developing islet autoantibodies in children born to mothers with type 1 diabetes was lowest in children of mothers with moderately uncontrolled glucose homeostasis during gestation. Based on these findings, we hypothesized that increased and varying glucose concentrations during pregnancy increases (pro)insulin production by the fetus already *in utero*, and that this provides protection in offspring of mothers with type 1 diabetes by increasing the efficiency of (pro)insulin-specific autoreactive T cells removal and their regulation. The aim of my thesis was to determine whether there are differences in (pro)insulin-specific $CD4^+$ T cells in offspring who have been exposed to a type 1 diabetes maternal environment. I specifically asked if there are fewer numbers of (pro)insulin-specific $CD4^+$ T cells, and whether there are additional features that are consistent with a less inflammatory and more regulatory T cell response to these autoantigens.

I first profiled cells from cord blood obtained from newborns who had a mother or father with type 1 diabetes. My main focus was on the proinsulin-responsive $CD4^+$ T cells. I, therefore, established a new multi-well, multi-dye proliferation assay to detect rare autoreactive T cell frequencies with more confidence and markedly less cells than previously possible. I showed that $CD4^+$ T cell reactivity against proinsulin and insulin were increased in neonates born to fathers with type 1 diabetes as compared to neonates of non-diabetic parents, and, importantly, that the frequency of (pro)insulin-responsive $CD4^+$ T cells from neonates born to mothers with type 1 diabetes was not increased and similar to those in control neonates. I then asked whether the responding cells also differed in their response profile. I applied single cell gene

expression profiling to the antigen-responsive $CD4^+$ T cells found at birth, and showed that the responses in neonates from mothers with type 1 diabetes also differed in their gene expression with few cells acquiring strong Th1 or Th17 profiles. Finally, I asked whether the children from mothers with type 1 diabetes showed increased regulation against $CD4^+$ T cell responsiveness to proinsulin and addressed this by performing T cell suppression assays in the children at median age of 9 months. I found that the regulatory T cells from children of mothers with type 1 diabetes were stronger suppressors of the $CD4^+$ T cells response to proinsulin than the regulatory T cells from fathers with type 1 diabetes, and that this increased suppression was not observed on polyclonally stimulated T cells. This suggests that there is more regulation against proinsulin-responsive T cells in children born to mothers with type 1 diabetes. I additionally developed a multi-parameter flow cytometry assay to extensively describe autoreactive $CD4^+$ T cell phenotypes and their cytokine profiles and applied this to patients at onset of type 1 diabetes. I confirmed previously described increased frequencies of autoantigen-reactive $CD4^+$ T cells in patients and additionally demonstrated pro-inflammatory GM-CSF signatures in the proinsulin- and GAD65-responsive $CD4^+$ T cells. With my work, I reported GM-CSF producing $CD4^+$ T cells as a novel phenotype in the repertoire of T helper cell subsets in type 1 diabetes.

In my thesis, I introduced new multi-parametric *in vitro* tools to extensively describe phenotypes of (auto)antigen-specific T cells. With respect to protection against islet autoimmunity in children conferred by maternal type 1 diabetes environment, I demonstrated cellular mechanisms pointing at increased (pro)insulin production by the fetus *in utero* to favor central and peripheral tolerance to these key targets of autoimmunity in type 1 diabetes.

Zusammenfassung

Der Typ 1 Diabetes zählt wie alle anderen Diabetesformen zu den metabolischen Erkrankungen (Stoffwechselerkrankungen). Der Typ 1 Diabetes ist jedoch gleichzeitig und primär eine Autoimmunerkrankung. Bevor es zu pathologischen Veränderungen der Blutglukose kommt, lassen sich Diabetes-spezifische Betazell-Autoantikörper im Blut der betroffenen Personen nachweisen. Die Phase der asymptomatischen Autoimmunität wird heute auch als Stadium 1 des Typ 1 Diabetes bezeichnet. Prospektive Kohortenstudien, die Kinder mit einem erhöhten genetischen Risiko für Typ 1 Diabetes von Geburt an nachuntersuchten, haben entscheidend zur Aufklärung der Pathogenese des Typ 1 Diabetes beigetragen. Eines der wichtigen Erkenntnisse dieser Studien war das frühe Auftreten der Betazell-Autoantikörper mit einem Inzidenzgipfel im ersten Lebensjahr. Das heißt Kinder, die irgendwann in ihrer Kindheit Typ 1 Diabetes entwickelten, entwickelten Betazell-Autoantikörper und demzufolge eine Störung der Immuntoleranz bereits in ihrem ersten Lebensjahr. Diese Befunde haben dazu geführt, dass neuere Studien zur Pathogenese der Autoimmunität bei Typ 1 Diabetes sich heute insbesondere mit den ersten Lebensjahren und frühkindlichen Risikofaktoren, sowie Umwelteinflüssen beschäftigen. Ein Faktor ist hierbei von besonderem Interesse, weil er der bisher einzige, beschriebene Umweltfaktor ist, der nachgewiesener Weise das Risiko Typ 1 Diabetes zu entwickeln reduziert: In unterschiedlichen nationalen und internationalen Studien konnte gezeigt werden, dass Kinder von Müttern mit Typ 1 Diabetes ein geringeres Erkrankungsrisiko im Vergleich zu Kindern mit einem anderen erstgradigen Verwandten mit Typ 1 Diabetes besitzen. Dieses reduzierte Risiko spiegelte sich nicht nur in einen reduzierten Typ 1 Diabeteshäufigkeit, sondern auch in einer ungefähren Halbierung der Antikörperinzidenz, sowie in einer verzögerten Antikörperentstehung wieder. Die Bildung der Insulin-Autoantikörper ist dabei am deutlichsten reduziert. Des Weiteren ist der beschriebene Effekt am stärksten bei Kindern von Müttern ausgeprägt, die während der Schwangerschaft keine zu strenge Stoffwechseleinstellung und somit mäßig unkontrollierte Blutzuckerwerte aufweisen.

Ausgehend von diesen Befunden habe ich in meiner Promotionsarbeit die Hypothese aufgestellt, dass mäßig erhöhte Glukosekonzentrationen bei der Mutter während der Schwangerschaft die fetale (Pro-)Insulin-Produktion erhöht, und dass die erhöhten (Pro-) Insulinspiegel der Feten zu einer optimierten Immuntoleranz gegenüber (Pro-)Insulin führen, welche das Kind einer Mutter mit Typ 1 Diabetes vor Typ 1 Diabetes schützen. Das Ziel meiner

Arbeit war deshalb, Methoden zu entwickeln, die es erlauben, (Pro-)Insulin-reaktive $CD4^+$ T Zellen bei Kindern in der frühen Lebensphase zu detektieren und zu phänotypisieren.

Insbesondere wurden in der vorliegenden Arbeit neue, multi-parametrische *in vitro* Methoden entwickelt. Es wurden Nabelschnurblut-Proben von Neugeborenen mit Müttern oder Vätern mit Typ 1 Diabetes auf (Pro-)Insulin-spezifische $CD4^+$ T Zellen untersucht. Hierfür etablierte ich einen neuartigen Proliferationsassay unter Verwendung multipler Farbstoffe und Replikate, welcher mir ermöglichte, sehr geringe Frequenzen von autoreaktiven T Zellen in wenig Ausgangsmaterial mit hoher Sensitivität detektieren zu können. Ich konnte zeigen, dass die Reaktivität von $CD4^+$ T Zellen gegenüber Proinsulin und Insulin im Nabelschnurblut bei Kindern von Vätern mit Typ 1 Diabetes und Kindern mit Geschwistern mit Typ 1 Diabetes im Vergleich zu Kindern gesunder Eltern erhöht war. Dieser Befund zeigt erstmals, dass bereits bei Geburt bei Kindern mit einer familiären Typ 1 Diabetes Belastung eine Immunpathologie vorhanden ist, die möglicherweise die Grundlage für ein erhöhtes Typ 1 Diabetes-Risiko liefert. Diese Immunpathologie fand sich nicht in Kindern von Müttern mit Typ 1 Diabetes. Analysen von Genexpressionssignaturen in einzelnen (Auto)Antigen-spezifischen $CD4^+$ T Zellen zeigten zudem, dass die fetale Exposition in ein maternales Typ 1 Diabetes Umfeld zu einer Reduktion von Th1- und Th17-spezifischen Profilen in T Zellen von Neugeborenen führte. Die regulatorische Kapazität von Proinsulin-spezifischen $CD4^+$ T Zellen von neun Monate alten Kindern von Müttern mit Typ 1 Diabetes wurde in T Zell-spezifischen, *in vitro* Suppressions-Experimenten evaluiert. Hierbei konnte ich zeigen, dass im Vergleich zu Kindern von Vätern mit Typ 1 Diabetes, regulatorische T Zellen von Kindern von Müttern mit Typ 1 Diabetes eine erhöhte suppressive Kapazität gegen Proinsulin-spezifische $CD4^+$ T Zellen besitzen. Dieser Antigen-spezifische Effekt konnte nicht für die Suppression polyklonal-stimulierter T Zellen gezeigt werden. Diese Ergebnisse deuten darauf hin, dass ein maternaler Typ 1 Diabetes während der Schwangerschaft eine erhöhte Regulation von (Auto)Antigen-spezifischen T Zellen in Neugeborenen induziert. Um diesen potentiell, protektiven Mechanismus zu validieren, entwickelte ich ein multi-parametrisches Durchflußzytometrie-Experiment, um vorliegende $CD4^+$ T Zell Phänotypen und deren Zytokin-Signaturen zum Zeitpunkt der klinischen Manifestation des Typ 1 Diabetes zu charakterisieren. Hierbei bestätigte ich bereits beschriebene, erhöhte Autoantigen-spezifische Frequenzen von $CD4^+$ T Zellen in Patienten, und wies zusätzlich eine pro-inflammatorische GM-CSF Signatur in Proinsulin- und GAD65-spezifischen $CD4^+$ T Zellen nach. GM-CSF produzierende, Autoantigen-spezifische $CD4^+$ T Zellen können sowohl einem

Th1, als auch einem Th17 Zell Phänotyp zugeordnet werden und wurden im Rahmen meiner Arbeit zum ersten Mal in der Pathogenese des Typ 1 Diabetes beschrieben.

In dieser Arbeit wurden neuartige, multi-parametrische *in vitro* Methoden zur detaillierten Phänotypisierung von (Auto)Antigen-spezifischen T Zellen etabliert und erstmals frühkindliche Einflußfaktoren am Modell des maternalen Typ 1 Diabetes auf zelluläre Mechanismen der Immuntoleranz untersucht. Die gezeigten Ergebnisse lassen vermuten, dass eine vermehrte (Pro-)Insulin Produktion während der Schwangerschaft durch den Fötus *in utero* eine erhöhte zentrale, sowie periphere Toleranz gegenüber Schlüssel-Antigenen des Typ 1 Diabetes induziert.

1. Introduction

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease characterized by destruction of insulin-producing beta cells in the pancreas (Cnop et al., 2005). Population-based studies have shown a rise in the incidence of T1D over the last decades and a doubling of new cases of T1D in children aged <15 years in Europe is predicted (Patterson et al., 2009). This increase in cases has heightened the search for genetic and environmental factors that accelerate disease progression and manifestation (Todd, 2010) and to develop strategies that may prevent T1D (Todd et al., 2011).

An understanding of the cellular mechanisms that are involved in the pathogenesis of T1D is needed to develop potential prevention and intervention therapies. T1D is characterized by insulitis within the pancreatic islets. Pancreatic biopsies from early stages of the disease is, however, not readily available to study this process (Campbell-Thompson et al., 2012), and we must rely on alternative samples such as peripheral blood, which may harbor immune cells that are involved in the pathogenesis. Although these immune cells will be infrequent, autoreactive T cells have been detected at low frequencies in various autoimmune diseases, including T1D (Monti et al., 2007; Girolamo et al., 2014; Tracy et al., 2017). It is, therefore, possible to examine the relationship between the numbers or characteristics of autoreactive T cells and T1D risk. The assessment of autoreactive T cells that recognize pancreatic beta cell antigens was the theme of my doctoral thesis. In particular, I asked how they were affected by an established protective factor, which is maternal T1D.

1.1. Type 1 Diabetes Pathogenesis

T1D is characterized by autoimmune destruction of beta cells in the pancreas, resulting in an absolute insulin deficiency and represents around 90% of all diabetes mellitus cases in children and adolescents and 5-10% in adults (Achenbach et al., 2010). T1D is a chronic autoimmune disease and an early model by Eisenbarth (Figure 1; (Eisenbarth, 1986) divides the pathogenesis into 3 stages:

1. Genetic predisposition
2. Pre-diabetes: Appearance and spreading of islet-specific autoantibodies against beta cell derived antigens followed by gradual destruction of insulin-producing beta cells
3. Clinical manifestation of type 1 diabetes with >80% - total loss of beta cell mass

The pre-diabetes stage is identified by circulating islet autoantibodies to beta cell antigens. The progression from the first appearance of islet autoimmunity (seroconversion) to clinical manifestation varies substantially between different patients from a few months to decades (Ziegler et al., 2013). Of importance, there is a peak incidence of seroconversion that appears already in early childhood before 2 years of age (Ziegler and Nepom, 2010; Ziegler and Bonifacio, 2012). In addition to strong genetic contributions to the susceptibility for T1D, the onset of autoimmunity is hypothesized to be influenced by environmental factors (Ziegler and Nepom, 2010). These modifying factors still remain unknown and may be a combination of different environmental events, which include prenatal exposures such as maternal enteroviral infections, cesarean section, maternal age and postnatal factors such as persistent and recurrent enteroviral infections, frequent respiratory and enteric infections and infant diet (reviewed in Rewers and Ludvigsson, 2016). Subsequent preclinical stages of T1D are characterized by progressive beta cell destruction via macrophages and effector T cells in a progress called insulitis (reviewed in Cnop et al., 2005), resulting in cumulative loss of insulin release to regulate blood glucose levels.

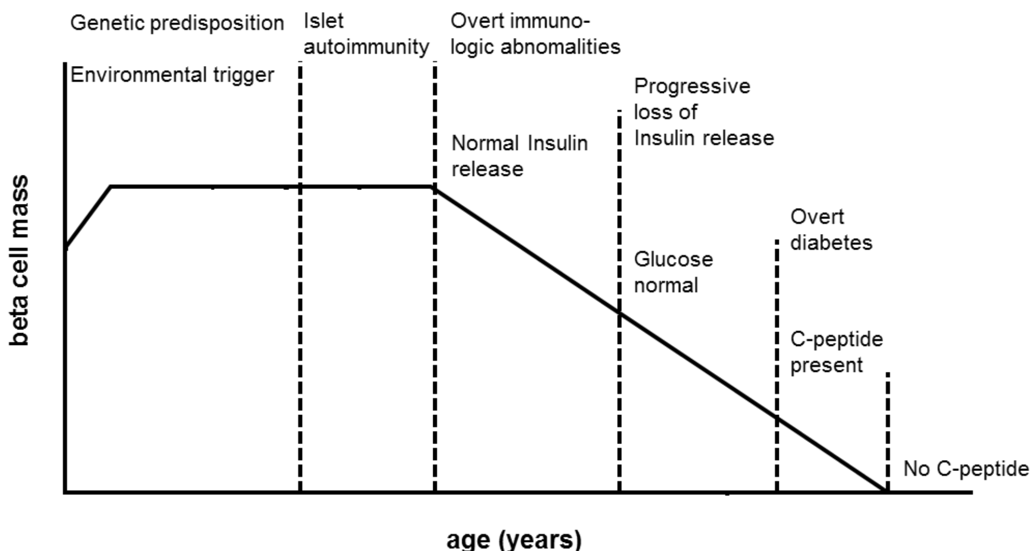


Figure 1: Different stages in the pathogenesis of type 1 diabetes.

1.1.1. Genetic susceptibility

Genetic susceptibility strongly contributes to the risk of developing autoimmunity and T1D. The background population risk is around 0.3 to 0.5%. In contrast, children who have a first degree relative with T1D have a 5% risk of T1D (Redondo et al., 2001; Bonifacio and Ziegler, 2010). Susceptibility genes include those that encode a) Human Leukocyte Antigen complex (HLA; encoding for Major Histocompatibility Complex I and II), b) proteins important for T (and B) cell immunity and c) proteins of pancreatic target tissue (Fernando et al., 2008).

1.1.1.1. HLA and type 1 diabetes susceptibility

Several studies have shown that gene clusters in the HLA region on chromosome 6p21.31 confer a strong susceptibility for T1D. HLA genotypes are used in combination with autoantibodies to identify individuals with a high risk of developing T1D (Bonifacio, 2015). HLA genes encode glycoproteins of the major histocompatibility complex (MHC), which are relevant for the immune system to distinguish between self and non-self (Murphy and Weaver, 2018). Products of the HLA loci encode for three class I (A, B and C) and three class II (DR, DQ and DP) cell surface proteins, which are similar in structure and bind antigenic-peptides for presentation to $CD8^+$ or $CD4^+$ T cells, respectively (Noble and Erlich, 2012). Genetic susceptibility for T1D is mainly conferred by haplotypes formed by the HLA class II DRB1,

DQA1, and DQB1 loci. Strong susceptibility is provided by the HLA-DRB*03-DQA1*0501-DQB1*0201 (DR3-DQ2) and the HLA-DRB1*04-DQA1*0301-DQB1*0302 (DR4-DQ8) haplotypes. The strongest genetic susceptibility is provided by the DR3-DQ2/DR4-DQ8 heterozygous genotype, which is present in around 35% of patients with T1D and 2% to 3% of healthy individuals (reviewed in van Belle et al., 2011). In contrast, haplotypes such as DRB1*1501-DQA1*0102-DQB1*0602 are associated with protection to T1D and are found in 20% of general population compared with only 1% of patients with T1D (Baschal and Eisenbarth, 2008; Erlich et al., 2008). Of particular relevance, it is reported that there is no compatibility difference in the HLA haplotypes found in children of mothers with T1D and children with fathers with T1D (Bronson et al., 2009).

Table 1: Type 1 diabetes risk associated with HLA-DR and HLA-DQ haplotypes (adapted from Atkinson and Eisenbarth, 2001).

Risk	**HLA genotype**		
	HLA DRB1	HLA DQA1	HLA DQB1
High risk	0401, 0402, 0405	0301	0302
	0301	0501	0201
Moderate risk	0801	0401	0402
	0101	0101	0501
	0901	0301	0303
Weak or moderate	0401	0301	0301
protection	0403	0301	0302
	0701	0201	0201
	1101	0501	0301
Strong protection	1501	0102	0602
	1401	0101	0503
	0701	0201	0303

The susceptibility for T1D conferred by HLA class II genotypes is most likely due to mechanisms involving the thymic selection of T cells (Kishimoto and Sprent, 2001). Each T cell that enters the thymus has a unique T cell receptor (TCR). T cells are selected or deleted in the thymus on the basis of the avidity of their TCR for peptides of self-proteins presented by HLA molecules (Klein et al., 2014). T cells bearing TCRs that do not bind will die as will T cells with TCR that have a very strong avidity for HLA/self-peptide complexes as these will pose too high a risk for autoimmunity. It is thought that the MHC molecules of the T1D susceptible HLA genotypes

inefficiently present peptides of T1D-related autoantigens to the potentially autoreactive T cells leading to an increased number of pancreatic beta cell reactive T cells in individuals with susceptible genotypes (reviewed in Wagner, 2016).

1.1.1.2. Non-HLA type 1 diabetes loci

The application of genome wide association studies (GWAS) using single nucleotide polymorphisms (SNPs) has identified more than 40 additional genetic loci contributing to the genetic susceptibility of T1D (Rich et al., 2009). Although the risk conferred by any one of these susceptibility regions is small in comparison to the risk conferred by HLA, combinations of these can be used to increase our ability to predict T1D (Steck et al., 2014; Winkler et al., 2014) or distinguish type 1 and type 2 diabetes (Oram et al., 2016). While HLA genotypes are known to interfere with antigen presentation and therefore mainly influence the initiation of islet autoimmunity, various characterized non-HLA type 1 diabetes loci also modify the rate of progression from autoimmunity to manifest diabetes (Achenbach et al., 2013).

One of the first discovered non-HLA T1D susceptibility genes was the insulin gene (*INS*) on chromosome 11p15.5. Here a variable number of tandem repeats (VNTR) were described in the *INS* gene promoter region (IDDM2; Bennett et al., 1996). *INS* gene polymorphisms confer substantial risk for T1D (Meigs et al., 2005) and autoantibodies (Walter et al., 2003). The polymorphisms are associated with differences in the expression of insulin in the thymus and, similar to HLA, are thought to modify T1D risk via thymic selection of insulin-specific T cells (Pugliese, 2005).

The third strongest T1D susceptibility gene is *PTPN22* gene, located on chromosome 1p13.2 and, which encodes the protein tyrosine phosphatase, non-receptor 22. PTPN22 is present on all lymphoid cells and functionally suppresses T cell activation by dephosphorylating mediators in T cell receptor signaling (Bottini et al., 2004). Alterations in the *PTPN22* gene product are linked to several autoimmune diseases including rheumatoid arthritis, Grave’s disease, juvenile idiopathic arthritis, systemic lupus erythematosus, and vitiligo (reviewed in Noble and Erlich, 2012).

Susceptibility to T1D that is comparable in risk for the *PTPN22* was described for the *IL2RA* gene, located on chromosome 10p15.1, which encodes the α-chain of the interleukin (IL) 2 receptor also known as cluster of differentiation (CD) 25 (Vella et al., 2005). IL-2 signaling plays a major role in activation, proliferation and regulation of all T cell subsets and has been

repeatedly linked to the pathogenesis of T1D (reviewed in Hulme et al., 2012). CD25 is constitutively expressed on forkhead box P3 (FoxP3) positive regulatory T cells that are essential in orchestrating and maintaining self-tolerance. Impaired function of regulatory T cells have been repeatedly linked to T1D (Rosenzwajg et al., 2014) and other autoimmune diseases including multiple sclerosis (Maier et al., 2009), vitiligo (Jin et al., 2010) and rheumatoid arthritis (van Steenbergen et al., 2015).

1.1.2. Early appearance of autoimmunity

Immune mediated beta cell destruction could begin months or even decades before the clinical manifestation of the disease (Eisenbarth, 1986). This symptomless phase is commonly identified through serological markers of islet autoimmunity, characterized as autoantibodies against specific beta cell antigens. Screening of serum samples to identify existing islet autoimmunity includes detection of autoantibodies against insulin (IAA), the 65-kDa isoform of glutamate decarboxylase (GAD65), protein tyrosine phosphatase related molecule islet antigen-2 (IA-2), and zinc transporter 8 (ZnT8) (Atkinson and Eisenbarth, 2001; Achenbach et al., 2009). Prospective studies of genetically high-risk children at birth have used the appearance of beta cell autoantibodies to better understand the etiology and natural history of T1D (Parikka et al., 2012; Ziegler and Bonifacio, 2012). Although individual autoantibodies may appear at different ages in a child (Figure 2; Bonifacio, 2015), the German BABYDIAB study, the Finnish DIPP study and the multi-center TEDDY study showed that seroconversion to autoantibody positivity has a peak incidence at around 1 year of age (Ziegler and Bonifacio, 2012; Krischer et al., 2015; Pöllänen et al., 2017). IAA was shown to be the dominant autoantibody first appearing at this age period in each of the studies, and the frequency of IAA is highest in patients with childhood onset of T1D (Ziegler et al., 1999). In contrast, autoantibodies against GAD65 and IA-2 are less frequent in the youngest age group and show a more age-independent appearance (Achenbach et al., 2005). Of importance, the order of autoantibody appearance is additionally associated with different HLA-DR-DQ genotypes (Krischer et al., 2015).

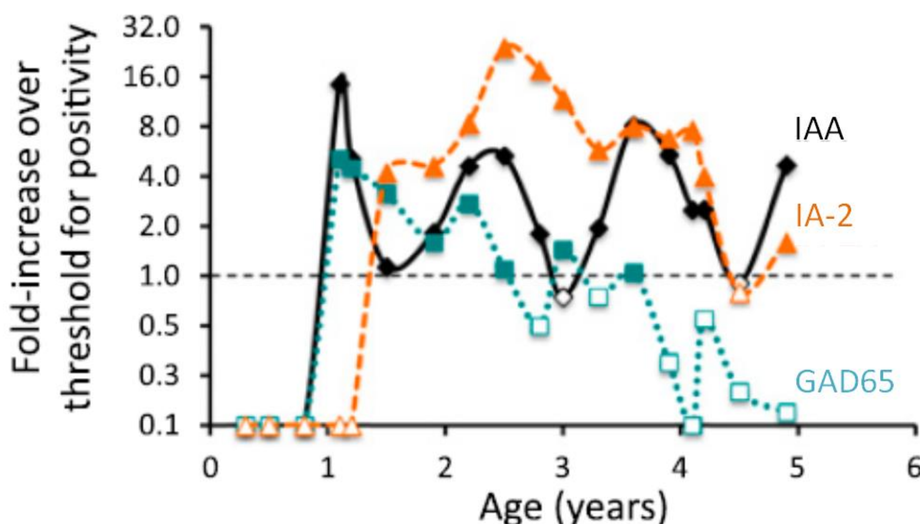


Figure 2: Time course of different islet autoantibodies in a child that develops type 1 diabetes (IAA, black; IA-2, orange; GAD65, green; modified from Bonifacio, 2015).

Although the appearance of circulating islet-specific autoantibodies before disease diagnosis is a characteristic of human T1D, no changes in the peripheral B cell compartment (the cellular source of antibodies) concerning activation status and cytokine secretion have been associated with autoimmune diabetes so far (Thompson et al., 2014).

1.1.3. Environmental exposures that modify the risk for beta cell autoimmunity

Evidence for environmental factors contributing to the risk for T1D includes the observation that monozygotic twins have a T1D concordance rate of only 30-50% (Hirschhorn, 2003), and the rapidly rising incidence of T1D over the last decades (Forouhi and Wareham, 2014).

Viral infections represent one of the most frequently discussed environmental factors to trigger pathogenesis of T1D (Christy et al., 1979). Various viral strains have been positively and negatively associated with development of T1D, especially Coxsackie and Echo viruses belonging to the enterovirus family. Findings are inconsistent, however. In contrast to the US DAISY study (Graves et al., 2003) and the German BABYDIAB study (Füchtenbusch et al., 2001), Laitinen and colleagues identified a positive association between Coxsackie virus B1 and the induction of beta cell autoimmunity in a longitudinal sample series from a large prospective

birth-cohort study (Laitinen et al., 2014). As a possible cellular mechanism, cross reactivity between the nonstructural Coxsackie virus antigen protein 2C and the beta cell autoantigen GAD65 was postulated (Tian et al., 1994), but were not confirmed in T1D patients (Vreugdenhil et al., 1999) and in islet autoantibody positive at risk children (Lee et al., 2013).

Similarly, vaccination in early childhood has been discussed as a trigger, but studies to date have failed to find an association between vaccination and the development of beta cell autoantibodies (Hummel et al., 2000; DeStefano et al., 2001; Beyerlein et al., 2017). Exposure to bacteria, including the microbiome has been controversially reported to affect the risk of beta cell autoimmunity (Atarashi and Honda, 2011; Wu and Wu, 2012; Chervonsky, 2013; Kostic et al., 2015; Endesfelder et al., 2016). Some of these effects may be linked to differences in nutrition in early life, especially duration of breast feeding as well as introduction of food in the perinatal phase that have been inconsistently associated with the risk of developing T1D (Norris et al., 2003; Ziegler et al., 2003; Pflüger et al., 2010; Sorkio et al., 2010; Cardwell et al., 2012).

1.1.4. Influence of maternal and paternal type 1 diabetes during gestation

The development of T1D in offspring is influenced by parental genetics and maternal factors that affect gestation. The latter is best evidenced by the almost 50% reduction in the risk to develop T1D in children who have a mother with T1D as compared to children who have a father or a sibling with T1D. The protection by maternal T1D occurs in the first two years of life at the stage of seroconversion to islet autoimmunity (Figure 3a). The most prominent feature of the decreased incidence of islet autoimmunity was shown in the reduction of the probability to develop antibodies against insulin.

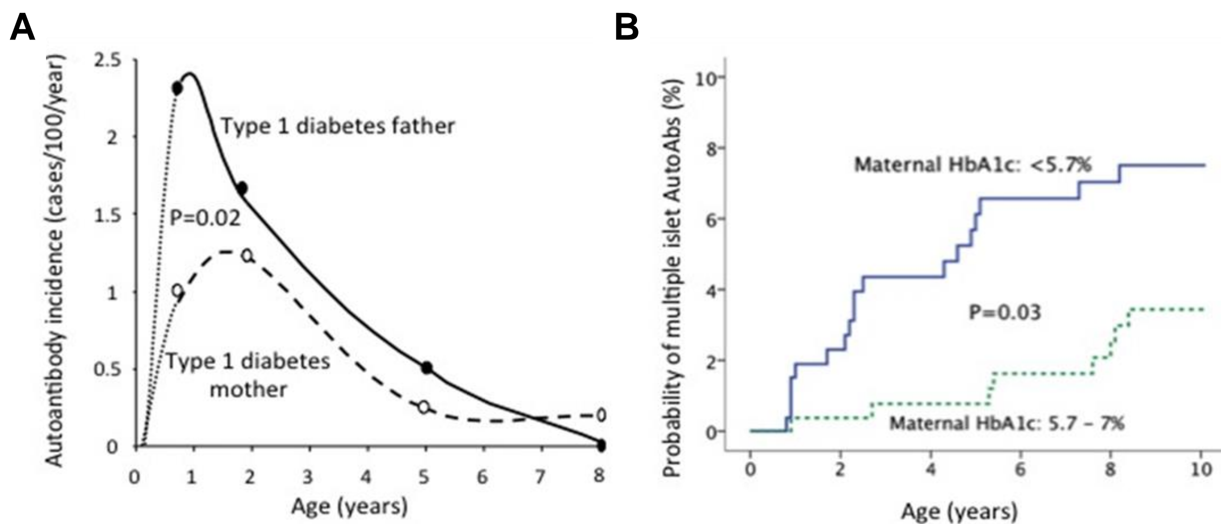


Figure 3: Maternal type 1 diabetes provides strong protection against type 1 diabetes (modified from Bonifacio et al., 2008).

1.1.4.1. Hyperglycemia during maternal type 1 diabetes gestation

Normal pregnancy is characterized by insulin resistance similar to that found in type 2 diabetes, and increases progressively until delivery (Catalano, 2010). Cumulative insulin resistance during gestation is mandatory to mediate transfer of glucose across the placental tissue between the mother and the child resulting in normal fetal development and growth (Farrar, 2016). This fetal development is disordered if resistance to maternal insulin becomes pathogenic in terms of gestational diabetes (GDM) or if insulin secretion is disturbed *per se* as is the case for maternal T1D during gestation. In both diseases, maternal hyperglycemia occurs and effects the development of the child *in utero*. Meta-analysis identified a positive linear association between elevated maternal glucose as measured by oral glucose tolerance tests (OGTT) and various important short-term perinatal risks, including cesarean section, large for gestational age (LGA), induction of labor to stimulate delivery, macrosomia (defined as birthweight >4000g), and infant adiposity (Farrar et al., 2016). During the prenatal phase, exposure to increased glucose concentrations results in an increased beta cell stimulus as indicated by elevated C peptide levels (Sosenko et al., 1979). Increased and varying glucose concentration will lead to increased proinsulin and insulin production by the fetus *in utero* (Lindsay et al., 2003). Of relevance, evaluating the risk of islet autoimmunity according to maternal HbA_{1c} values of the expectant mothers in the last trimester of pregnancy, classified as near normal (<5.7%)

compared to moderately elevated (5.7-7%), showed, that the risk of islet autoimmunity is specifically decreased in children of mothers with moderately uncontrolled glucose homeostasis during gestation (Figure 3b, Bonifacio et al., 2008).

1.1.4.2. Autoantibody transfer during maternal type 1 diabetes pregnancy

It has been shown, that offspring of mothers with T1D are exposed to maternal transfer of IgG beta cell autoantibodies across the placental barrier. Strong correlation between the presence of beta cell antibodies in the cord blood of neonates and in maternal circulation was shown at birth, but autoantibody positivity was lost during development of the child (Ziegler et al., 1993; Naserke et al., 1999). The transmission was suggested to prime autoimmunity in animal models of autoimmune diabetes (Greeley et al., 2002). However, in a study of children born to mothers with T1D, the presence of beta cell autoantibodies from the mother at birth was mildly protective in children who do not have the high risk HLA DRB1*03/DRB1*04-DQB1*0302 genotype (Koczwara et al., 2004).

1.1.4.3. Increased prevalence of caesarian section following maternal type 1 diabetes

Pregnancies in women with T1D more frequently result in delivery by caesarian section than non-diabetic pregnancies (Bonifacio et al., 2008). Birth by caesarian section is associated with a 20% increase in the risk for T1D as compared to vaginal delivery (Cardwell et al., 2008), and some of this effect appears to be via a faster progression to diabetes after the development of beta cell autoantibodies (Bonifacio et al., 2011). These associations are counter-intuitive to the protection offered by maternal T1D.

1.2. Hematopoiesis and T cell development

The formation of cellular blood components, known as hematopoiesis, occurs during early human embryogenesis in the yolk sac and temporarily in the liver before this cellular renewal process is finally localised to the bone marrow. The process of blood cell development is divided into two different stages, the primitive and definitive hematopoiesis. The transient, primitive hematopoiesis plays an important role during early embryonic development and primarily

ensures sufficient tissue oxygenation by involving erythroid progenitor cells that differentiate into erythrocytes and macrophages. These progenitor cells are not pluripotent or capable of self-renewal. The definitive hematopoiesis is characterized by cell differentiation from multipotent, hematopoietic stem cells (HSCs), which have self-renewal characteristics and are capable of differentiating into all blood cell lineages (reviewed in Jagannathan-Bogdan and Zon, 2013). In the developing embryo, HSCs are formed in the aorta-gonad-mesonephros region (Ivanovs et al., 2011) and migrate to the bone marrow as their resident target tissue (Cumano and Godin, 2007). Wnt signaling, which is essential for several embryonic development steps, as well as the Notch signaling pathway have been repeatedly described to promote self-renewal and expansion of HSCs in mice and humans (Reya et al., 2003; Guruharsha et al., 2012). In addition to intrinsic pathways, the microenvironment in the bone marrow is crucial for the maintenance of HSCs. Here, the chemokine CXCL12, which is produced by endothelial (osteoblasts) and perivascular cells (Kiel and Morrison, 2006) and binds to its specific chemokine receptor CXCR4 on HSCs (Peled et al., 1999), is particularly important. To differentiate into mature blood cells, HSCs lose their self-renewal capacity and become multi- to unipotent precursor cells, which are regulated by intrinsic transcription factors as well as external signals such as cytokines in the mircoenvironment (Zhu and Emerson, 2002). The classical model describes the differentiation of multipotent progenitor cells (MPP) into common myeloid (CMP) and common lymphoid progenitors (CLP), which give rise to macrophages, granulocytes, megakaryocytes and erythrocytes or natural killer (NK) cells and T and B lymphocytes, respectively. A more recent model describes the lymphoid lineage commitment as a step-wise differentiation of MPPs and as a consequence myeloid and lymphoid progenitors arise asymmetrically from different precursor cells (Lai and Kondo, 2008). In both cases, it has been demonstrated that the entire process of commitment and maintenance of mammalian hematopoiesis is tightly regulated by the expression of runt-related transcription factor 1 (*RUNX1*) in HSCs (Ichikawa et al., 2013).

Formed lymphocyte precursors can remain in the bone marrow and differentiate to B lymphocytes (Nuñez et al., 1996) or migrate to thymic tissue in a C-C-chemokine receptor 9 (CCR9)-dependent manner in order to proliferate and differentiate into functional T lymphocytes (Cyster, 2009). The entire process of T cell commitment from progenitor cells in the thymic cortex and later medulla is shaped through interactions with the thymic stroma inducing Notch- and IL-7 signaling pathways (Petrie and Zúñiga-Pflücker, 2007). Thymus-entering lymphoid precursors lack surface expression of TCRs as well as CD4 or CD8 co-receptors, defining these cells as double-negative (DN) thymocytes (Figure 4). The development of DN thymocytes is

divided into four sub-stages (DN1-DN4), which are based upon the surface expression of the adhesion molecule CD44, the α-chain of the IL-2 receptor (CD25) and the receptor (c-Kit) for the hematopoietic cytokine stem cell factor (SCF), (reviewed in Famili et al., 2017). The $CD44^{+}CD25^{-}$ DN1 stage is highly heterogeneous and can differentiate into T cell and non-T cell lineages (Porritt et al., 2004). T cell lineage commitment occurs in the late $CD44^{+}CD25^{+}$ DN2 stage, where thymocytes rearrange their β-chain locus of the T cell receptor. $CD44^{low}CD25^{+}$ DN3 thymocytes will survive if their expressed β-chains are capable of pairing with corresponding pre-TCR α-chains to form a pre-TCR on their surface. Subsequent formation of complexes of pre-TCRs and co-stimulatory CD3 surface molecules leads to the proliferation of DN4 thymocytes, followed by termination of β-chain rearrangement and the simultaneous expression of CD4 and CD8 co-receptors. In this double positive (DP) stage, thymocytes repeatedly rearrange their TCR α-chain locus in a recombination-activating gene (RAG)-dependent manner in order to form functional α/β T cell receptors. The affinity of expressed TCRs towards self-peptides that are embedded in MHCs by cortical thymic epithelial cells (cTECs) defines the fate of αβ T cells during positive selection in the thymic cortex. Already weak TCR-MHC interactions induce prevention of B-cell lymphoma 2 (BCL-2)-dependent apoptosis mechanisms and surviving cells further differentiate either into single-positive $CD4^{+}CD8^{-}$ (CD4SP) or $CD4^{-}CD8^{+}$ (CD8SP) thymocytes. Thymocytes lacking interactions with presented self-peptides and their expressed TCRs, will miss intrinsic signaling and will undergo apoptosis as 'death by neglect'. In a following central tolerance mechanism, positive selected SP thymocytes enter the thymic medulla by directed migration and interact with self-peptide/MHC complexes of several antigen-presenting cells (APCs), such as medullary thymic endothelial cells (mTECs), resident and migratory conventional dendritic cells (cDCs), plasmacytoid DCs and B cells. Here, high affinity of TCRs with presented self-peptide/MHC class I or II complexes lead to thymocyte depletion by apoptosis in order to eliminate potential autoreactive T cells. Only non-reactive thymocytes survive this negative selection in the medulla and up-regulate the sphingosine-1-phosphate receptor that controls the exit of naïve T cells from the thymus (Allende et al., 2004). It has been shown that only approximately 2% of DP thymocytes survive positive and negative selection in the thymus to contribute to peripheral T cell repertoire (Murphy and Weaver, 2018). In peripheral lymphoid organs, naïve T cells will be primed by binding of their specific TCR repertoire to presented foreign-peptide/MHC complexes on APCs in order to induce maturation and proliferation of antigen-specific, differentiated effector T cells (Obst, 2015).

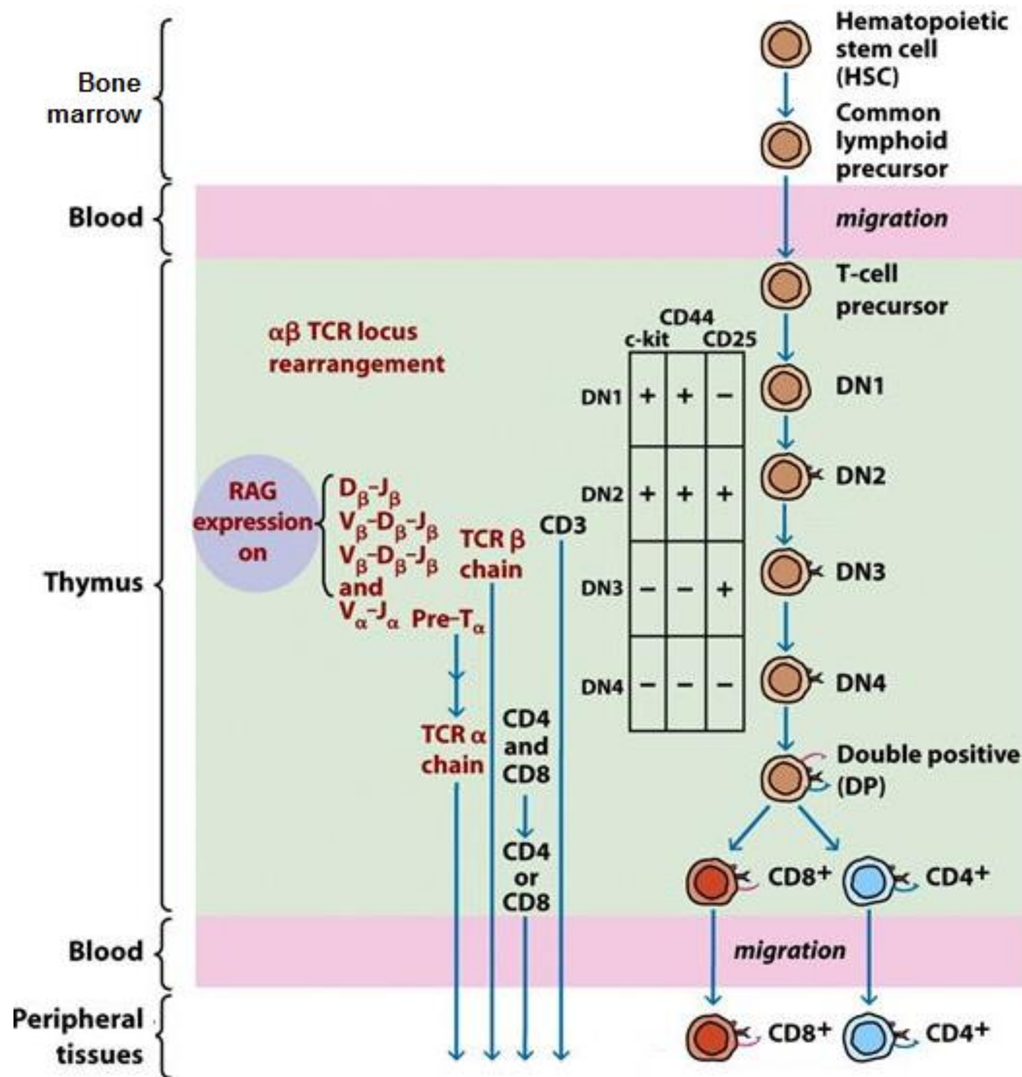


Figure 4: T cell development in the thymus (adapted from Kindt et al., 2006).

1.3. Sensitive methodology for characterizing cellular mechanisms in the pathogenesis of type 1 diabetes

T1D is a T cell mediated autoimmune disease, characterized by autoreactive CD4$^+$ T cells that have been repeatedly described to orchestrate the autoimmune response and cytotoxic CD8$^+$ T cells involved in the direct destruction of the insulin-producing beta cells in the pancreas in a process called insulitis (Bach and Chatenoud, 2001). The frequencies of circulating autoreactive T cells against beta cell antigens are very low and human pancreatic material for research proposes is still rare (Campbell-Thompson et al., 2012). These infrequent T cells can be identified and isolated using state of the art flow methods coupled with antigen bound MHC

class I dextramers and tetramers (Cernea and Herold, 2010) or MHC class II multimers (Massilamany et al., 2015; Serr et al., 2016). Other methods include the use of HLA-A*0201 expressing K562 cells for antigen presentation to phenotype antigen-specific $CD8^+$ T cells (Fuchs et al., 2015) or the detection of beta cell autoreactive $CD8^+$ T cells by islet-specific $CD8^+$ T cell interferon-γ enzyme-linked immunospot (ISL8Spot) assays (Mallone et al., 2007). Enzyme-linked immunosorbent spot (ELISPOT) methods can be used to identify cells producing other cytokines and can be used for both, $CD8^+$ or $CD4^+$ T cells (Alleva et al., 2001; Arif et al., 2004; Bradshaw et al., 2009; Fuchs et al., 2017). The detection of circulating autoreactive $CD4^+$ T cells is often performed using in vitro proliferation response methods. These methods commonly use cell labelling dyes such as Carboxyfluorescein diacetate succinimidyl ester (CFSE) and measure dye dilution as an indicator of cells that have undergone proliferation (Mannering et al., 2004). Many of these methods can be combined with further analyses of the responsive cells such as TCR sequencing of gene expression profiling (Fuchs et al., 2017; Heninger et al., 2017).

Sensitization against beta cell antigens leads to the activation of antigen-specific, naïve $CD4^+$ T cells, which have been reported to be found already at birth in children genetically at risk for T1D (Heninger et al., 2013). Conversion to memory $CD4^+$ T cells is an essential stop in the process of autoimmunity. Memory $CD4^+$ T cells of T helper (Th) 1 and Th17 subsets have been described (Arif et al., 2004; Arif et al., 2011; Reinert-Hartwall et al., 2015). Heninger and colleagues longitudinally followed immune cell profiling of naïve $CD4^+$ T cells in children with strong genetic susceptibility for T1D in the BABYDIET birth cohort study (Heninger et al., 2017). They showed that children who developed beta cell autoantibodies had naïve $CD4^+$ T cells that responded to proinsulin or GAD65 with a pro-inflammatory pre-Th1/Th17/follicular T helper cell gene expression profile at 6 months of age and prior to the development of autoantibodies. A memory $CD4^+$ T cell response with a Th1 profile was observed to be synchronous with the appearance of beta cell autoantibodies. These findings indicate that defined immune signatures are present in children with increased genetic risk for T1D before sensitization and suggest a dynamic shaping of autoimmune responses during T1D pathogenesis.

1.3.1. $CD8^+$ T cells in type 1 diabetes

A major function of $CD8^+$ T cells in host defense is fulfilled by T cell-mediated cytotoxicity (Murphy and Weaver, 2018), which has also been linked to several autoimmune diseases (reviewed in Walter and Santamaria, 2005). In human T1D, both B and T cells are reported

during insulitis. However, $CD8^+$ T cells represent the major immune cell population infiltrating pancreatic islets (Foulis et al., 1986). The islets express elevated surface levels of MHC class I molecules, which present antigens to the $CD8^+$ T cells. These features have been described for pancreatic samples obtained from adult autoantibody positive organ donors (In't Veld et al., 2007), patients at recent onset (Bottazzo et al., 1985) as well as in long-term T1D patients (Coppieters et al., 2012). For further mechanistic insight into $CD8^+$ T cell infiltration during T1D, recent studies of human pancreatic tissue from organ donors identified Hyaluronan and Hyaluronan-binding proteins as possible mediators of $CD8^+$ T cell insulitis in human type 1 diabetic islets (Bogdani et al., 2014). $CD8^+$ T cells against beta cell autoantigens have also been identified and isolated from the peripheral blood of patients. Panina-Bordignon and colleagues showed cytotoxic activity against APCs presenting GAD65-epitopes by T lymphocytes isolated from patients carrying the HLA-A*0201 allele (Panina-Bordignon et al., 1995). Circulating, pre-proinsulin-specific (PPI) $CD8^+$ T cells were detected in T1D patients (Skowera et al., 2008). Combinatorial MHC multimers were used to simultaneously detect increased frequencies of peripheral $CD8^+$ T cells against HLA-A2-restricted beta cell epitopes of insulin, IA-2, the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and PPI in recent-onset diabetic patients (Velthuis et al., 2010). $CD8^+$ T cells against epitopes of ZnT8 were also recently reported (Culina et al., 2018).

1.3.2. $CD4^+$ T cells in type 1 diabetes

$CD4^+$ T cells orchestrate immune responses against pathogens. Following recognition of presented foreign peptides by APCs via MHC class II molecules, naïve $CD4^+$ T cells can become activated and differentiate into different Th subsets according to the cytokine milieu of the microenvironment. In addition to the classical Th1 and Th2 subsets that are characterized by secretion of the IFNγ inflammatory cytokine or the IL-4, IL-5 and IL-13 cytokines, respectively, further Th subsets have been identified with respect to their characteristic cytokine profile. Differentiated T cells subsets include Th17 cell that secrete pleiotropic IL-17 and granulocyte-macrophage colony-stimulating factor (GM-CSF), follicular helper T cells that act via IL-21 signaling and Th9 or Th22 cells that produce IL-9 or IL-22 as their signature cytokine (Murphy and Weaver, 2018). There is also a regulatory subset of $CD4^+$ T cells (T_{reg}) that produce transforming growth factor beta (TGFβ) and, to a lesser extent, IL-10. An important role for various $CD4^+$ T cells subsets have been identified in several autoimmune diseases such as rheumatoid arthritis (Cope et al., 2007), multiples sclerosis (Dittel, 2008) and Crohn’s disease

(Chao et al., 2014). In T1D, autoreactive CD4$^+$ T cells against various diabetes-related antigens have been repeatedly identified. In human T1D, autoreactive CD4$^+$ T cells have been mainly characterized as Th1 (Szebeni et al., 2005; Vaseghi and Jadali, 2016). Moving away from an impaired Th1-Th2 paradigm in T1D, recent studies focused on additional Th subsets. Pathogenic Th17 cells are observed and an increase in Th17 cells secreting IL-17 and IFNγ in autoantibody-positive patients has been reported (Reinert-Hartwall et al., 2015). Increased frequencies of antigen-specific Th17 cells were identified in the presence of proinsulin, insulinoma-associated protein, and GAD65 peptides and were further characterized by simultaneously expression of IFNγ (Arif et al., 2011). Previous findings were supported by detection of upregulated Th17 immunity in pancreatic lymph nodes originating from type 1 diabetic patients who had undergone a pancreatic transplant (Ferraro et al., 2011). In addition, it has been reported that monocytes secrete pro-inflammatory cytokines to induce Th17 maturation in patients with T1D (Bradshaw et al., 2009). Increased frequencies of circulating CXCR5$^+$ follicular helper T cells have been shown in patients with T1D with a shift towards an antigen-experienced phenotype (Kenefeck et al., 2015). Ryba-Stanisławowska and colleagues identified increased plasma levels of IL-9 and IL-22 as well as Th9 and Th22 cell subsets using flow cytometry in children at onset of T1D (Ryba-Stanislawowska et al., 2016).

1.3.2.1. Cytokine signaling of CD4$^+$ T cells in type 1 diabetes

CD4$^+$ T cells shape immune responses by secretion of pro- and anti-inflammatory cytokines according to exogenous stimuli (Figure 5). In T1D, the CD4$^+$ T cell compartment is expected to orchestrate and drive the immune response resulting in inflammation within the pancreatic tissue. Pathogenesis of T1D has been repeatedly linked with a polarization to pro-inflammatory cytokine production by CD4$^+$ T cells. Th1-type cytokines, such as IFNγ, are typically associated with T1D and Th2-type cytokines, such as IL-4, are linked with protection against T1D (reviewed in Rabinovitch and Suarez-Pinzon, 2007). Using an IFNγ-specific ELISPOT assay, Nagata and colleagues described increased IFNγ production by T cells that recognise diabetes-associated antigens (GAD65, insulin and IA-2 peptides) in type 1 diabetic patients (Nagata et al., 2004). A similar study using IFNγ and IL-10 specific ELISPOT in the presence of different proinsulin and IA-2 epitopes supported the hypothesis that islet destruction in T1D is characterized by a strong shift towards a pro-inflammatory CD4$^+$ T cell phenotype. In this study, the circulating CD4$^+$ T cell compartment of patients with T1D had increased frequencies of beta cell antigen-specific CD4$^+$ T cells producing IFNγ, whereas non-diabetic healthy control donors had autoreactive CD4$^+$ T

cells that were characterized by the production of IL-10 (Arif et al., 2004). More recently, an increased production of IFNγ in peripheral $CD4^+$ T cells was not observed in an antigen-unspecific manner (Ferreira et al., 2015). In addition to the classical Th1 (IFNγ) cytokines, various cytokines that may have pleiotropic characteristics have gained increasing interest in studies of T1D. Potential pathogenic production of IL-17 by $CD4^+$ T cells has been described in several autoimmune diseases (reviewed in Sundrud and Trivigno, 2013). In T1D, increased frequencies of circulating IL-17-producing $CD45RA^-CCR6^+$ $CD4^+$ T cell subsets have been described in patients (Ferreira et al., 2015). In addition, Honkanen and colleagues could identify increased IL-17 protein production and increased real-time PCR expression levels of IL-17 in T cells of T1D patients following polyclonal anti-CD3/28 stimulus as well as in circulating memory $CD4^+$ T cells (Honkanen et al., 2010). Increased frequencies of IL-17 producing $CD4^+$ T cells following anti-CD3/CD28 have been confirmed in another set of patients at onset of T1D (Marwaha et al., 2010). Recent studies further identified increased frequencies of IL-21-producing, circulating follicular T helper cells in patients with T1D (Ferreira et al., 2015; Kenefeck et al., 2015). Clinical trials for other autoimmune diseases, such as Rheumatoid arthritis, Crohn's disease and Systemic lupus erythematosus have been established to target IL-21 signal pathway in disease (Spolski and Leonard, 2014).

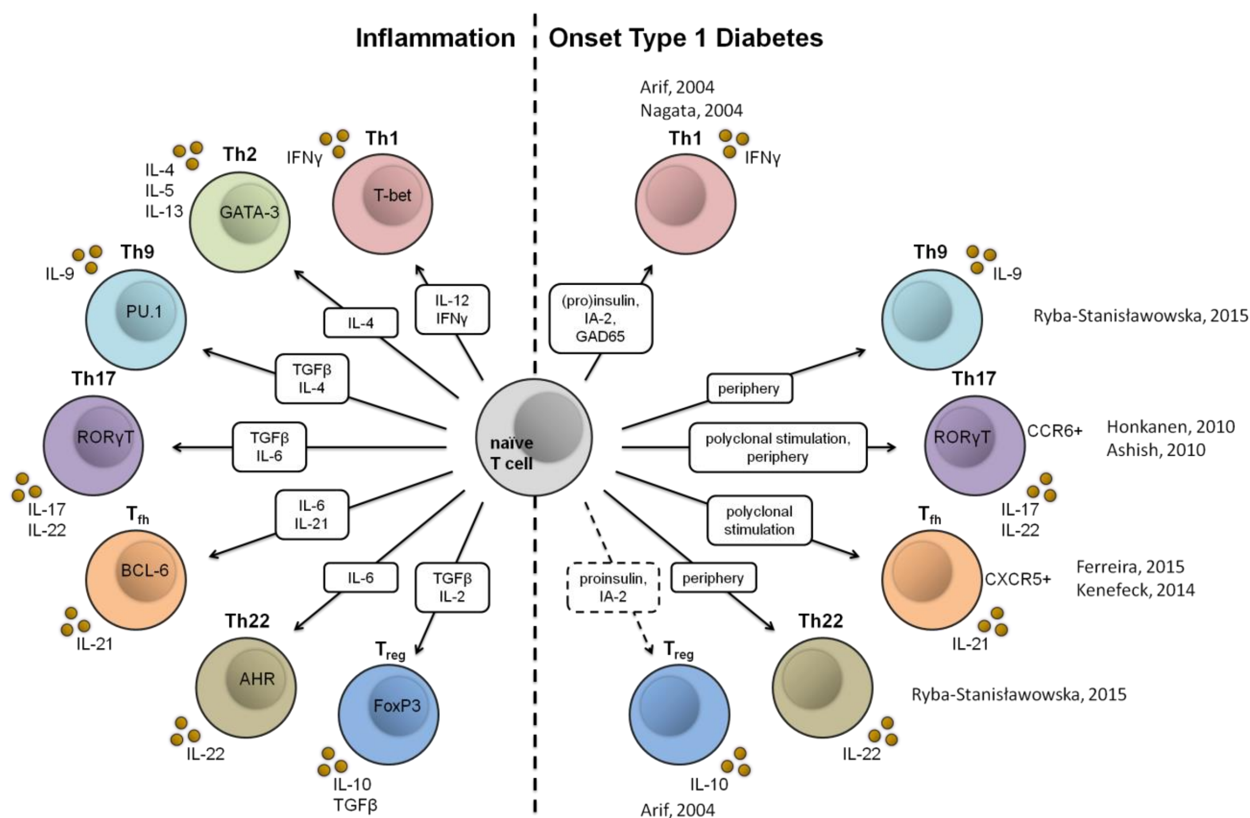


Figure 5: Differentiation of naïve T cells into Th cell subsets in healthy individuals and T1D patients. Naïve T cells are primed by APCs and differentiate into different Th1, Th2, Th9, Th17, Th22, follicular helper T cells (T_{fh}) or T_{reg} subsets depending on the microenvironment. Activation profiles of different Th subsets are characterized by signature cytokine production and upregulation of transcription factors (left side; modified from Wu et al., 2014). *In vitro* studies of T cell differentiation in PBMCs isolated from patients at onset of T1D were characterized in absence (periphery) or presence of antigen-specific (proinsulin, insulin, IA-2 and GAD65) and polyclonal stimulation (right side; references indicated).

1.3.3. Regulatory T cells in type 1 diabetes

Regulatory $CD4^+$ T cells play a key role in maintaining peripheral tolerance against foreign pathogens, preventing autoimmune diseases and limiting chronic inflammatory diseases (Vignali et al., 2008). T_{reg} are commonly characterized by constitutive surface expression of the α-chain of the IL-2 receptor (CD25) and intracellular expression of the signature transcription factor FoxP3, as well as low expression of the IL-7 receptor (CD127). FoxP3 suppresses signaling of nuclear factor of activated T cells (NFAT) and nuclear factor kappa light chain enhancer of activated B-cells (NFkB), leading to reduced expression of effector cytokines, such as IL-2, and T cell specific surface molecules important for interaction with other immune

compartments (Kim, 2009). Of importance, expression of FOXP3 is essential for $CD4^{+}CD25^{+}$ T_{reg} development and mutations in the *FOXP3* locus lead to T_{reg} deficiency in Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. IPEX patients are characterized by impaired immune homeostasis leading to severe disease including autoimmune enteropathy, thyroiditis, dermatitis, and T1D (van der Vliet and Nieuwenhuis, 2007). T_{reg} numbers and function are inconsistently shown to be imbalanced in T1D (Kukreja et al., 2002; Brusko et al., 2005; Lindley et al., 2005; Putnam et al., 2005; Brusko and Atkinson, 2007). Although previous studies in humans draw a controversial picture of how T_{reg} are involved in T1D (reviewed in Tree et al., 2006), these cells clearly have a therapeutic effect in animal models (Tarbell et al., 2007), and a safety phase I clinical trial of *ex vivo* expanded T_{reg} transfer in 14 patients with T1D was conducted (Bluestone et al., 2015).

1.4. Protection against type 1 diabetes in offspring of mothers with type 1 diabetes

It has been repeatedly demonstrated, that maternal T1D provides strong protection against T1D pathogenesis in offspring. Compared to children who have a father or sibling with T1D, offspring of mothers with T1D have around half the risk to develop autoimmune diabetes later in life (Warram et al., 1984). These findings imply that maternal T1D gestation confers relative protection for the neonates. The mechanisms operating to orchestrate this protection are unknown. Therefore, understanding why the risk is reduced in children of mothers with T1D may provide the opportunity to recapitulate protection in genetically at risk infants. I propose that exposure to increased levels of (pro)insulin during fetal development and soon after birth could favor early tolerance to this key target of T1D autoimmunity.

1.5. Objective

Increasing incidence of T1D raises interest of developing strategies to prevent the development and progression of islet autoimmunity (Ziegler et al., 2016). This requires an understanding of mechanisms of protection. It has been repeatedly demonstrated, that children born to mothers with T1D are protected against this autoimmune disease and in particular in the first two years of life at the stage of insulin autoimmunity. Within the framework of this thesis, I focused on possible cellular mechanisms that may act on the presentation of (pro)insulin to T cells during thymic education or during peripheral tolerance. I used the elevated production of (pro)insulin by

the fetus during maternal hyperglycemic gestation as my model with the notion that increased antigen exposure and/or potentially glucose modified (pro)insulin could lead to an increased tolerance to these key targets of autoimmunity. In the process, it became clear that methodology to quantify and phenotype proinsulin-responsive T cells needed to be developed. Moreover, there was relatively little information regarding the phenotype of proinsulin-responsive T cells in T1D. I, therefore, included objectives to address these gaps in my thesis.

The overall objectives of this thesis were to determine mechanisms of immunological proinsulin exposure that may account for maternal protection against T1D in the offspring. To address this, I developed methods and carried out experimentation that allowed me to:

a. Profile immune cells at birth in relation to maternal type 1 diabetes.
b. Determine whether maternal type 1 diabetes leads to reduced precursor frequencies of naïve (pro)insulin-responsive $CD4^+$ T cells in offspring.
c. Assess responses to antigens in a glycated form as may be expected during hyperglycemic conditions found in type 1 diabetes pregnancies.
d. Determine whether maternal type 1 diabetes leads to increased regulation of proinsulin-responsive $CD4^+$ T cells in the offspring.
e. Examine HLA-DR expression as an additional potential mechanism of regulating the amount of antigen presentation.
f. Define the phenotype of proinsulin-responsive $CD4^+$ T cells.

2. Material and methods

2.1. Materials

2.1.1. Chemicals and reagents

Used chemical reagents in this PhD study are listed in table 2.

Table 2: Chemicals and reagents.

Chemical name	Manufacturer
1x PBS	Gibco, Invitrogen Ltd., Paisley, UK
BD FACS™ Clean Solution	BD Biosciences; San Jose, CA, USA
BD FACS™ Rinse Solution	BD Biosciences; San Jose, CA, USA
BD FACS™ Sheath Solution	BD Biosciences; San Jose, CA, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, Inc., St. Louis, MO, USA
Diethylpyrocarbonate (DEPC) PBS	Ambion Inc.; Austin, TX, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Inc., St. Louis, MO, USA
DNA*Zap*™ Solutions	Life Technologies, Carlsbad, CA, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Inc., St. Louis, MO, USA
Ethanol	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Ethanol absolute	Carl Roth GmbH + Co.KG, Karlsruhe, Germany
Formalin (10%, neutral buffered)	Sigma-Aldrich, Inc., St. Louis, MO, USA
Histopaque-1077 Hybri-Max	Sigma-Aldrich, Inc., St. Louis, MO, USA
Luminex® xMAP® sheath fluid	Luminex Corp.; Austin, TX, USA
Lymphoprep™	STEMCELL Technologies, Vancouver, Canada
MUSE Guava Instrument Cleaning (ICF) solution	Merck Millipore, Darmstadt, Germany
RNaseZap® spray	Ambion Inc.; Austin, TX, USA
Trypan blue stain	Gibco, Invitrogen Ltd., Paisley, UK

2.1.2. Cell culture media and supplements

Cell culture media used for this work is listed in table 3. Serum used for cell culture was previously heat inactivated at 56°C for 30 minutes, subsequently filtered through a 0.45µm steritop filter (Millipore) and aliquots were stored at -20°C. Culture media was prepared fresh directly before experiments, additionally filtered through a 0.22µm steritop filter (Millipore) and kept in the fridge at 4°C for no longer than 4 weeks.

Table 3: Cell culture media and supplements.

Name	Manufacturer
DMEM 4,5g/l glucose w/o L-Glutamine	Lonza; Verviers, Belgium
X-VIVO 15	Lonza; Verviers, Belgium
RPMI 1640 medium, no glutamine	Life Technologies, Carlsbad, CA, USA
CellGro DC Dendritic Cell Medium	CellGenix GmbH, Freiburg, Germany
Human serum AB (HS)	PAA Laboratories GmbH; Pasching, Austria
L-Glutamine 200 mM (L-Glu)	Lonza; Verviers, Belgium
LPS (1 mg)	Sigma-Aldrich, Inc., St. Louis, MO, USA
Penicillin - Streptomycin (P/S)	Lonza; Verviers, Belgium
recombinant human GM-CSF (50 µg)	R&D Systems; Minneapolis, MN, USA
recombinant human IL-4 (50 µg)	R&D Systems; Minneapolis, MN, USA
recombinant human IL-7 (25 µg)	R&D Systems; Minneapolis, MN, USA
recombinant human IFNγ (100 µg)	R&D Systems; Minneapolis, MN, USA

2.1.3. Commercial kits

All commercially available kits used in this PhD thesis are listed in table 4.

Table 4: Commercial kits.

Name	Catalogue number	Manufacturer
Cell culture stimulation		
Dynabeads® Human T-Activator CD3/CD28	111-31D	Invitrogen; Oslo, Norway

Human insulin	-	Eli Lilly and Co., Indianapolis, IN, USA
Human proinsulin	-	Biomm S. A., Belo Horizonte, Brazil
Keyhole lympet hemocyanin (KLH)	9013-72-3	Sigma-Aldrich, Inc., St. Louis, MO, USA
Tetanus Toxoid zur Injektion	02/232	Andreae Noris Zahn-AG, Ottendorf-Okrilla, Germany
Tcell GAD (rhGAD65)	10-65702-16-01	Diamyd Medical AB, Stockholm, Sweden
Cell separation		
$CD4^+$ T Cell Isolation Kit II, human	130-096-533	Miltenyi; Bergisch-Gladbach, Germany
CD8 MicroBeads, human	130-045-201	Miltenyi; Bergisch-Gladbach, Germany
CD14 MicroBeads, human	130-050-201	Miltenyi; Bergisch-Gladbach, Germany
CD25 MicroBeads II, human	130-092-983	Miltenyi; Bergisch-Gladbach, Germany
Flow cytometry staining		
BD Cytofix/Cytoperm Fixation/Permeabilization Solution	554714	BD Biosciences; San Jose, CA, USA
CS&T Research Beads	650621	BD Biosciences; San Jose, CA, USA
Foxp3 / Transcription Factor Staining Buffer Set	00-5523-00	eBioscience; San Diego, CA USA
FoxP3 Fix/Perm Buffer Set	421403	BioLegend; San Diego, CA USA
BD™CompBeads Anti-Mouse Ig,κ/Negative Control Compensation Particles Set	552843	BD Biosciences; San Jose, CA, USA
Leukocyte activation cocktail (LAC) with GolgiPlug	550583	BD Biosciences; San Jose, CA, USA

2.1.4. Buffers

Prepared buffers for cell culture and flow cytometry for this PhD thesis are listed in table 5.

Table 5: Prepared buffers.

Buffer name	Composition
FACS buffer	0.1% BSA in 1x PBS
MACS buffer	0.5% BSA ; 2 mM EDTA in 1x PBS
FACS sort buffer	1 % human serum in 1x PBS

2.1.5. Consumables

Cell culture material used in this PhD thesis is listed in table 6.

Table 6: Cell culture material.

Name	Specification	Manufacturer
Cell culture plates	Nunclon™ 6 well and 96 well	Nunc, Thermo Fisher Scientific; Roskilde, Denmark
Cell culture plates	Costar® 48 wells	Corning Inc., Corning, NY USA
Cell culture flasks	Nunclon™ 25 cm²	Nunc, Thermo Fisher Scientific, Roskilde, Denmark
Cell preparation tubes (CPT™)	BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube, Sodium Heparin 8 ml	BD Biosciences; San Jose, CA, USA
Cell strainers	Falcon™ Cell Strainers, 40 µm nylon	Corning Inc., Corning, NY USA
Cryotubes	Cryo.s™, 2.0 ml	Greiner bio-one GmbH; Frickenhausen, Germany
FACS tubes	BD Falcon™ Polystyrene round bottom tubes 5 ml	BD Biosciences Discovery; Labware, Bedford MA USA
	Tube 4.5 ml, 75x12 mm, conical base, PS	Sarstedt, Wedel, Germany
FACS tube filter caps	Cell strainer cap	BD Biosciences Discovery; Labware, Bedford MA USA
Falcon tubes	15, 50 ml	Greiner bio-one GmbH;

		Frickenhausen, Germany or BD Biosciences Discovery; Labware, Bedford MA USA
Heparin blood collection tubes	BD Vacutainer® Glass Sodium Heparin Plasma tube 10ml	BD Biosciences; San Jose, CA, USA
MACS column filters	Pre-separation filters, 30 µm	Miltenyi Biotech; Bergisch-Gladbach, Germany
MACS columns	MS columns LS columns	Miltenyi Biotech; Bergisch-Gladbach, Germany
Pasteur pipettes	3 ml	VWR, Darmstadt, Germany
PCR tubes	0.2 ml	VWR, Darmstadt; Germany
Pipette tips	Without filter, 0.1-10 µl and 50-1000 µl	Eppendorf AG, Hamburg, Germany
	Without filter ,10-200 µl	Greiner bio-one GmbH;Frickenhausen, Germany
	ART® Aerosol resistent	Sorenson BioScience Inc., Salt Lake City, Utah, USA
Safelock reaction tubes	0.5 ml, 1.5 ml, 2.0 ml	Eppendorf AG, Hamburg, Germany
Serological pipettes	Cellstar®, 5 ml, 10 ml and 25 ml	Greiner bio-one GmbH;Frickenhausen, Germany
Stericup and Steritop	Vacuum filter bottles, PES Membrane 0.22 µm	Merck Millipore, Darmstadt, Germany
Syringe filters	Syringe filter units with 0.22 µm and 0.45 µm with cellulose acetate membrane	Corning Inc., Corning, NY USA
Tips for Multipette	2.5 ml, 5 ml Combitips® plus	Eppendorf AG, Hamburg, Germany
96 well plate seals	Adhesive PCR sealing sheets	Thermo Fisher Scientific; Hudson, NH, USA

2.1.6. Technical equipment

Used technical equipment for this PhD thesis is listed in table 7.

Table 7: Technical equipment.

Device	Name	Manufacturer
Cell counter	MUSE Cell Analyzer	Merck Millipore, Darmstadt, Germany
Cell sorter	ARIA II and ARIA III	BD Biosciences; San Jose, CA, USA
Centrifuge (molecular biology, fixed angle rotor)	Centrifuge 5415 R	Eppendorf AG; Hamburg, Germany
Centrifuge (cell culture, swing-out rotor)	Heraeus Multifuge X3R	Thermo Fisher Scientific; Hudson, NH, USA
Flow Cytometer	LSR II and LSR Fortessa	BD Biosciences; San Jose, CA, USA
Incubator	BBD 6220 CO_2-Incubator	Thermo Fisher Scientific; Hudson, NH, USA
Inverted microscope	Leica DM IL LED	Leica Microsystems GmbH, Wetzlar, Germany,
Luminex	Luminex® 200™ System	Luminex Corp.; Austin, TX, USA
MACS magnets and stands	MiniMACS Separator QuadroMACS™ Seperator	Miltenyi Biotech; Bergisch-Gladbach, Germany
Single cell profiling	Biomark™ HD	Fluidigm, South San Francisco, CA, USA
Sterile work bench	ScanLaf Mars Safety Class 2	LaboGene ApS, Allerød. Denmark
Waterbath	Incubation/Inactivation Water Bath 1008	GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany

2.2. Study populations

Specific questions in this PhD thesis have been addressed in different cohort studies. Antigen-specific, naïve immune responses with respect to relative protection conferred by maternal T1D during gestation have been investigated in samples obtained from the ImmunDiabRisk study and characterization of antigen-specific responses in the adaptive immune compartment in later life have been addressed using samples from participants in the Bavarian diabetes register DiMelli study and German-wide TEENDIAB study.

2.2.1. ImmunDiabRisk study

Human cord blood samples were obtained within the ImmuDiabRisk study, which investigated the maturation of the immune system in children in relation to genetic and familial risk for T1D. The ImmuDiabRisk study recruited pregnant women in Germany, who have gestational or T1D as well as healthy women with or without 1st degree relatives who have T1D. Immediately after delivery, human cord blood samples were collected by double venal puncture using a cord blood collection bag system (Macopharma) from the placental side of the umbilical cord vein. Information on child's sex, birth weight, delivery mode and gestational age at birth was obtained from birth certificates. Neonates were subsequently followed for 1 year by blood withdrawal every 3 months using standardized protocols in sodium-heparinized whole-blood tube collection systems. All samples were collected after obtaining signed informant content by the parents and the IDR study was approved by the Ethics Committee of the Technical University Munich (No. 5293/12).

2.2.2. DiMelli study

The incidence DiMelli cohort study recruits children and adolescents with recent onset of any type of diabetes mellitus in Bavaria, Germany. The cohort registers and recruits patients younger than 20 years, within six months after diagnosis of clinical diabetes. Recruitment of the patients is conducted mainly by children's hospitals and pediatric practices, where one blood draw is obtained using standardized protocols in sodium-heparinized whole-blood tube collection systems (BD Vacutainer) as well as questionnaires concerning demography, anthropometry, familiar diabetes mellitus history, diagnosis and medication (Thümer et al., 2010). All samples were obtained with informed consent as part of the DiMelli study and

protocols were approved by the Ethics Committee of the Bayerischen Landesärztekammer (No. 08043).

2.2.3. TEENDIAB study

The German-wide, observational TEENDIAB study recruits children between the age of 6 to 10 years who have a 1 first degree relative with T1D. The TEENDIAB study examines genetic and environmental factors contributing to the development of T1D in puberty and early childhood. Blood, serum and stool samples as well as anthropometric and socio-economic questionnaires of enrolled children are collected every 6 months until the age of 14 years and yearly after (Ziegler et al., 2012). Blood draws in this study for isolation of peripheral mononuclear cells (PBMCs) were conducted using standardized protocols of vacutainer mononuclear cell preparation tubes (CPT; BD Bioscience) and all samples were collected after obtaining signed informant content. Protocols were approved by the Ethics Committee of the faculty of medicine of the Technical University Munich (No. 2149/08).

2.2.4. Characteristics of samples for investigation of naïve and memory $CD4^+$ T cell responses in T1D

To analyze immune cell compartments and antigen-specific characteristics of naïve $CD4^+$ T cells with respect to paternal diabetes, cord blood and follow up samples were obtained from children with healthy parents, of children of mothers with GDM or T1D and of neonates who have a father or sibling with T1D. Samples processed for the different aims of this PhD thesis are summarized in table 8 and 9.

Table 8: Characteristics of samples for analysis of various immune compartments and antigen-specific, naïve CD4$^+$ T cell responses at birth.

	healthy parents	maternal T1D	gestational DM	paternal/sibling T1D	both parents T1D
Phenotype of cord blood mononuclear cells (n=82)					
sample set, n	21	25	16	20	-
sex, n (m/f)	(7/14)	(12/13)	(8/8)	(11/9)	-
HLA DR3 n (%, frequency of subjects)	3 (14.3)	8 (32)	2 (12.5)	4 (20)	-
HLA DR4 n (%, frequency of subjects)	1 (4.8)	9 (36)	4 (25)	9 (45)	-
Non-HLA DR3orDR4 n (%, frequency of subjects)	6 (28.6)	7 (28)	8 (50)	7 (35)	-
unknown genotype n (%, frequency of subjects)	11 (52.4)	3 (12)	2 (12.5)	1 (5)	-
Phenotype of cord blood-derived CD14$^+$ monocytes (n=50)					
sample set, n	15	17	3	15	-
sex, n (m/f)	(7/8)	(12/5)	(1/2)	(8/7)	-
HLA DR3 n (%, frequency of subjects)	0 (0)	5 (29.4)	1 (33.3)	5 (33.3)	-
HLA DR4 n (%, frequency of subjects)	1 (6.7)	9 (52.9)	0 (0)	8 (53.3)	-
Non-HLA DR3orDR4 n (%, frequency of subjects)	2 (13.3)	4 (23.5)	2 (66.7)	4 (26.7)	-
unknown genotype n (%, frequency of subjects)	12 (80)	0 (0)	0 (0)	0 (0)	-

	healthy parents	maternal T1D	gestational DM	paternal/sibling T1D	both parents T1D
Precursor frequencies of antigen-responsive, naïve CD4$^+$ T cells (n=45)					
sample set, n	16	15	-	14	-
sex, n (m/f)	(8/8)	(10/5)	-	(7/7)	-
HLA DR3 n (%, frequency of subjects)	0 (0)	6 (40)	-	4 (28.6)	-
HLA DR4 n (%, frequency of subjects)	1 (6.3)	10 (66.7)	-	5 (35.7)	-
Non-HLA DR3orDR4 n (%, frequency of subjects)	3 (18.8)	2 (13.3)	-	6 (42.9)	-
unknown genotype n (%, frequency of subjects)	12 (75)	0 (0)	-	0 (0)	-
Gene expression profile of antigen-responsive, naïve CD4$^+$ T cells (n=15)					
sample set, n	4	5	-	4	2
sex, n (m/f)	(2/2)	(4/1)	-	(3/1)	(1/1)
HLA DR3 n (%, frequency of subjects)	0 (0)	3 (60)	-	1 (25)	1 (50)
HLA DR4 n (%, frequency of subjects)	0 (0)	3 (60)	-	3 (75)	0 (0)
Non-HLA DR3orDR4 n (%, frequency of subjects)	0 (0)	0 (0)	-	1 (25)	1 (50)
unknown genotype n (%, frequency of subjects)	4 (100)	0 (0)	-	0 (0)	0 (0)

	healthy parents	maternal T1D	gestational DM	paternal/sibling T1D	both parents T1D
Suppressive capacity of antigen-specific, regulatory CD4$^+$ T cells (n=13)					
sample set, n	-	7	-	6	-
sex, n (m/f)	-	(5/2)	-	(6/0)	-
median age, years (range)	-	0.76 (0.24 – 0.83)	-	0.76 (0.49 – 0.83)	-
HLA DR3 n (%, frequency of subjects)	-	2 (28.6)	-	2 (33.3)	-
HLA DR4 n (%, frequency of subjects)	-	2 (28.6)	-	3 (50)	-
Non-HLA DR3orDR4 n (%, frequency of subjects)	-	2 (28.6)	-	1 (16.7)	-
unknown genotype n (%, frequency of subjects)	-	1 (14.3)	-	0 (0)	-

In order to transfer and interpret antigen-specific responses of naïve CD4$^+$ T cell compartment at birth to cytokine signatures in memory CD4$^+$ T cell responses at onset of T1D, PBMCs isolated from patients at onset of T1D and healthy children matched for age, sex and HLA genotype have been polyclonaly stimulated or in presence of diabetes associated antigens. Gene expression signatures have been additionally analyzed in three different patients at onset of T1D (Table 9).

Table 9: Characteristics of samples for analysis of antigen-specific, memory CD4$^+$ T cell responses at onset of T1D.

	Cytokine producing CD4$^+$ T cells after polyclonal stimulation		**Cytokine production of antigen-responsive CD4$^+$ T cells**		**Single cell gene expression of proinsulin-responsive CD4$^+$ T cells**
sample group	controls	T1D patients	controls	T1D patients	T1D patients
sex (m/f)	(12/11)	(8/10)	(17/19)	(17/16)	(1/2)
median age, years (range)	10.8 (7.0 - 13.7)	10.1 (7.4 - 13.7)	13.2 (10.5 - 15.3)	14.1 (5.2 - 31.4)	11.5 (10.7 - 13.3)
median time after onset, days (range)	-	11 (3 - 31)	-	14 (4 - 343)	7 (5 - 14)
HLA DR3 (%, frequency of subjects)	32	33	31	50	0
HLA DR4 (%, frequency of subjects)	64	60	54	60	100

2.3. Methods

2.3.1. Isolation of mononuclear cells and cryopreservation

Cord blood mononuclear cells (CBMCs) were isolated and processed within 72 hours following birth and PBMCs were isolated within 48 hours following blood draw. Mononuclear cells were isolated by density gradient centrifugation over Lymphoprep (Axis-Shield) according to the manufacturer´s protocol. Cell numbers as well as viability of isolated CBMCs and PBMCs were obtained with a hemocytometer following staining with trypan blue in a 1:2 or 1:10 ratio to discriminate between live and dead cells or by using standardized Muse count and viability solution on the Muse cell analyzer (Merck Millipore). Isolated CBMCs or PBMCs were used fresh or stored in liquid nitrogen in human serum AB (PAA) in the presence of 10% Dimethylsulfoxide (DMSO; SIGMA Aldrich). Cryopreservation of CBMCs was supplemented with 0.1 ng/ml IL-7 (R&D Systems).

2.3.2. Thawing of cryopreserved mononuclear cells

Frozen mononuclear cells were thawed with pre-warmed DMEM (4,5g/l Glucose, w/o L-Glutamine; Lonza) supplemented with Benzonase (25 Units/ml; Novagen 99% purity) and washed twice with pre-warmed DMEM without Benzonase supplementation. Cell yield and viability was assessed by trypan blue staining on a hemocytometer or by using Muse count and viability solution (Merck Millipore).

2.3.3. Magnetic activated cell sorting as pre-purification of immune cell subsets

Magnetic activated cell sorting (MACS) was used to purify immune cell subsets from CBMCs or PBMCs by positive selection using two different kits from Miltenyi Biotec. CD14 Microbeads (Miltenyi Biotec) were used to purify $CD14^+$ monocytes from whole CBMCs in order to obtain monocyte-derived dendritic cells. CD25 Microbeads II (Miltenyi Biotec) were used to purify $CD25^+$ PBMCs prior to florescence activated cell sorting of regulatory T cells or for depletion of $CD25^+$ $CD4^+$ T cells in proliferation assays. Both kits were used on whole mononuclear cells fractions according to manufacturer’s protocols and according to estimated cell numbers; up to $1x10^7$ or $1x10^8$ labelled cells were passed through MS or LS columns (Miltenyi Biotec),

respectively. Purification of isolated cell subsets were subsequently validated via flow cytometry by staining $0.5x10^5$ isolated cell subsets with anti-CD14 FITC (clone HCD14; Biolegend) or anti-CD25 PE (clone M-A251; BD Pharmingen) for 20 min at 4°C in $PBS^{-/-}$ (Gibco) containing 0.5% BSA.

2.3.4. Flow cytometry

2.3.4.1. Instrumental set up and device stability

Multi-parameter flow cytometry is a powerful tool to characterize individual cells as well as cell populations in a high-throughput manner. Complex measurements of multiple characteristics of single immune cell subsets alone or in context of other immune cell compartments are enabled by the detection of cell-bound fluorochrome-conjugated antibodies to surface and intracellular proteins. For this purpose, stained cell suspensions are aligned and separated by a focused fluid stream, which passes beams of different laser sources individually in a flow cell. Following excitation of the fluorochrome-conjugated antibodies, the resulting light emission is focused by fibers and optical filters and finally detected by photomultipliers (PMT) in a laser-dependent manner. PMTs enable the transformation of light to electrical signals, resulting in the resolution of specific markers on and in the detected cells. In dependency of their emitted wavelength, different lasers are capable to excite various sets of fluorochrome-conjugated antibodies. In my thesis, a LSR Fortessa (BD Bioscience) equipped with violet (405nm), blue (488nm), yellow-green (561nm) and red (633nm) lasers was used for detection of up to 11 different markers simultaneously (Table 10). In addition, fluorochrome independent detection of particle size and granularity is enabled by the forward scatter (FSC) and side scatter (SSC) detectors, respectively. Area, height and width parameters of the FSC and SSC detectors were used for doublet exclusion. To increase stable performance of the flow cytometric device, cytometer setup and tracking (CS&T) settings using CS&T beads (BD Bioscience) were calibrated routinely every week.

Table 10: Configuration of LSR Fortessa.

Laser	longpass filter	bandpass filter	detected fluorochromes by PMT
405nm	600LP	610/20	Brillant Violet 605, Qdot605
	505LP	525/50	Brillant Violet 510, AmCyan
		450/50	eFluor450, Pacific Blue
488nm	685LP	710/50	PerCP, PerCP-Cy5.5
	505LP	530/30	Alexa Fluor 488, CFSE, FITC
		488/10	SSC
561nm	750LP	780/60	PE-Cy7
	685LP	710/50	PE-Cy5.5 (not used)
	635LP	670/30	PE-Cy5
	600LP	610/20	PE-Texas Red (not used)
		585/15	PE
640nm	750LP	780/60	APC-Cy7
	690LP	730/45	APC-Alexa 700
		670/14	eFluor670, APC

For the analysis of multicolor panels using flow cytometry, spectral overlap among used fluorochromes have to be compensated. For this purpose, cells of interest or polystyrene microbeads coated with Anti-Mouse Immunoglobulin κ (CompBeads, BD Bioscience), which unspecifically bind any mouse κ light chain-bearing antibody, were stained with only one of the used fluorochrome-conjugated antibodies. Instrument PMT voltages were set using unstained CompBeads and subsequently single staining was acquired and recorded. Here, CompBeads were used for intra- and extracellular markers and 1:1 mixtures of labeled and unlabeled cells as well as alive and heat shock treated dead cells (10 minutes at 65°C) for proliferation dyes and live/dead staining, respectively. Compensation of spectral overlap was calculated using an automatic tool within the BD FACSDiva software routinely prior measurements of experimental samples, which were recorded at a speed of $1x10^3$ events/second. Raw data was exported as 3.0 fcs-files and subsequently analyzed using FlowJo software (Version 10; TreeStar Inc.).

2.3.4.2. Fluorescence activated cell sorting on single cell level

Distinct immune cell subsets were sorted at high purity based on their fluorescent characteristics by fluorescence activated cell sorting (FACS) on an ARIA II and III containing a violet (405nm), a blue (488nm) and a red laser (633nm). Single cells were sorted into 96 well

PCR plates preloaded with 5 µl Diethylpyrocarbonate PBS (DEPC; Ambion) at a speed of 200 events/second.

2.3.5. Glycation of antigens

Antigens were experimentally glycated by 7 days incubation in the dark at 37 °C in the presence of 100 mM D-glucose (Sigma-Aldrich) or $PBS^{-/-}$ as control as previously described (Monti et al., 2013).

2.3.6. Phenotyping of cord blood mononuclear cells

To characterize cord blood-derived innate and adaptive immune cell subsets related to parental diabetes status, surface markers of isolated CBMCs were stained using the following monoclonal antibodies: anti-CD3 FITC (clone HIT3α; BioLegend), anti-CD4 Pacific Blue (clone RPA-T4; BD Pharmingen), anti-CD8a Brilliant Violet 605 (clone RPA-T8), anti-CD14 PE-Cy7 (clone HCD14), anti-CD19 PE (clone HIB19), anti-CD34 APC (clone 561) and anti-CD56 PerCP-Cy5.5 (clone HCD56; all BioLegend). $5x10^5$ cells were stained for 20 min at 4°C in phosphate-buffered saline without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$; Gibco) containing 0.5% BSA, washed in $PBS^{-/-}$ and stained for 15 minutes at room temperature with Zombie NIR (BioLegend) to evaluate the viability of the cells. CBMCs were fixed with 1.5% Formalin in $PBS^{-/-}$ and the samples were acquired within 24 hours on a Becton Dickinson LSR Fortessa flow cytometer and analyzed with FlowJo software (Version 10; TreeStar Inc.). For multi-dimensional tSNE analysis, data for each immune cell subset were ranked by descending frequencies. A representative FACS plot of cord blood-derived mononuclear cells of a neonate from a mother with T1D is shown in figure 6 and various immune subsets are expressed as percentage of the total alive, mononuclear cells.

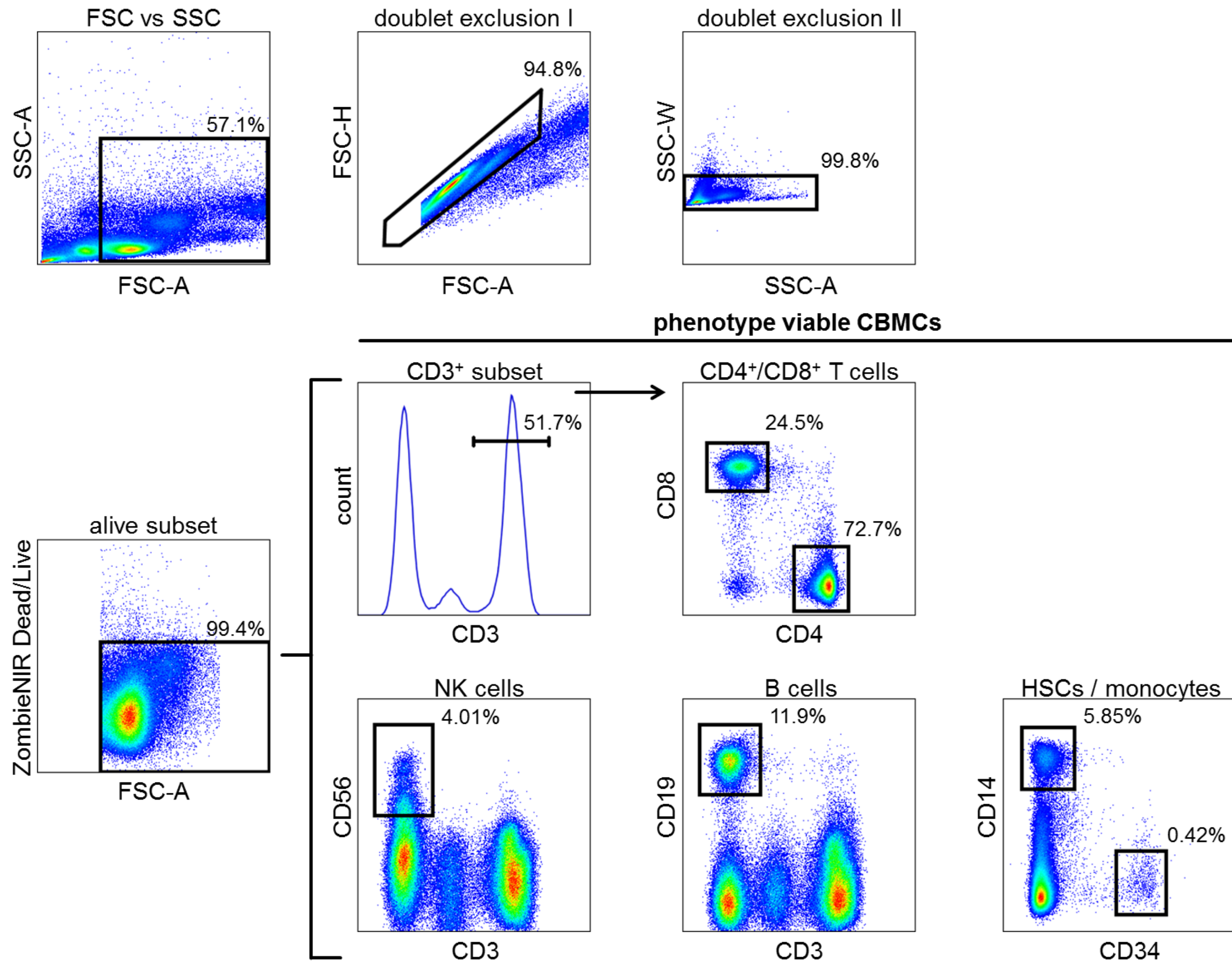


Figure 6: Phenotyping of cord blood mononuclear cells via flow cytometry. Representative FACS plot following doublet and dead cells exclusion in order to phenotype distinct immune cell subsets by their expression of signature surface markers as $CD3^+$ lymphocytes, $CD4^+$ or $CD8^+$ T cells, $CD3^-CD56^+$ NK cells, $CD19^+$ B cells, $CD34^+$ hematopoietic stem cells (HSC) and $CD14^+$ monocytes.

2.3.7. Phenotyping $CD4^+$ T cells at onset of type 1 diabetes following polyclonal stimulation

Frozen PBMCs of patients at onset of T1D and matched healthy children were thawed as previously described and $2x10^6$ cells were polyclonally stimulated with Phorbol myristate acetate (PMA) and ionomycin in the presence of Brefeldin A (Leukocyte activation cocktail with GolgiPlug; final dilution 1:500; BD Bioscience) for 6 hours. Following polyclonal stimulation, samples were incubated for 5 minutes in the presence of 40µl DNase I (Final concentration $6x10^4$ U/ml; Sigma-Aldrich) at 37°C, 5% CO_2 and 95% humidity, washed in $PBS^{-/-}$ and stained on ice for 30 minutes with UV Viability Dye eFluor455UV (eBioscience). All samples were

permeabilized and blocked in 50 µl of $PBS^{-/-}$ containing 0.1 % (w/v) Saponin, 1% BSA, 0.05% NaN_3 and 5% (w/v) Nonfat dried milk powder (PBS-S milk buffer) for 2 hours on ice. For simultaneous surface and intracellular staining, the following monoclonal antibodies were used: anti-CD3 Horizon V500 (clone UCHT1; BD Bioscience), anti-CD4 Qdot605 (clone S3.5; Life Technologies), anti-CD8 Brilliant Violet 785 (clone RPA-T8; BioLegend), anti-IFNγ APC-eFluor780 (clone 4S.B3; eBioscience), anti-IL-17A PE-Cy7 (clone eBio64DEC17; eBioscience), anti-IL-22 PE (clone 22URTI; eBioscience), anti-IL-2 Brilliant Violet 650 (clone MQ1-17H12, BioLegend), anti-IL-4 Alexa Fluor 488 (clone MP4-25D2, BioLegend), anti-TNFα Brilliant Violet 711 (clone Mab11; BioLegend), anti- GM-CSF PerCP-Cy5.5 (clone BVD2-21C11, BD Bioscience), anti-IL-21 Alexa Fluor 647 (clone 3A3-N2.1; BD Bioscience). For intracellular cytokine staining, the following respective isotype control antibodies were used: APC-eFluor780 (Mouse; IgG1; κ; clone P3.6.2.8.1; eBioscience), PE-Cy7 (Mouse; IgG1; κ; clone MOPC-21; BioLegend), PE (Mouse; IgG1; κ; clone MOPC-21; BioLegend), Brilliant Violet 650 (Rat; IgG2a; κ; clone RTK2758; BioLegend), Alexa Fluor 488 (Rat; IgG1; κ; clone RTK2071; BioLegend), Brilliant Violet 711 (Mouse; IgG1; κ; clone MOPC-21; BioLegend), PerCP-Cy5.5 (Rat; IgG2a; κ; clone RTK2758; BioLegend) and Alexa Fluor 647 (Mouse; IgG1; κ; clone MOPC-21; BioLegend). In parallel, an aliquot of $2x10^6$ cells of all samples were stained without intracellular cytokines using the same surface marker panel extended to anti-CD45RA APC-H7 (clone HI100; BD Bioscience) monoclonal antibody. Each staining was conducted for 30 minutes on ice and antibody staining was terminated by washing the cells in 2ml $PBS^{-/-}$-Saponin buffer. All samples were acquired on a BD LSR-II within 24 hours and cytokine expression of $CD4^+$ T cells was analyzed with FlowJo software (Version 10; TreeStar Inc.). A representative FACS plot is shown in figure 7. In addition, expression of respective cytokine producing $CD4^+$ T cells were expressed as the fraction of memory $CD45RA^-$ $CD4^+$ T cells obtained from parallel surface staining.

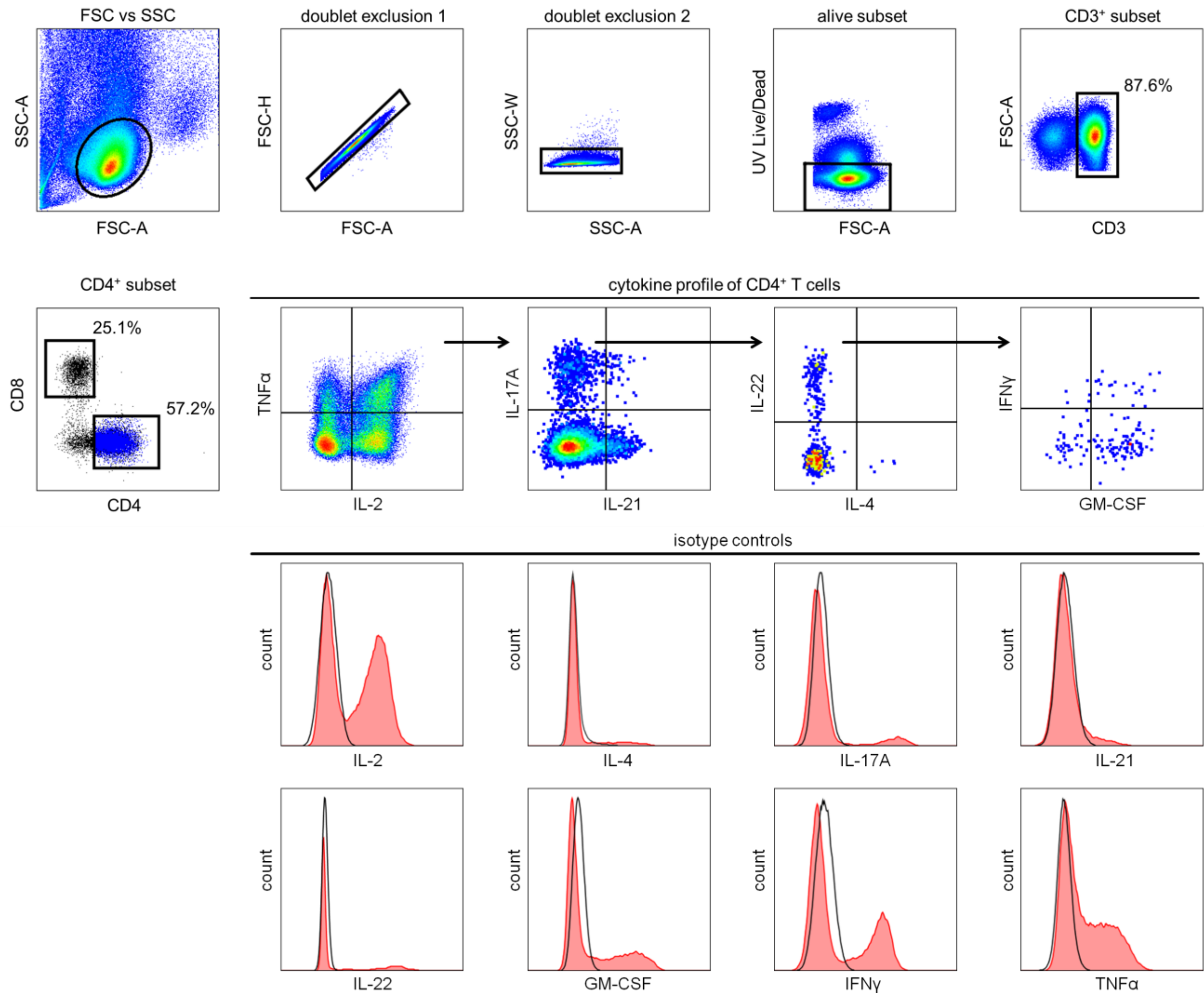


Figure 7: Representative FACS plot and gating strategy of intracellular cytokine staining in cryo-conserved PBMCs following polyclonal stimulation. Single lymphocytes were identified by forward and side scatter, live cells gated as viability dye eFluor455UV negative, and $CD3^+$ and $CD4^+$ gated cells were analyzed for IL-2, IL-4, IL-17A, IL-21, IL-22, GM-CSF, IFNγ, and TNFα (red) according to respective isotype controls (white). Arrows in the cytokine staining plots indicate the quadrant used in the analysis of the adjacent plot.

2.3.8. HLA-DR surface protein expression on cord blood derived monocytes

$CD14^+$ monocytes were enriched from CBMCs by positive selection using MACS (Miltenyi Biotec) according manufacturer's protocol. $1x10^5$ purified $CD14^+$ monocytes were subsequently stained for 20 min at 4°C in $PBS^{-/-}$ containing 0.5% BSA using the following monoclonal

antibodies: anti-CD11c APC-Cy7 (clone Bu15), anti-CD14 FITC (clone HCD14), anti-CD83 PE (clone HB15e), anti-CD86 PerCP-Cy5.5 (clone IT2.2), anti-CD209 PE-Cy7 (clone 9E9A8) and anti-HLA-DR APC (clone L243; all BioLegend). Cells were washed twice with $PBS^{-/-}$ and stained for 15 minutes at 4°C with violet fluorescent fixable dead cell stain (Life Technologies) for cell viability. Cells were fixed with 1.5% Formalin in $PBS^{-/-}$ and all samples were acquired within 24 hours on a Becton Dickinson LSR Fortessa flow cytometer and analyzed with FlowJo software (Version 10; TreeStar Inc.). Representative surface expression of markers important for peptide presentation (HLA-DR), adhesion (CD11c) and co-stimulation (CD83 and CD86) on cord blood-derived, enriched $CD14^+$ monocytes of a neonate from a mother with T1D are shown in figure 8 and were expressed as median fluorescence intensities (MFI) gated on alive, $CD14^+$ monocytes.

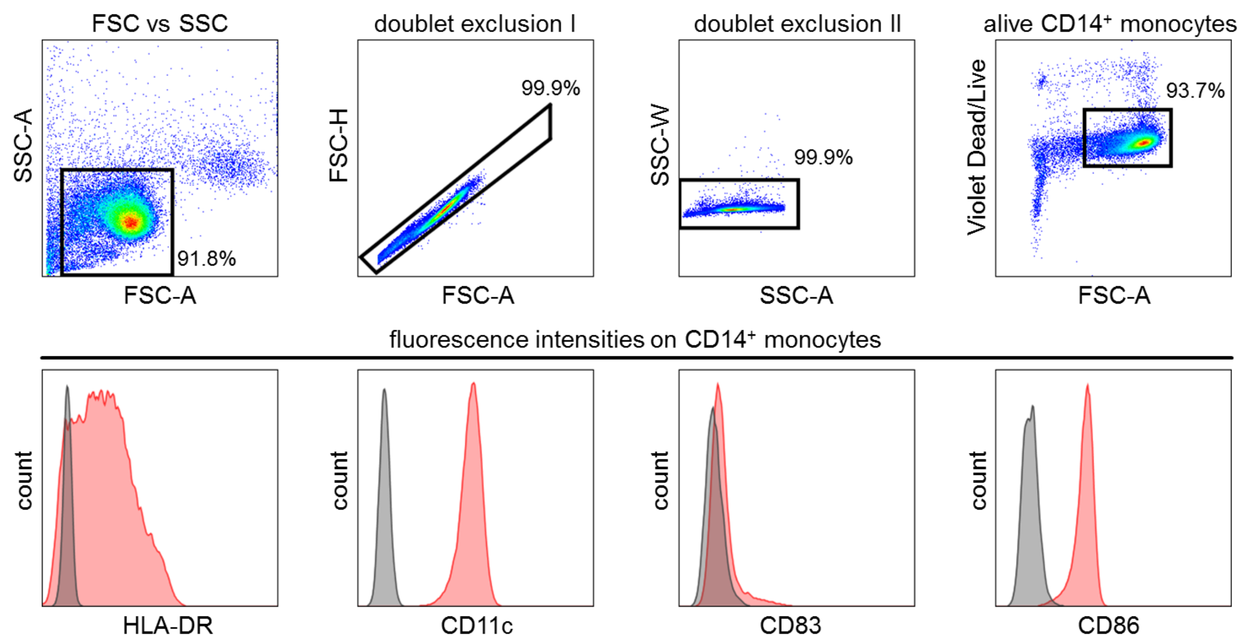


Figure 8: Representative FACS plots and histograms of surface markers important for peptide presentation, adhesion and co-stimulation on cord blood-derived $CD14^+$ monocytes. Median fluorescence intensity analysis of surface expression of HLA-DR, CD11c ,CD83 and CD86 on cord blood-derived $CD14^+$ monocytes (red) via flow cytometry. Unstained cord blood-derived $CD14^+$ monocytes (black) were set as controls.

2.3.9. Precursor frequencies of antigen-specific, naïve CD4^{+} T cells

Cord blood-derived CD14^{+} monocytes were isolated by positive isolation using MACS (Miltenyi Biotec) as previously described. The CD14^{+} cell-depleted CBMC component was frozen in human serum AB (PAA) containing 10% DMSO (SIGMA Aldrich) and 0.1 ng/ml IL-7 (R&D Systems), and stored in liquid nitrogen until monocyte-derived dendritic cells (MO-DCs) were prepared and matured in the presence of antigens of interest. DCs were generated from purified CD14^{+} monocytes in DC medium (CellGenix) supplemented with 3% human serum AB (PAA), 2 mM L-glutamine (Lonza) and 100 U/ml Penicillin/Streptomycin (Lonza) at 37°C, 5% CO_2 and 95% humidity in the presence of 50 ng/ml GM-CSF and 10 ng/ml IL-4 (both R&D Systems) as previously described (Heninger et al., 2013). After 6 days, immature DC were harvested and pulsed with 100 µg/ml insulin (Sigma-Aldrich), proinsulin (Biomm), or Keyhole limpet hemocyanin (KLH; Calbiochem) or the glycated forms of the antigens. MO-DCs were matured overnight with 100 units/ml IFN-γ (R&D Systems) and 10 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich). As described in section 2.3.8., monitoring of MO-DC maturation status was conducted by surface staining of purified CD14^{+} monocytes, unloaded MO-DCs after 6 days and fully matured MO-DCs in presence of antigen after 7 days post isolation of CBMCs.

Following successful maturation of MO-DCs, cryovials of the same cord bloods were thawed as previously described and CD25 positive cells were depleted by magnetic activated cell sorting using CD25 Microbeads II (Miltenyi Biotec) according to manufacturer’s protocols. CD25^{+} cell-depleted CBMCs were washed twice with PBS$^{-/-}$ and subsequently divided into two or three fractions and each fraction was labelled with a different proliferation dye. Respective cells were labelled by priority with 10 µM eFluor450 for 30 minutes at room temperature, 5 µM eFluor670 for 10 minutes at 37°C in the dark (both eBioscience) or 1 µM CFSE (BioLegend) for 4 minutes at 37°C according to the manufacturer´s protocol. Labelling with all proliferation dyes was terminated by washing cells with RPMI 1640 (Gibco) containing 15% human serum AB at 4˚C. $5x10^4$ of each eFluor450-, eFluor670- and CFSE-labelled CBMCs were added to $3x10^3$ autologous mature DCs (1:50 ratio) loaded with or without the antigens of interest for a total of 10 replicates of round-bottom 96-well plates. Mixed co-cultures were incubated in RPMI 1640 (Gibco) supplemented with 5% human serum AB (PAA), 2 mM L-glutamine (Lonza) and 100 U/ml Penicillin/Streptomycin (Lonza) at 37°C, 5% CO^2 and 95% humidity in the presence of 0.1 ng/ml IL-7 (R&D Systems) for 5 days. Cells were harvested and stained for 20 min at 4°C in PBS$^{-/-}$ containing 0.5% BSA using the following monoclonal antibodies: anti-CD4 PerCP (clone

SK3; BD Bioscience), anti-CD25 PE (clone M-A251; BD Pharmingen), anti-CD45RO PE-Cy7 (clone UCHL1; BD Bioscience). Cells were washed twice in $PBS^{-/-}$ and stained for 15 minutes at room temperature with Zombie NIR (BioLegend) to evaluate the viability of the cells. CBMCs were fixed with 1.5% Formalin in $PBS^{-/-}$ and all samples were acquired within 24 hours on a Becton Dickinson LSR Fortessa flow cytometer and analyzed with FlowJo software (Version 10; TreeStar Inc.). For each antigen stimulus, and each proliferation dye, 10 technical replicates were analyzed and responsiveness of $CD4^+$ T cells was identified as decline for each proliferation dye and upregulation of CD25 and CD45RO. Work flow and representative FACS plots are shown in figure 9 and responsive $CD4^+$ T cells in presence of antigen-loaded MO-DCs are expressed as the proportion of total proliferation dye labelled $CD4^+$ T cells after subtraction of the median of values obtained from unloaded MO-DCs control wells.

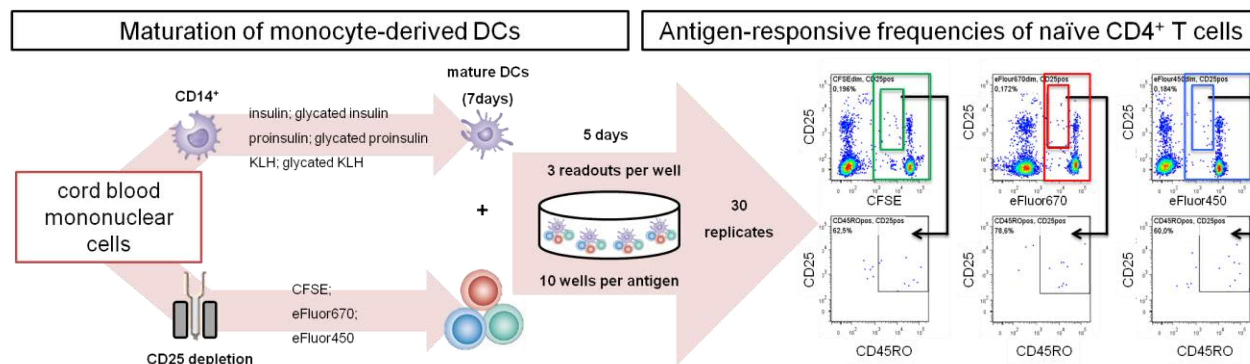


Figure 9: Workflow and representative FACS plots of newly designed multi-dye, multi-well proliferation assay to identify autoreactive, naïve $CD4^+$ T cells at birth. The established multi-dye proliferation assay consists of two parts: 1) maturation of MO-DCs from cord blood and 2) multi-parameter detection of antigen-specific, naïve $CD4^+$ T cells. This newly designed assay enabled assessment of antigen-responsive frequencies of naïve $CD4^+$ T cells with up to 30 replicates using relatively few responder cells (1.5×10^6).

In terms of sufficient cell numbers, parallel proliferation assays of $1.5x10^5$ labeled, CD25-depleted CBMCs with only one proliferation dye and $3x10^4$ antigen-loaded MO-DCs were identically treated in 4 technical replicates as described for the multi-dye proliferation assays and assed for single cell sort via FACS as described in section 2.3.12.

2.3.10. Suppressive capacity of antigen-specific, regulatory $CD4^+$ T cells in follow up samples

Frozen PBMCs obtained from follow up visits at the age of 3 to 9 months in offspring of mothers or fathers with T1D were thawed as previously described. $CD25^+$ positive cells were isolated by MACS using CD25 Microbeads II (Miltenyi Biotec). $CD25^+$ cells were stained for 20 min at 4°C in $PBS^{-/-}$ containing 0.5% BSA for FACS using the following monoclonal antibodies: anti-CD4 Brilliant Violet 510 (clone SK3; BD Bioscience), anti-CD25 PE (clone M-A251; BD Pharmingen) and anti-CD127 PE-Cy7 (clone A019D5; BioLegend). Cell viability was assessed by Via Probe (BD Bioscience) and $CD4^+$ regulatory T cells were identified as CD25high and CD127low. Cells were acquired and sorted on the BD FACS ARIA III cell sorting system using FACSDiva software. The $CD25^+$ cell-depleted fraction was separated in to $CD8^+$ and $CD8^-$ cells by MACS using CD8 Microbeads II (Miltenyi Biotec) and both cell fractions were labelled with 10 µM eFluor450 for 30 minutes at room temperature, terminated by washing cells with X-Vivo15 media (Lonza) containing 10% human serum AB at 4˚C. In case of sufficient cell numbers, $1.5x10^5$ eFluor450-labelled CD8-depleted or CD8 positive cells were co-cultured with $CD4^+CD25^+CD127^{low}$ sorted T_{reg} cells at a T_{reg}:$T_{responder}$ ratio of 1:2 and 0:1 (no T_{reg}) in X-Vivo15 media (Lonza) supplemented with 10% human serum AB (PAA), 2 mM L-glutamine (Lonza) and 100 U/ml Penicillin/Streptomycin (Lonza) at 37°C, 5% CO_2 and 95% humidity in the presence of 0.1 ng/ml IL-7 (R&D Systems). Each ratio was tested in up to 4 wells depending upon T_{reg} cell yield. Mixed co-cultures containing CD8-depleted cells were incubated for 5 days in presence of 10 µg/ml proinsulin (Biomm) and co-cultures including CD8 positive T cells were stimulated with anti-CD3/anti-CD28-coupled beads (Dynabeads; Invitrogen) at a 1:50 bead/cell ratio for 3 days in 96-well plates (Figure 10). Cells were stained with anti-CD4 Brilliant Violet 510 (clone SK3), anti-CD8 PerCP (clone SK1; both BD Bioscience), anti-CD25 PE (clone M-A251), anti-CD45RO APC (clone UCHL1; both BD Pharmingen) and anti-CD127 PE-Cy7 (clone A019D5; BioLegend). Cells were washed with $PBS^{-/-}$, stained for 15 minutes at room temperature with Zombie NIR (BioLegend) to evaluate the viability of the cells and fixed with 1.5% Formalin in $PBS^{-/-}$. All samples were acquired within 24 hours on a Becton Dickinson LSR Fortessa flow cytometer and analyzed with FlowJo software (Version 10; TreeStar Inc.). Responsiveness of $CD4^+$ or $CD8^+$ T cells respectively was identified as proliferation dye decline and upregulation of CD25 and CD45RO and percentage of T_{reg}-induced suppression was calculated for each stimulus and T_{reg} to $T_{responder}$ cell ratio.

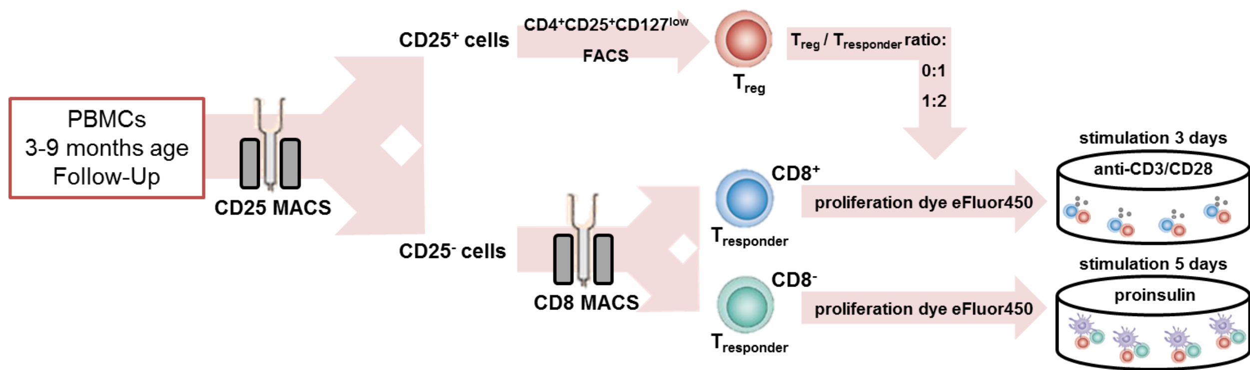


Figure 10: Representative workflow of the established suppression assay in children at the age of 3 to 9 months. Frozen PBMCs from follow up visits of children between 3 and 9 months of age were thawed as previously described and separated by CD25 positive MACS. CD25-depleted PBMCs were further processed by CD8 positive MACS and respective cell fractions were labeled with the proliferation dye eFluor450. In parallel, $CD25^+$ cells were purified by FACS to obtain $CD4^+CD25^+CD127^{low}$ T_{reg} and co-cultures including $CD8^+$ or $CD8^-$ cells with 2 different ratios of T_{reg} were performed for 3 days in the presence of anti-CD3/CD28 Dynabeads or for 5 days in the presence of 10µg/ml proinsulin, respectively.

2.3.11. Cytokine phenotyping of antigen-responsive, memory $CD4^+$ T cells at onset of type 1 diabetes

For in-depth phenotyping of antigen-responsive, memory $CD4^+$ T cells, thawed PBMCs were washed twice with $PBS^{-/-}$ and labeled with Cell Proliferation Dye eFluor670 (5 µM in $PBS^{-/-}$ for 10 min at 37°C; eBioscience). Staining was terminated by adding RPMI 1640 (Gibco) containing 15% human serum AB at 4˚C. $2x10^5$ eFluor670-labeled PBMCs were added to each well of a round-bottom 96-well microtiter plate and incubated in RPMI 1640 (Gibco) supplemented with 5% human serum AB (PAA), 2 mM L-glutamine (Lonza) and 100 U/ml Penicillin/Streptomycin (Lonza) at 37°C, 5% CO_2 and 95% humidity in the presence of proinsulin (10 µg/ml, Biomm), GAD65 (10 µg/ml; Diamyd Diagnostics), insulin (50 µg/ml; Lilly) or, as control antigen, tetanus toxoid (1 µl/ml; Novartis) for 5 days. For amplification of antigen-induced cytokine production, all cells were subsequently stimulated with pre-diluted leukocyte activation cocktail with GolgiPlug (final dilution 1:500; BD Bioscience) for additional 5 hours. Cells were harvested and stained using the following monoclonal antibodies: anti-CD4 Brilliant Violet 510 (clone SK3; BD Horizon) and anti-CD45RA PE-Cy5 (clone HI100; eBioscience). Cells were stained for 20 min at 4°C in $PBS^{-/-}$ containing 0.5% BSA, washed in $PBS^{-/-}$ and stained for 15 minutes at room temperature with Zombie NIR (BioLegend) for cell viability. Cells were permeabilized and fixed for 20 minutes at 4°C using Cytofix/Cytoperm buffer solution (BD Bioscience) and subsequently

stained intracellularly in Perm/Wash buffer (BD Bioscience) with monoclonal anti-IL-10 Alexa Fluor 488 (clone JES3-9D7; BioLegend), anti-GM-CSF PerCP-Cy5.5 (clone BVD2-21C11; BioLegend), anti-IL-17A Brilliant Violet 605 (clone BL168; BioLegend), anti-IL-21 PE (clone 3A3-N2; eBioscience), anti-IL-22 eFluor450 (clone 22URTI; eBioscience) and anti-IFNγ PE-Cy7 (clone B27; BD Bioscience) for 20 minutes at room temperature. All samples were washed with $PBS^{-/-}$ containing 0.5% BSA and acquired within 24 hours on a Becton Dickinson LSR Fortessa flow cytometer. For each stimulus at least two replicates were analyzed with FlowJo software (Version 10; TreeStar Inc.) and responsiveness of $CD4^{+}CD45RA^{-}$ T cells was identified as proliferation dye decline. Proliferation to antigen is expressed as the proportion of dye-diluted $CD4^{+}CD45RA^{-}$ T cells after subtraction of values from medium alone control wells. Antigen-specific cytokine production in proliferating cells is expressed as the proportion of total $CD4^{+}$ $CD45RA^{-}$ T cells that had dye-diluted after subtraction of values from medium alone control wells and were positive for the respective cytokine (representative FACS plot shown in figure 11).

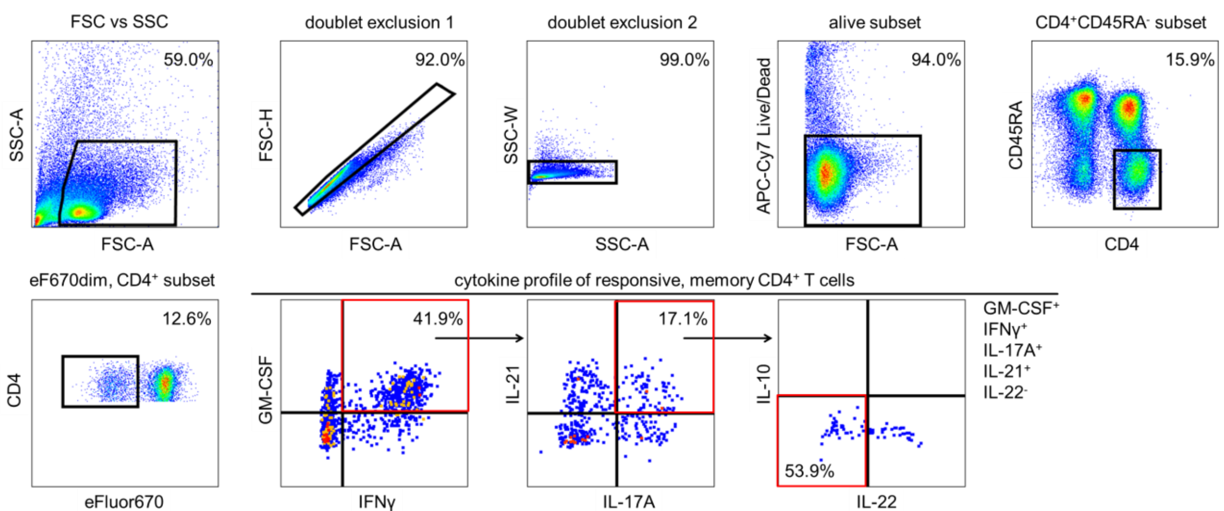


Figure 11: Representative FACS plot and gating strategy of the established cytokine staining of antigen-responsive $CD4^{+}CD45RA^{-}$ T cells. Applied boolean gating approach to identify specific GM-CSF, IFNγ, IL-17A, IL-21, and IL-22 cytokine profiles in antigen-responsive $CD4^{+}CD45RA^{-}$ T cells following stimulation with proinsulin or GAD65. Following doublet and dead cell exclusion, antigen-responsiveness was identified as proliferation dye decline in memory $CD4^{+}$ T cells. The gated antigen-responsive cells were subsequently analyzed for their cytokine production. The example shows the identification of $GM\text{-}CSF^{+}IFN\gamma^{+}IL\text{-}17A^{+}IL\text{-}21^{+}IL\text{-}22^{-}$ proinsulin-responsive $CD4^{+}CD45RA^{-}$ T cells.

2.3.12. Single cell gene expression profiling of antigen-specific CD4$^+$ T cells

Antigen-responsive, naïve CD4$^+$ T cells collected at birth and memory CD4$^+$ T cells collected at onset of T1D were single cell sorted into 96-well PCR plates. Sorted single cell plates were stored at -80°C and gene expression analysis in antigen-responsive CD4$^+$ T cells at birth (Table 11) and at onset of T1D (Table 9) was performed as previously described (Eugster et al., 2013; Heninger et al., 2017). All cells were processed and analyzed by quantitative real-time PCR using the Fluidigm Biomark HD on an already established set of genes (listed in table 12) in cooperation with Dr. Anne Eugster from the Single Cell Facility of the Center for Regenerative Therapies Dresden (CRTD, Germany).

In brief, qScript complementary DNA (cDNA) Supermix (Quanta Biosciences, Gaithersburg, MD) was used to generate cDNA from single cells, which was subsequently amplified with the TATAA GrandMaster Mix (TATAA Biocenter, Göteborg, Sweden) in the presence of primers for 48 genes of interest. Following exonuclease I digestion (Thermo Fisher Scientific, Waltham, MA), quantitative real-time PCR was performed on the 96.96 Dynamic Array IFC using the Biomark HD System (Fluidigm Corporation, San Francisco, CA) and primers as indicated (Table 12). Raw values from qPCR were analyzed and pre-processed for statistical analysis by using the real-time PCR analysis software from Fluidigm.

Table 11: Sample set for single cell gene expression profiles in antigen-specific, naïve CD4$^+$ T cells at birth.

Antigen	KLH		gly. KLH		insulin		gly. Insulin		proinsulin		gly. proinsulin	
	donors	total cells	donors	total cells	donors	total cells	donors	total cells	donors	total cells	donors	total cells
healthy parents	**3**	**165**	**-**	**-**	**1**	**30**	**1**	**24**	**3**	**90**	**2**	**60**
maternal T1D	**5**	**157**	**1**	**25**	**2**	**57**	**1**	**30**	**3**	**81**	**2**	**46**
both parents DM	**-**	**-**	**-**	**-**	**2**	**60**	**2**	**60**	**2**	**60**	**2**	**60**
paternal T1D	**4**	**128**	**1**	**24**	**2**	**59**	**3**	**94**	**4**	**120**	**3**	**83**

Table 12: Primers of genes for single cell gene expression profiling.

1st PCR

Gene	PCR1 Primer 5'	qPCR 5' Sequence 5'→ 3'	PCR1 Primer 3'	qPCR 3' Sequence 5'→ 3'
AHR	2-5'	TAAAGCCAATCCCAGCTGAA	1-3'	GACGCTGAGCCTAAGAACTGA
BCL6	2-5'	AGCCGTGAGCAGTTTAGAGC	1-3'	AAGTCCAGGAGGATGCAGAA
CCR10	6-5'	GCTGCTCTTCAGCCAGGAT	6-3'	GCGTAGCAGGCTACCATGAC
CCR3N	4-5'	TGTCTCGTTCTCCCTCTGCT	4-3'	AGCCACATTGTAGGGTGTCC
CCR4	2-5'	CAAATACAAGCGGCTCAGGT	1-3'	AGCCCACCAAGTACATCCAG
CCR5	1-5'	GGCCATCTCTGACCTGTTTTT	1-3'	AAACACAGCATGGACGACAG
CCR6	2-5'	TCAGCGATGTTTTCGACTCC	1-3'	CACCAGAATATTCCCCAGGA
CCR7	1-5'	CAATGAAAAGCGTGCTGGT	1-3'	ATAGGGAGGAACCAGGCTTT
CD127	1-5'	CTGAGGCTCCTTTTGACCTG	1-3'	CTGCAGGAGTGTCAGCTTTG
CD134	2-5'	AAGCCTGGAGTTGACTGTGC1-3'	1-3'	GGTCCCTGTCCTCACAGATT
CD3E	1-5'	GCACTCACTGGAGAGTTCTGG	1-3'	CCTCATCACCGCCTATGTTT
CD4	1-5'	ACCGGGGAGTCCCTTTTAG	1-3'	CATTCAGCTTGGATGGACCT
CD40	2-5'	GTGAGAGCTGTGTCCTGCAC	1-3'	GCTTGTCCAAGGGTGACATT
CD52	1-5'	GCGCTTCCTCTTCCTCCTAC	1-3'	CTGAAGCAGAAGAGGTGGATT
CD8	3-5'	GCTGGACTTCGCCTGTGATAT	4-3'	TTGTCTCCCGATTTGACCAC
CMAF	2-5'	GGACGCGTACAAGGAGAAAT	1-3'	GCTTCCAAAATGTGGCGTAT
CTLA4	1-5'	TGACAGCCAGGTGACTGAAG	1-3'	GTTGCCTATGCCCAGGTAGT
CXCR5	2-5'	1-3AAATGGACCTCGAGAACCTG'	1-3'	CTTGAAGGAGGCCATGAGG
EGR2	2-5'	TGGAGAGAAGAGGTCGTTGG	1-3'	GTTGAAGCTGGGGAAGTGAC
EOMES	1-5'	CACAAATACCAACCCCGACT	1-3'	GGGACAATCTGATGGGATGA
FOXP3	1-5'	GTAGCCATGGAAACAGCACAT	3'	GCGTGTGAACCAGTGGTAGAT
GATA3	1-5'	CCGCCCTACTACGGAAACTC	1-3'	TTGGAGAAGGGGCTGAGAT
GITR	5'	GAGTGGGACTGCATGTGTGT	3'	TGCAGTCTGTCCAAGGTTTG
GMCSF	1-5'	CACTGCTGCTGAGATGAATGA	1-3'	AGGGCAGTGCTGCTTGTAGT
HELIOS	2-5'	CGAAAGGGAGCACTCCAATA	1-3'	ATGGCCCCTGATCTCATCT
ICOS	1-5'	GGACCATTCTCATGCCAACT	1-3'	TCGTGCACACTGGATGAATA
IL10	1-5'	TGCTGGAGGACTTTAAGGGTTA	1-3'	GCCTTGCTCTTGTTTTCACAG
IL13	1_5'	GGTCAACATCACCCAGAACC	1-3'	TTTACAAACTGGGCCACCTC
IL17A	3-5'	TGGGAAGACCTCATTGGTGT	3-3'	CCGGTTATGGATGTTCAGGT
IL17fF	1-5'	TCCAAAAGCCTGAGAGTTGC	1-3'	ATGCAGCCCAAGTTCCTACA
IL18RAP	2-5'	TTGCAGGAGAGCGAATTAAA	1-3'	GGTGAGAGTCGATTTCTGTGG
IL2	3-5'	TGGAGCATTTACTGCTGGATT	3-3'	GCACTTCCTCCAGAGGTTTG
IL21	2-5'	TCGCCACATGATTAGAATGC	1-3'	AAGCAGGAAAAAGCTGACCA
IL22	2-5'	TCCAGCAGCCCTATATCACC	1-3'	GTTCAGCACCTGCTTCATCA
IL4	1-5'	TGCCTCCAAGAACACAACTG	1-3'	CTCTGGTTGGCTTCCTTCAC
IL9	2-5'	CTCATCAACAAGATGCAGGAAG	1-3'	TGTTTGCATGGTGGTATTGG
INFGAMMA	1-5'	CTGTTACTGCCAGGACCCAT	1-3'	TGGATGCTCTGGTCATCTTT
NFATC2	1-3'	AACTCGTCTTTGGCGAGGA	2-5'	AGACGGTAATCCATGATGTGG
PDCD1	1-5'	ACCTGCAGCTTCTCCAACAC	1-3'	GCAGTTGTGTGACACGGAAG
RANTES	1-5'	CGCTGTCATCCTCATTGCTA	1-3'	ACACACTTGGCGGTTCTTTC
REL	1-5'	ACAAATGTGAAGGGCGATCA	1-3'	CCGTCTCTGCAGTCTTTTCC
RGS16	2-5'	CACGCTTTCCTGAAGACAGA	1-3'	GACCTCTTTAGGGGCCTCAC
RORA	4-5'	CACCAGCATCAGGCTTCTTT	4-3'	GGTCTGCCACGTTATCTGCT
RORC	4-5'	TCCCGAGATGCTGTCAAGTT	3-3'	TCCCTCTGCTTCTTGGACAT
SRP14	5'	TATGACGGTCGAACCAAACC	3'	GCTGCTGCTTTGGTCTTCTT
TBET	2-5'	CCGTGACTGCCTACCAGAAT	1-3'	ATCTCCCCCAAGGAATTGAC
TGFBETA	5'Taq-1	TACCTGAACCCGTGTTGCT	1-3'	CACAACTCCGGTGACATCAAA
TNFALPHA	1-5'	CCCCAGGGACCTCTCTCTAA	1-3'	TGAGGTACAGGCCCTCTGAT

Quantitative PCR

Gene	qPCR Primer 5'	qPCR 5' Sequence 5'→ 3'	qPCR Primer 3'	qPCR 3' Sequence 5'→ 3'
AHR	2-5'	TAAAGCCAATCCCAGCTGAA	1-3'	GACGCTGAGCCTAAGAACTGA
BCL6	2-5'	AGCCGTGAGCAGTTTAGAGC	1-3'	AAGTCCAGGAGGATGCAGAA
CCR10	6-5'	GCTGCTCTTCAGCCAGGAT	6-3'	GCGTAGCAGGCTACCATGAC
CCR3N	4-5'	TGTCTCGTTCTCCCTCTGCT	4-3'	AGCCACATTGTAGGGTGTCC
CCR4	2-5'	CAAATACAAGCGGCTCAGGT	1-3'	AGCCCACCAAGTACATCCAG
CCR5	2-5'	GTCCCCTTCTGGGCTCACTA	1-3'	AAACACAGCATGGACGACAG
CCR6	2-5'	TCAGCGATGTTTTCGACTCC	1-3'	CACCAGAATATTCCCCAGGA
CCR7	2-5'	GTGGTGGCTCTCCTTGTCAT	1-3'	ATAGGGAGGAACCAGGCTTT
CD127	1-5'	CTGAGGCTCCTTTTGACCTG	1-3'	CTGCAGGAGTGTCAGCTTTG
CD134	1-5'	CCACACAGGACACAGTCTGC	1-3'	GGTCCCTGTCCTCACAGATT
CD3E	1-5'	GCACTCACTGGAGAGTTCTGG	1-3'	CCTCATCACCGCCTATGTTT
CD4	1-5'	ACCGGGGAGTCCCTTTTAG	1-3'	CATTCAGCTTGGATGGACCT
CD40	2-5'	GTGAGAGCTGTGTCCTGCAC	1-3'	GCTTGTCCAAGGGTGACATT
CD52	1-5'	GCGCTTCCTCTTCCTCCTAC	1-3'	CTGAAGCAGAAGAGGTGGATT
CD8	3-5'	GCTGGACTTCGCCTGTGATAT	4-3'	TTGTCTCCCGATTTGACCAC
CMAF	2-5'	GGACGCGTACAAGGAGAAAT	1-3'	GCTTCCAAAATGTGGCGTAT
CTLA4	2-5'	TGGGGAATGAGTTGACCTTC	1-3'	GTTGCCTATGCCCAGGTAGT
CXCR5	2-5'	AAATGGACCTCGAGAACCTG	1-3'	CTTGAAGGAGGCCATGAGG
EGR2	2-5'	TGGAGAGAAGAGGTCGTTGG	1-3'	GTTGAAGCTGGGGAAGTGAC
EOMES	1-5'	CACAAATACCAACCCCGACT	1-3'	GGGACAATCTGATGGGATGA
FOXP3	2-5’	ACATTCCCAGAGTTCCTCCAC	1-3’	GCGTGTGAACCAGTGGTAGAT
GATA3	1-5'	CCGCCCTACTACGGAAACTC	1-3'	TTGGAGAAGGGGCTGAGAT
GITR	3-5'	GAGTGGGACTGCATGTGTGT	3-3'	TGCAGTCTGTCCAAGGTTTG
GMCSF	1-5'	CACTGCTGCTGAGATGAATGA	1-3'	AGGGCAGTGCTGCTTGTAGT
HELIOS	2-5'	CGAAAGGGAGCACTCCAATA	1-3'	ATGGCCCCTGATCTCATCT
ICOS	2-5'	GGTTACCCATAGGATGTGCAG	1-3'	TCGTGCACACTGGATGAATA
IL10	2-5’	TTTAAGGGTTACCTGGGTTGC	1-3’	GCCTTGCTCTTGTTTTCACAG
IL13	2-5’	GTACTGTGCAGCCCTGGAAT	1-3’	TTTACAAACTGGGCCACCTC
IL17A	3-5'	GCCAAATTCTGAGGACAAG	1-3'	GGGGACAGAGTTCATGTGGT
IL17fF	2-5’	GCCTGTGCCAGGAGGTAGTA	1-3’	ATGCAGCCCAAGTTCCTACA
IL18RAP	2-5'	TTGCAGGAGAGCGAATTAAA	1-3'	GGTGAGAGTCGATTTCTGTGG
IL2	3-5'	TGGAGCATTTACTGCTGGATT	3-3'	GCACTTCCTCCAGAGGTTTG
IL21	2-5'	TCGCCACATGATTAGAATGC	1-3'	AAGCAGGAAAAAGCTGACCA
IL22	2-5'	TCCAGCAGCCCTATATCACC	1-3'	GTTCAGCACCTGCTTCATCA
IL4	2-5’	GGCAGTTCTACAGCCACCAT	1-3’	CTCTGGTTGGCTTCCTTCAC
IL9	2-5'	CTCATCAACAAGATGCAGGAAG	1-3'	TGTTTGCATGGTGGTATTGG
INFGAMMA	2-5’	GGTCATTCAGATGTAGCGGA	1-3’	TGGATGCTCTGGTCATCTTT
NFATC2	2-5'	AAGAAGAGCCGAATGCACATA	1-3'	AGAAACTTCTGCGGCCCTAC
PDCD1	1-5'	GCTTCCGTGTCACACAACTG	1-3'	GCACTTCTGCCCTTCTCTCT
RANTES	2-5'	ATCTGCCTCCCCATATTCCT	1-3'	ACACACTTGGCGGTTCTTTC
REL	2-5'	GGAGCACAGCACAGACAACA	1-3'	CCGTCTCTGCAGTCTTTTCC
RGS16	2-5'	CACGCTTTCCTGAAGACAGA	1-3'	GACCTCTTTAGGGGCCTCAC
RORA	4-5'	CACCAGCATCAGGCTTCTTT	4-3'	GGTCTGCCACGTTATCTGCT
RORC	4-5'	TCCCGAGATGCTGTCAAGTT	3-3'	TCCCTCTGCTTCTTGGACAT
SRP14	2-5’	TACTGTGGAGGGCTTTGAGC	3’	GCTGCTGCTTTGGTCTTCTT
TBET	2-5’	CCGTGACTGCCTACCAGAAT	1-3’	ATCTCCCCCAAGGAATTGAC
TGFBETA	5'Taq2	TACCTGAACCCGTGTTGCTT	3'Taq1	CAACTCCGGTGACATCAAAA
TNFALPHA	2-5'	CCCGAGTGACAAGCCTGTAG	1-3'	TGAGGTACAGGCCCTCTGAT

2.3.13. Statistical analysis

2.3.13.1. General data analysis

Data were analyzed with GraphPad Prism 4 (GraphPad), SPSS (IBM) or R Studio Version 3.2.2 (RStudio, Inc) software. Data characterized by Gaussian normal distribution are shown as scatter plots indicating the mean and comparisons between different groups were made using a parametric two-tailed, unpaired Student's t test. Non-Gaussian distributed data were shown as scatter plots indicating the median and different groups were compared using the two-tailed Mann Whitney U test. P-values lower than 0.05 were considered significant. For boolean analysis, which examined combinations of cytokine production in antigen-specific, memory $CD4^+$ T cells, significance was considered, when p-values were <0.05 / number of comparisons.

2.3.13.2. Analysis of gene expression on single cell level in antigen-responsive $CD4^+$ T cells

Analysis of gene expression profiles of single cells was done using KNIME 2.9.4 (Berthold et al., 2007) and R studio version 1.1.383 (RStudio, Inc) software. As previously described (Buettner et al., 2015), raw C_t values were pre-processed using a linear model to correct for potential confounding effects, which can mask biological variability. In brief, modeling of plate batch effects (by using dummy coding for each plate per batch) while maintaining dose effects allowed comparison between different plates. Corrected C_t values of 48 genes for each antigen-responsive $CD4^+$ T cell were used to identify multivariate gene expression profiles by t-distributed Stochastic Neighbor Embedding (tSNE). To find genes whose expression were varied significantly between groups, a semi-continuous modeling framework, the Hurdle model, was applied without outlier removal or single well adjustment to avoid over dispersion of regressed data. The distribution of antigen-responsive cells among identified clusters between different parental groups was analyzed using the Fischer's exact test.

3. Results

A major aim of my thesis was to understand mechanisms of protection against T1D offered by maternal T1D during gestation. I, therefore, examined phenotype and function of various immune cells in cord blood from neonates of mothers who had T1D.

3.1. Immune phenotype at birth in relation to maternal type 1 diabetes gestation

I helped to collect cord blood samples obtained from our ImmunDiabRisk study from 21 children born to parents who did not have diabetes (controls), 16 children from mothers with GDM, 25 children from mother with T1D and 20 children who have a father or sibling with T1D. In order to correlate changes at the immune phenotype at birth to parental diabetes status, I established multi-parameter surface marker staining panels for flow cytometry and analyzed these samples.

Increased frequencies of $CD34^+$ HSCs were observed in cord blood from children of mothers with gestational (p=0.023) or type 1 diabetes (p=0.025) as compared to children who have a father or sibling with T1D. Frequencies of HSCs were additionally increased in offspring of mother with gestational (p=0.037) or type 1 diabetes (p=0.040) as compared to controls (Figure 12a). Overall, the mean frequency of HSCs was 1.19% ± 1.14 (Standard deviation (SD)) in cord blood from neonates who had a mother with gestational or type 1 diabetes versus 0.58% ± 0.45 in cord blood from neonates with a non-diabetic mother (two-sided, unpaired Student’s t test: p=0.002; Figure 12b).

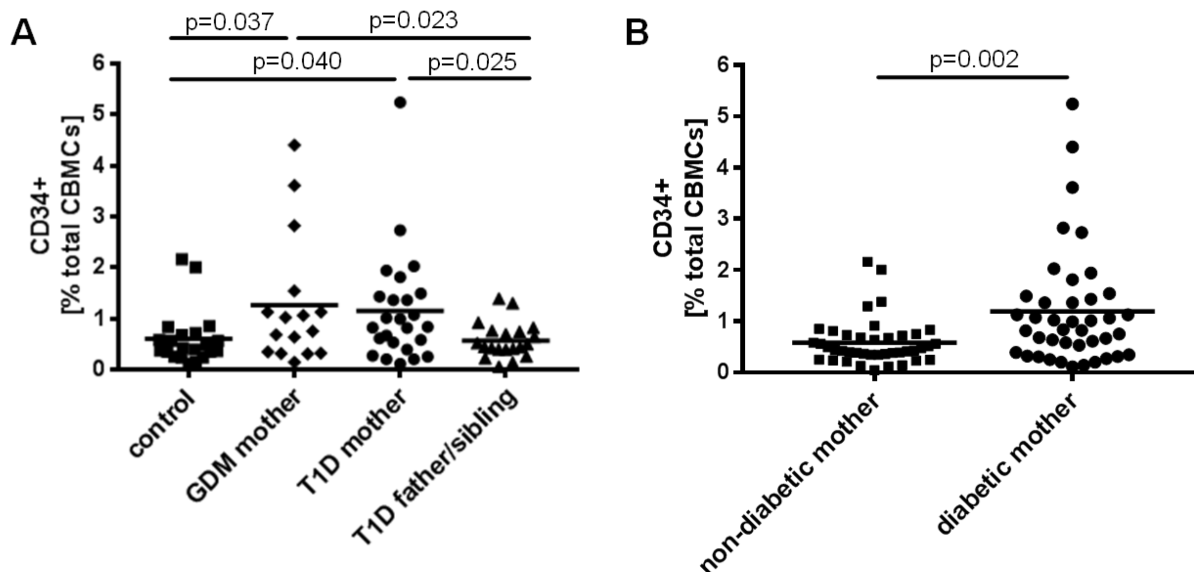


Figure 12: Phenotyping of hematopoietic stem cells in cord blood. a) Distribution of $CD34^+$ HSCs of total viable CBMCs (y axis) of children from healthy parents (squares, n=21), mother with GDM (diamonds, n=16), with T1D (circles, n=25) or father/sibling with T1D (triangles, n=20). b) The same data showing frequencies of HSCs of total viable CBMCs of children from mother without (squares, n=41) or with (circles, n=41) diabetes during pregnancy. Indicated in each scatter plot is the mean and p-values were obtained using the two-sided, unpaired Student's t test.

Children of mothers with T1D had decreased frequencies of $CD14^+$ monocytes compared to children who have a father or sibling with T1D (p=0.049; Figure 13a). No differences were observed for $CD3^-CD56^+$ NK cell and $CD19^+$ B cell frequencies in relation to parental diabetes status during gestation (Figure 13b and c).

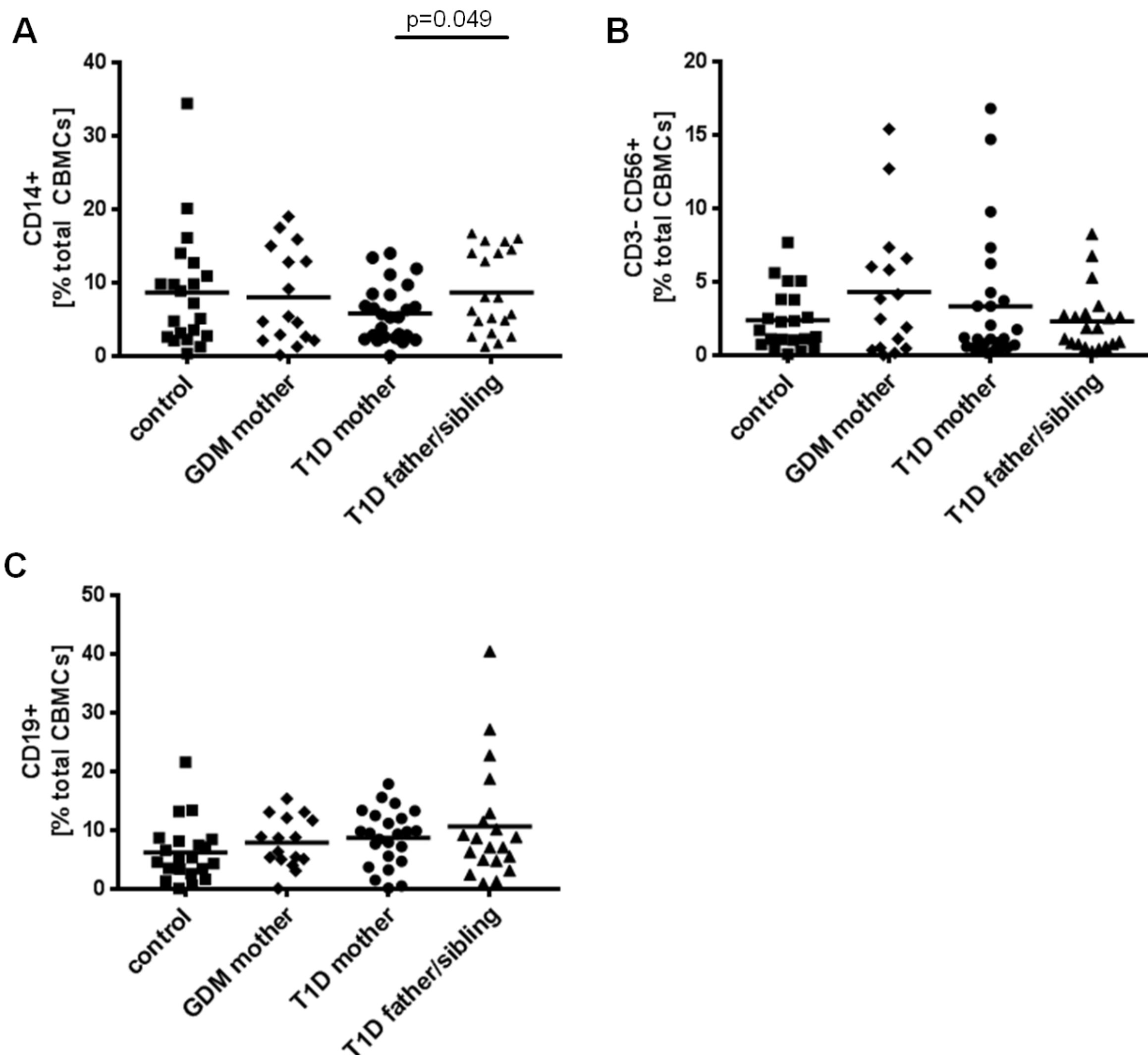


Figure 13: Phenotyping of different immune cell subsets in cord blood. Distribution of a) monocytes, b) NK cells and c) B cell subsets of total viable CBMCs of children from healthy parents (squares, n=21), mother with GDM (diamonds, n=16), with T1D (circles, n=25) or father/sibling with T1D (triangles, n=20). Indicated in each scatter plot is the mean and p-values were obtained using the two-sided, unpaired Student's t test.

No difference among children with or without T1D parents was observed within total $CD3^+$ lymphocyte compartment, or in the frequencies of $CD4^+$ and $CD8^+$ T cell subsets of gated $CD3^+$ lymphocytes as well as $CD4^+$ to $CD8^+$ T cell ratios at birth (Figure 14).

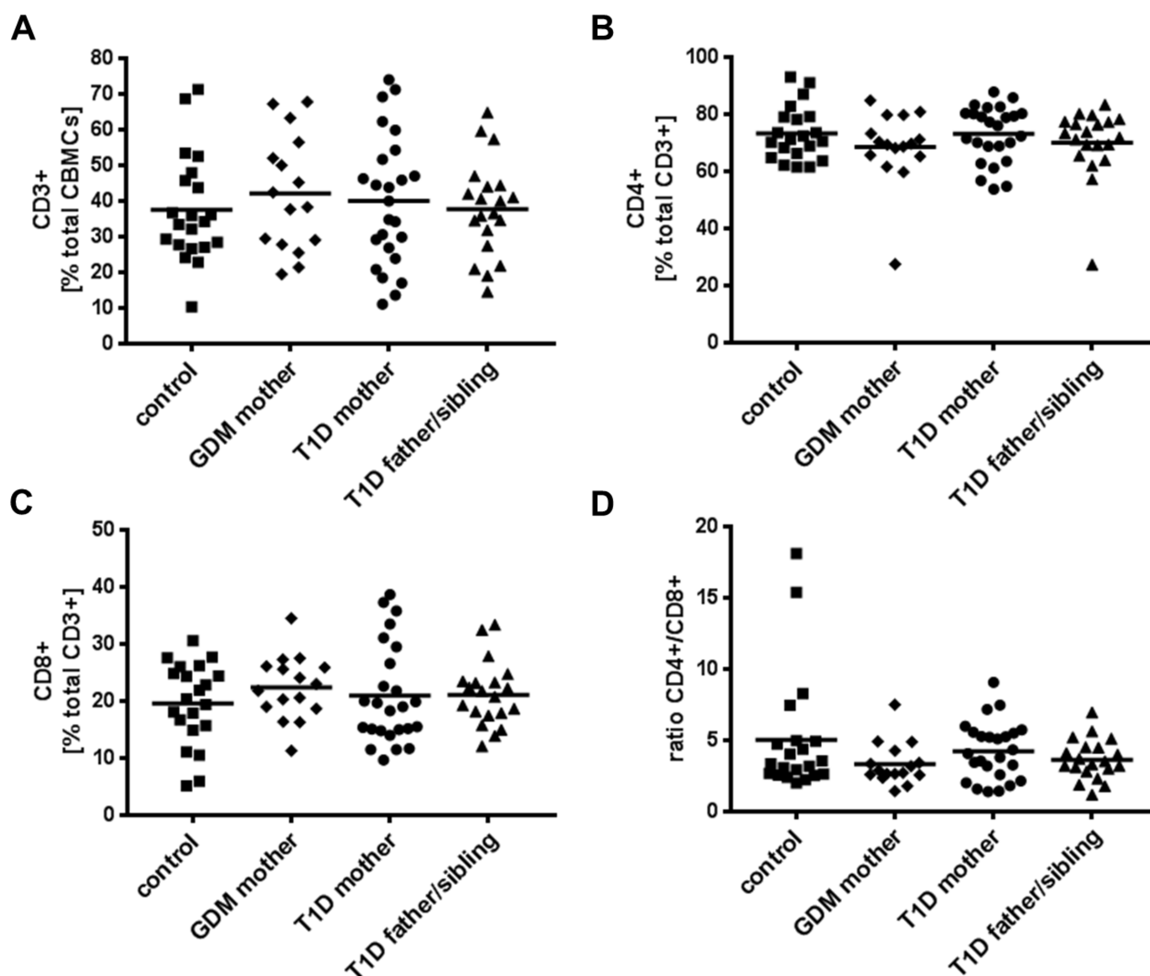
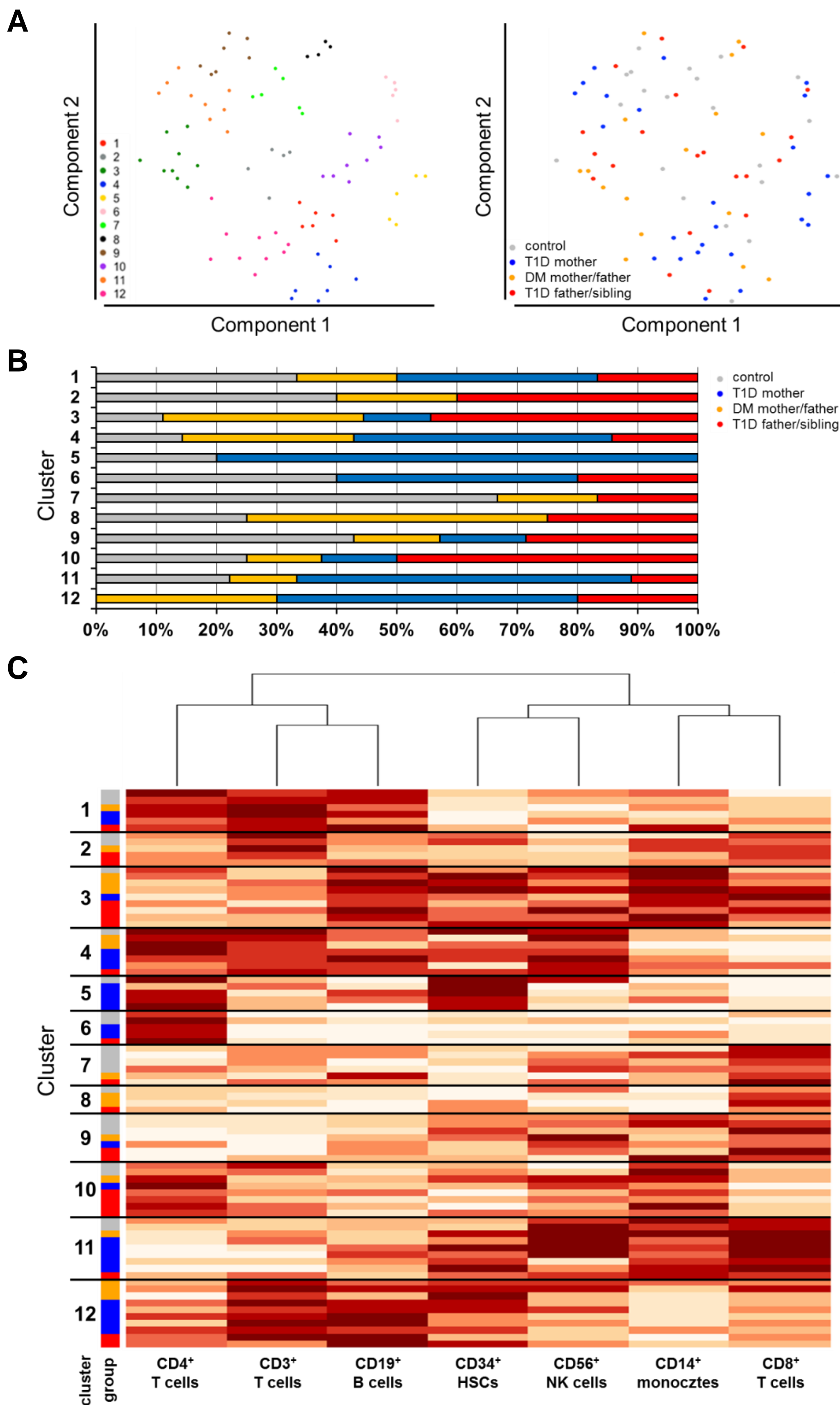


Figure 14: Phenotyping of T cell subsets in cord blood. Frequency of a) $CD3^+$ lymphocyte subset of total viable CBMCs and b) $CD4^+$ and c) $CD8^+$ T cell frequencies among $CD3^+$ lymphocytes of children from healthy parents (squares, n=21) mother with GDM (diamonds, n=16), with T1D (circles, n=25) or father/sibling with T1D (triangles, n=20). d) Ratios of $CD4^+$ to $CD8^+$ T cell fractions. Indicated in each scatter plot is the mean and no significant differences were observed between different groups (two-sided, unpaired Student's t test).

I performed a multi-dimensional analysis of the observed cell frequencies in 81 cord blood samples (Figure 15). The tSNE analysis showed 12 potential clusters. Clusters were defined as cluster 1 (red, n=6 samples), 2 (grey, n=5 samples), 3 (dark green, n=9 samples), 4 (blue, n=7 samples), 5 (yellow, n=5 samples), 6 (rose, n=5 samples), 7 (bright green, n=6 samples), 8 (black, n=4 samples), 9 (brown, n=7 samples), 10 (purple, n=8 samples), 11 (orange, n=9 samples) and 12 (pink, n=10 samples) and characterized according to parental diabetes status during gestation. Cells obtained from cord bloods of children with healthy parents dominated

cluster 7 (4 of 6; 66.7%), which was characterized by high frequencies of $CD8^+$ T cells. Phenotyped CBMCs of cord blood samples obtained from children with a father or sibling with T1D were primarily found in cluster 10 (4 of 8; 50%), featuring high frequencies of $CD4^+$ T cells and $CD14^+$ monocytes. In contrast, 4 clusters dominated by cord bloods obtained from children of a mother with gestational or type 1 diabetes were identified. Cluster 4 (5 of 7; 71.4%) was characterized by decreased frequencies of $CD14^+$ monocytes and $CD8^+$ T cells and high frequency of all other immune subsets. Cluster 11 (6 of 9; 66.7%) was characterized by increased frequencies of NK and $CD8^+$ T cells, while cluster 12 (8 of 10; 80%) had low frequencies of $CD14^+$ monocytes and high expression of $CD34^+$ HSCs, $CD3^+$ T and $CD19^+$ B cells. Cluster 5, which was exclusively dominated by cord bloods from children with maternal T1D during gestation (4 of 5; 80%), shared previously described low expression of monocytes and $CD8^+$ T cells, but featured high $CD4^+$ T cell and $CD34^+$ HSC frequencies as a prominent profile.


A
Component 2
Component 1
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12
control
T1D mother
DM mother/father
T1D father/sibling
B
Cluster
0%
10%
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30%
40%
50%
60%
70%
80%
90%
100%
C
cluster
group
CD4+ T cells
CD3+ T cells
CD19+ B cells
CD34+ HSCs
CD56+ NK cells
CD14+ monocztes
CD8+ T cells

Figure 15: Multidimensional data analysis of cell frequencies in cord blood. Frequencies of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes as well as $CD19^+$ B cells, $CD34^+$ HSCs, $CD3^-CD56^+$ NK cells and $CD14^+$ monocytes were each ranked from low to high abundance and used for multidimensional tSNE analysis. a) tSNE analysis visualized 12 major clusters, defined as clusters 1 (red, 6 samples), 2 (grey, 5 samples), 3 (dark green, 9 samples), 4 (blue, 7 samples), 5 (yellow, 5 samples), 6 (rose, 5 samples), 7 (bright green, 6 samples), 8 (black, 4 samples), 9 (brown, 7 samples), 10 (purple, 8 samples), 11 (orange, 9 samples) and 12 (pink, 10 samples). b) Distribution of cord bloods of children of mothers with gestational (yellow) or type 1 diabetes (blue), of children with fathers or siblings with T1D (red) or with healthy parents (grey) among different clusters. c) The ranked frequencies of each immune cell subsets are shown as a heatmap.

Overall, the findings show that there is a potential increase in HSCs in children born to mothers with diabetes. This could reflect exposure to factors that increase stem cell turnover and will be addressed beyond my thesis by transcriptomic analysis of isolated cord blood HSCs.

3.2. Precursor frequencies of antigen-responsive, naïve $CD4^+$ T cells in dependency of maternal type 1 diabetes pregnancy

I next wanted to test my hypothesis that the maternal T1D environment reduces the frequency of proinsulin- and insulin-reactive $CD4^+$ T cells, potentially as a result of the increased exposure to (pro)insulin during fetal life. In order to do this, I needed a method to provide antigen to naïve $CD4^+$ T cells. Since I expected very low frequencies of (pro)insulin-reactive T cells, I decided that an assay with many replicates might be required in order to increase the confidence of the values I obtain for each child. I chose proliferation with 10 well replicates and three dyes to measure proliferation in each well providing a total of 30 replicates per antigen. I also used a dendritic cell-based antigen presentation since Heninger and colleagues had previously shown that this was required in order to see responses at birth (Heninger et al., 2013). I removed potential regulatory T cells so that I could specifically look at response without confounding suppression. The resultant new assay (Figure 9) satisfactorily measured naïve $CD4^+$ T cells response to antigen. Since no differences in frequencies of peripheral T cell subsets at birth were shown in relation to parental T1D, I designed a new T cell proliferation assay involving antigen specificity against (pro)insulin as the first major autoantigen in T1D. This established new multi-dye, multi-well proliferation assay expanded previously used methodology to antigen specificity in order to determine very low frequencies of antigen-responsive, naïve $CD4^+$ T cells with more confidence and with markedly less cells than previously possible. This newly designed multi-dye assay was used as a powerful tool, to address the question, if maternal T1D

during gestation confers relative protection against T1D in neonates by inducing decreased frequencies of antigen-specific, naïve CD4$^+$ T cell at birth. A representative CD4$^+$ T cell response against diabetes-related antigens in cord blood of a neonate with a type 1 diabetic father is shown in figure 16.

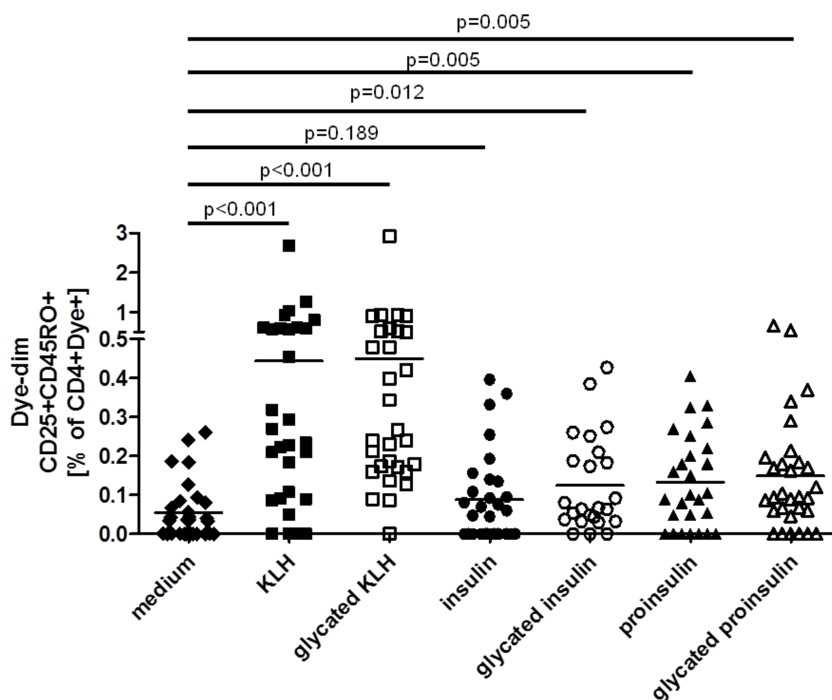


Figure 16: Frequencies of antigen-responsive, naïve CD4$^+$ T cells in one representative cord blood sample at birth. Naïve CD4$^+$ T cell response at birth indicated on the y axis as the proportion of proliferated (dye-dim), activated (CD25$^+$CD45RO$^+$) CD4$^+$ T cells in presence of antigen-unloaded (medium control) and antigen-loaded MO-DCs (x axis). Scatter plots indicate 30 replicates per condition originating from incubating responder cells with 3 different proliferation dyes in 10 well technical replicates. Indicated in each scatter plot is the mean. P-values were obtained using the two-sided, unpaired Student's t test to compare antigen loaded to medium responses.

I tested antigen responses in 16 cord bloods of control neonates from healthy parents, 15 cord bloods of neonates from T1D mothers and 14 cord bloods of neonates who have a father or sibling with T1D (Figure 17).

I used KLH as a positive, model antigen with various unspecific epitopes to induce immune activation as previously described (reviewed in Swaminathan et al., 2014). The majority of all cord bloods showed increased responsiveness of CD4$^+$ T cells in presence of KLH. Responses to KLH were not different in any of the subject groups and were, therefore, unaffected by

maternal diabetes status. Responses to proinsulin and insulin were lower than those against KLH, in the control cord bloods (two-sided, unpaired Student's t test; proinsulin p=0.037; insulin p=0.003) and in the cord bloods from children of mothers with T1D (two-sided, unpaired Student's t test; proinsulin p=0.027; insulin p=0.019). Increased responsiveness in cord bloods of neonates who have a father or siblings with T1D was observed in presence of insulin (against controls, p=0.0009; against maternal T1D, p=0.0046) and proinsulin (against controls, p=0.0046; against maternal T1D, p=0.0027). Consistent with our hypothesis of decreased frequencies of autoantigen-specific precursor frequencies, the responsiveness of naïve $CD4^+$ T cells of neonates from mothers with T1D was reduced to level of healthy controls.

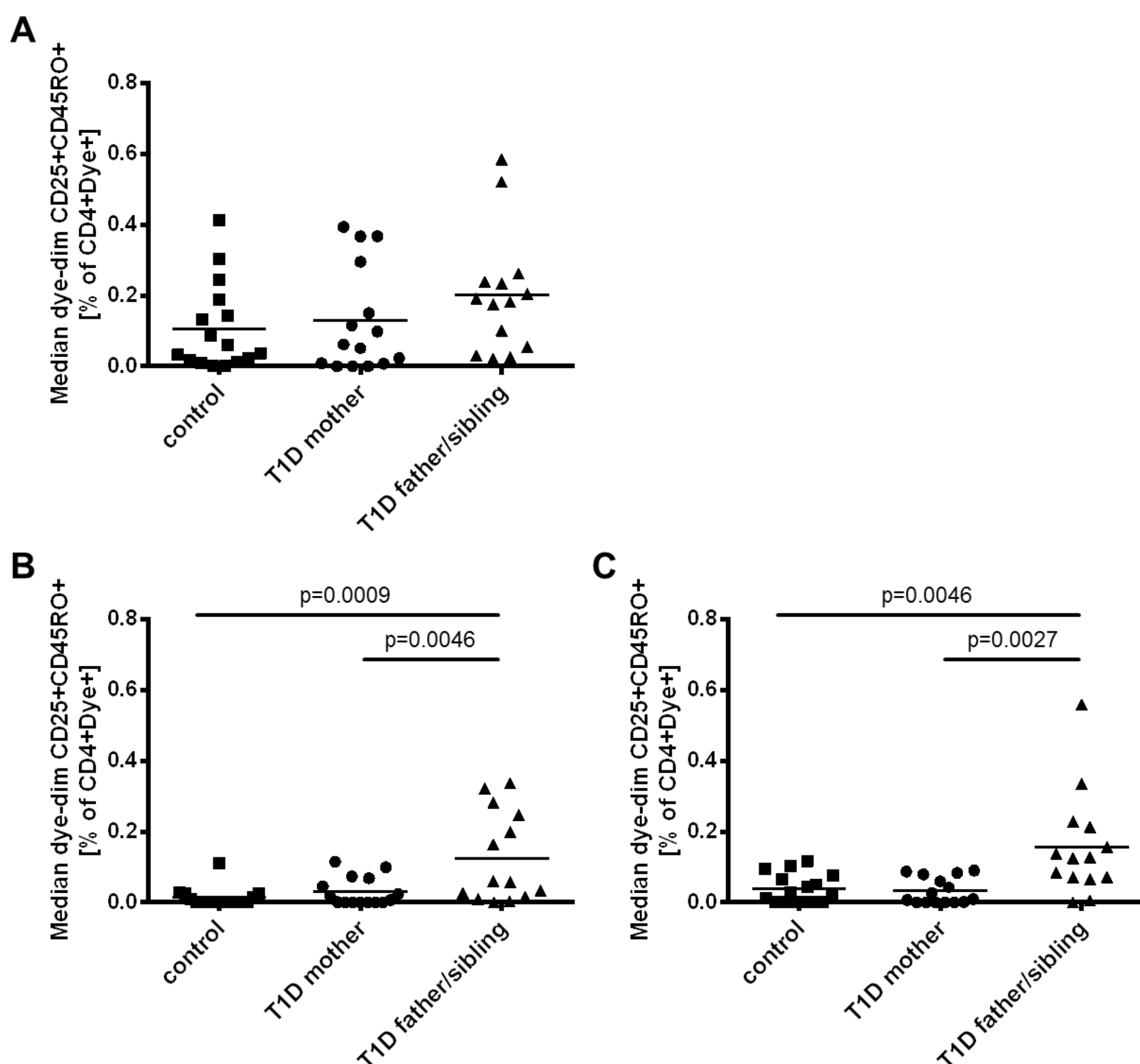


Figure 17: Antigen-responsive $CD4^+$ T cells in cord blood. Background-adjusted antigen-response of autoreactive $CD4^+$ T cells in presence of matured, monocytes-derived DCs loaded with a) KLH, b) insulin

and c) proinsulin. Indicated in each scatter plot is the mean; P-values were obtained using the two-sided, unpaired Student's t test.

3.2.1. Glycation of antigens during maternal type 1 diabetes gestation as a possible mechanism to modify antigen-specific, naïve CD4^{+} T cell responses

Children of mothers with T1D are exposed to increased glucose. Increased exposure to glucose may lead to glycation of proteins, which has been demonstrated to affect cellular mechanisms in T1D (Monti et al., 2013). I had suggested that if the child was exposed to increased glucose, there may also be increased glycation of insulin and proinsulin and this may affect antigenicity. To test this, I compared cord blood responses to glycated and non-glycated antigens in the T cell response assay (Figure 18). Similar to previously described responsiveness against unglycated forms of diabetes-related antigens, responsiveness of naïve CD4^{+} T cells was increased in children who have father or sibling with T1D against glycated forms of insulin (against controls, p=0.0107; against maternal T1D, p=0.0385) and proinsulin (against controls, p=0.0005; against maternal T1D, p=0.001). Responses to glycated insulin or proinsulin in cord blood from neonates of mothers with T1D were not different to those in cord bloods from controls.

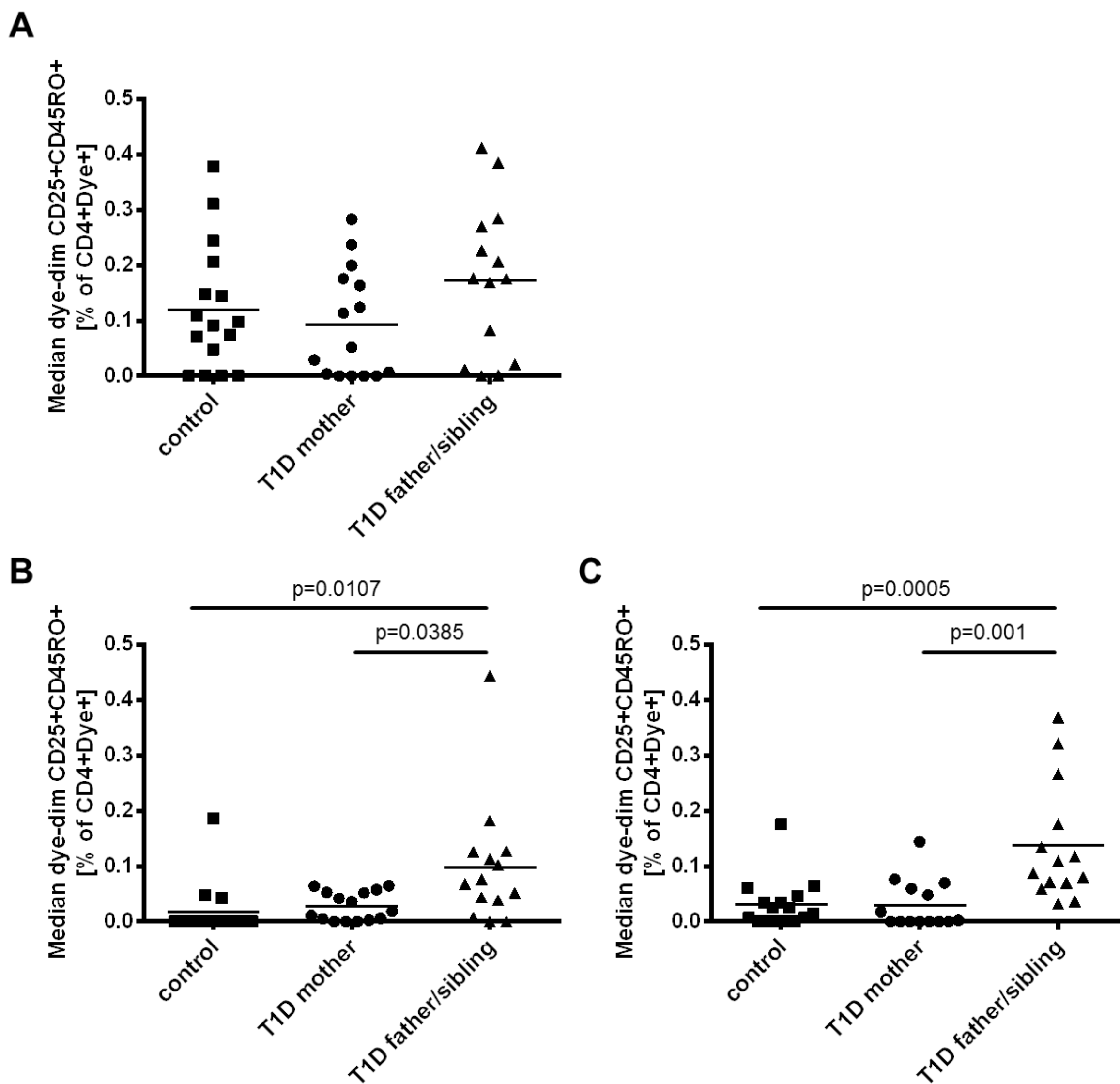


Figure 18: Responsive CD4$^+$ T cells against glycated antigens at birth. Background-adjusted antigen-response of autoreactive CD4$^+$ T cells in presence of matured, monocytes-derived DCs loaded with a) glycated KLH, b) glycated insulin and c) glycated proinsulin in children from healthy parents (squares, n=16), of mother with T1D (circles, n=15) or with a father or sibling with T1D (triangles, n=14). Indicated in each scatter plot is the mean; P-values were obtained using the two-sided, unpaired Student's t test.

I next examined whether the responses to glycated antigens were similar to non-glycated antigens. For each of the antigens examined, KLH, insulin and proinsulin, the responses to the non-glycated and glycated forms were correlated (Figure 19). I concluded that glycation of proinsulin or insulin did not increase or reduce its antigenicity in relation to the rise of

autoimmunity in T1D. This was also the case when only neonates from mothers with T1D were analyzed. I later examined whether the phenotype of the responsive cells was affected (see section 3.3.).

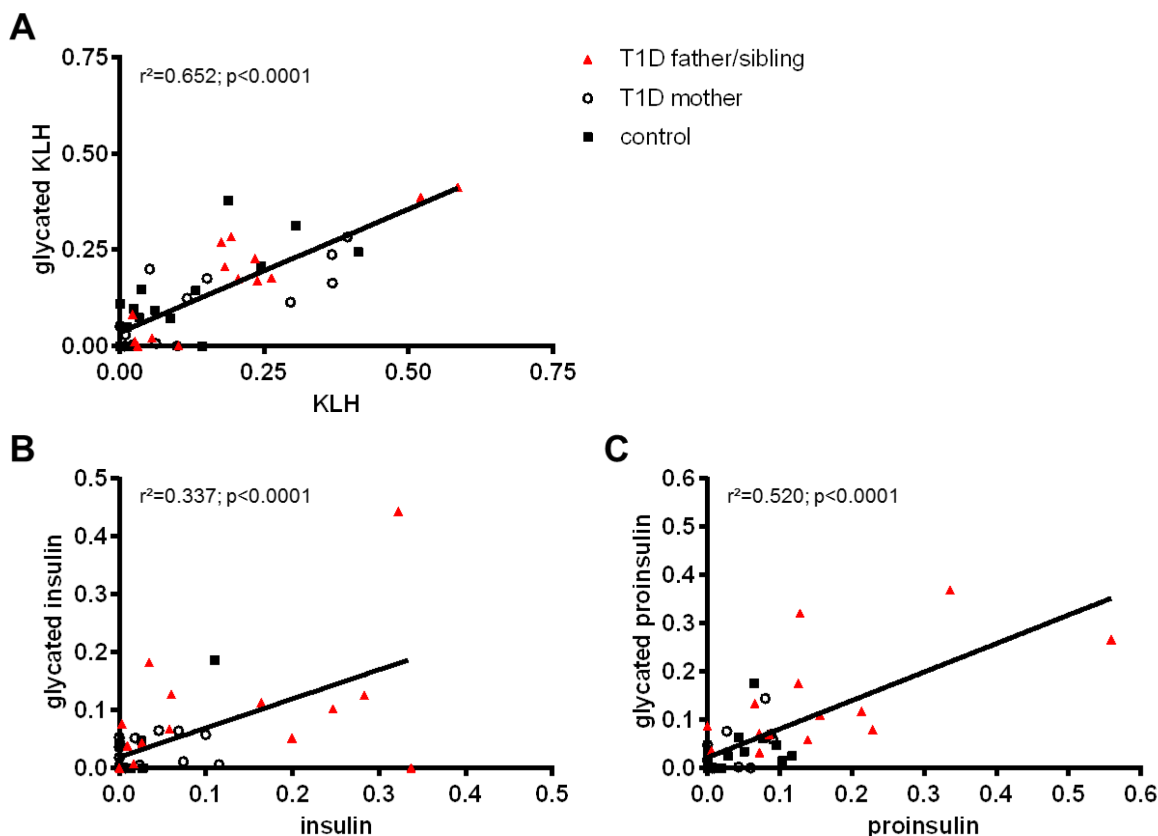


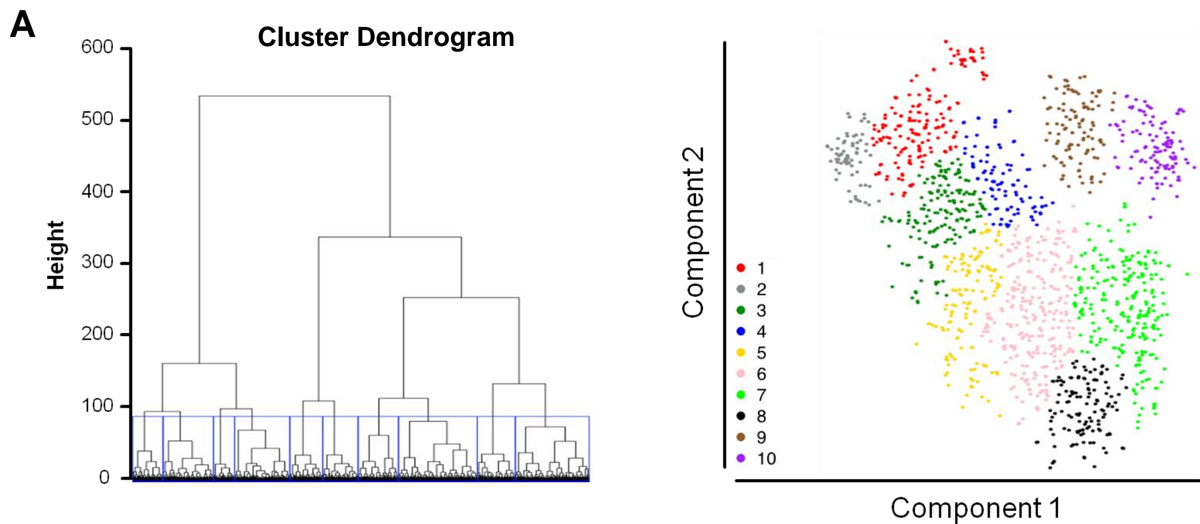
Figure 19: Responsive CD4⁺ T cells against glycated and unglycated antigens at birth. Background-adjusted antigen-response of autoreactive CD4⁺ T cells in presence of matured, monocytes-derived DCs loaded with glycated (y axis) and non-glycated (x axis) forms of a) KLH, b) insulin and c) proinsulin. Antigen response was characterized as previously described in children from healthy parents (squares, n=16), of mother with T1D (circles, n=15) or with a father or sibling with T1D (triangles, n=14). Indicated in each scatter plot is the coefficient of determination (r^2) and p-values were calculated using Pearson correlation.

3.3. Phenotype of (pro)insulin-responsive CD4⁺ T cells at birth

I had found that maternal T1D was associated with a reduced frequency of proinsulin- and insulin-reactive CD4⁺ T cells at birth. This is consistent with my hypothesis that the increased (pro)insulin production by the neonate of a mother with T1D could increase tolerance to these

antigens. It was also possible that not only numbers are reduced, but that the phenotype of the antigen-responsive cells would differ when they encountered antigen. To answer this, I single cell sorted 1513 antigen-responsive $CD4^+$ T cells from the previously described proliferation assay and examined their gene expression profiles using a panel of 48 genes.

tSNE analysis of multi-parameter gene expression was first performed on all 1513 antigen-responsive $CD4^+$ T cells at birth. This analysis showed a separation into 10 major clusters (Figure 20a). These clusters were defined as cluster 1 (red, 183 cells, 12.1% of total 1513 cells), cluster 2 (grey, 70 cells, 4.6%), cluster 3 (dark green, 167 cells, 11.0%), cluster 4 (blue, 101 cells, 6.7%), cluster 5 (yellow, 134 cells, 8.9%), cluster 6 (pink, 261 cells, 17.3%), cluster 7 (bright green, 245 cells, 16.2%), cluster 8 (black, 126 cells, 8.3%), cluster 9 (brown, 109 cells, 7.2%) and cluster 10 (purple, 117 cells, 7.7%, Figure 20b). Antigen-responsive $CD4^+$ T cells in cluster 1 and 2 showed uniformly low expression of *CD3*. While cluster 1 was further dominated by low overall expression of most analyzed genes, cluster 2 showed a distinct population of cells with high expression of C-C-chemokine receptors (*CCR3*, *CCR4*, *CCR5* and *CCR6*), cytotoxic T-lymphocyte antigen 4 (*CTLA4*) and the transcription factor Aryl hydrocarbon receptor (*AHR*). Clusters 3 and 4 had a similar low overall expression of most genes analyzed, but were not characterized by a lack of *CD3* expression. $CD4^+$ T cells in cluster 5 were characterized by high expression of *CD127* in absence of the expression of T_{reg} markers important for differentiation, such as *FOXP3* and Glucocorticoid-induced tumor necrosis factor receptor (*GITR*), and T_{reg} function, such as *CD134*, *IL10* and *CTLA4*. Antigen-specific $CD4^+$ T cells in cluster 6 and 7 were characterized by increased expression of *FOXP3* and *CTLA4*. Cells in cluster 7, but not cluster 6, additionally showed a high expression of C-C chemokine receptors (*CCR3*, *CCR4*, *CCR5* and *CCR6*) that are associated with Th1, Th17, and follicular helper T cell subsets. Although characterized by intermediate expression of *FOXP3*, cluster 8 resembles an activated T_{reg} cell subset with low expression of *CD127* and high expression of *GITR*, inducible costimulator (*ICOS*), *CD134* and *TGFBETA*. Cells in cluster 9 and 10 showed a highly pro-inflammatory phenotype, characterized by increased expression of *IL22*, *CD40*, T-box transcription factor (*TBET*), *CD134* and *CXCR5*. The pro-inflammatory phenotype of $CD4^+$ T cells in cluster 10 was further extended to high expression of C-C chemokine receptors (*CCR3*, *CCR4*, *CCR5* and *CCR6*), IL-18 receptor accessory protein (*IL18RAP*), Interferon gamma (*IFNG*) and *IL9*.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Cluster Dendrogram
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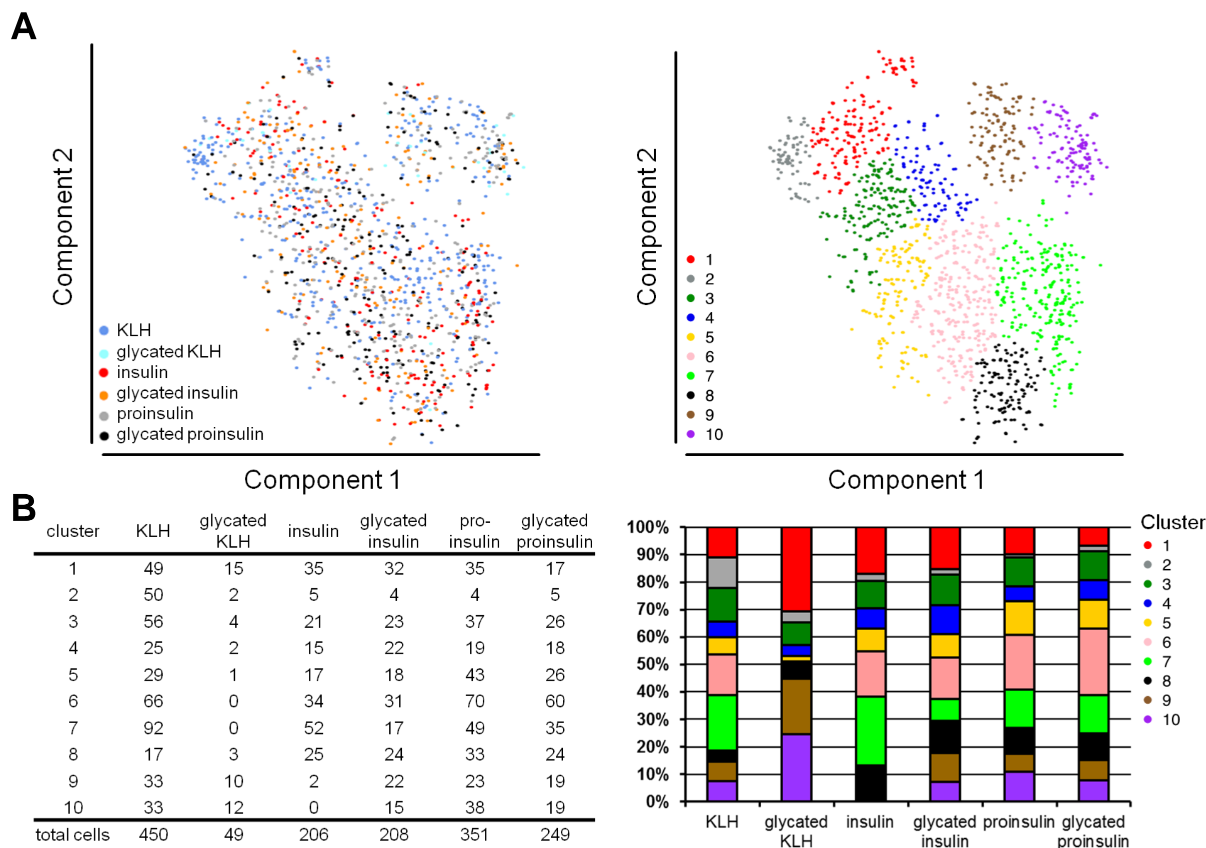
CD3E SRP14 CD62 RGS16 CCR6 CCR3 CCR5 IL18RAP EGR2 IL22 CD40 FOXP3 CD127 BCL6 GITR CMAF TBET CD134 EOMES IFNG CXCR5 PD1 TNFALPHA IL2 GMCSF IL9 NFATC2 CD8 RORC RANTES IL10 IL17A IL21 GATA3 IL4 CCR10 IL17F IL13 CCR4 CTLA4 AHR REL RORA CD4 CCR7 HELIOS ICOS TGFBETA

Figure 20: Cluster analysis of gene expression profiles of antigen-responsive $CD4^+$ T cells at birth. a) tSNE analysis of single cell gene expression of responsive $CD4^+$ T cells in presence of KLH, insulin, proinsulin and corresponding glycated forms identified 10 major clusters defined as cluster 1 (red, 183 cells), cluster 2 (grey, 70 cells), cluster 3 (dark green, 167 cells), cluster 4 (blue, 101 cells), cluster 5 (yellow, 134 cells), cluster 6 (pink, 261 cells), cluster 7 (bright green, 245 cells), cluster 8 (black, 126 cells), cluster 9 (brown, 109 cells) and cluster 10 (purple, 117 cells). b) Gene expression intensities (white, no expression; red, highest expression) were visualized as heatmap for detailed cluster characterization.

In a next step, I analyzed the distribution of the identified clusters among specific antigen responses in $CD4^+$ T cells at birth (Figure 21a). In the presence of our control stimulus KLH, antigen-specific $CD4^+$ T cell phenotypes were dominated by cluster 7 (92 cells, 20.4% of total cells per antigen) and showed the highest frequency of cells obtained in cluster 2 (50 cells, 11.1%) among all antigens tested. Both these clusters had high expression of the chemokine receptors. There were much fewer cells responsive to glycated KLH analyzed. Clusters 6 and 7 were absent and cluster 2 markedly reduced in these glycated KLH-responsive cells. Cluster 2 was virtually absent from the insulin and proinsulin responsive cells. No other major differences were observed when compared to the KLH responsive cells. The insulin response was dominated by cells originating to the *FOXP3*-elevated cluster 7 (52 cells, 25.2%), while glycation of this diabetes-related antigen led to a reduction of cells with this gene expression feature (17 cells, 8.2%). Further major clusters in the presence of glycated and unglycated insulin were cluster 6 (insulin, 34 cells, 16.5%; glycated insulin, 31 cells, 14.9%) and 8 (insulin, 25 cells, 12.1%; glycated insulin, 24 cells, 11.5%). Proinsulin-specific $CD4^+$ T cells at birth were dominated by features found in cluster 6 (70 cells, 19.9%), while glycation of proinsulin showed no major effect on the distribution of previously identified gene expression pattern in antigen-specific $CD4^+$ T cells. Clusters 3, 4 and 5 were uniformly present in all antigen-specific $CD4^+$ T cells at birth (Figure 21b).

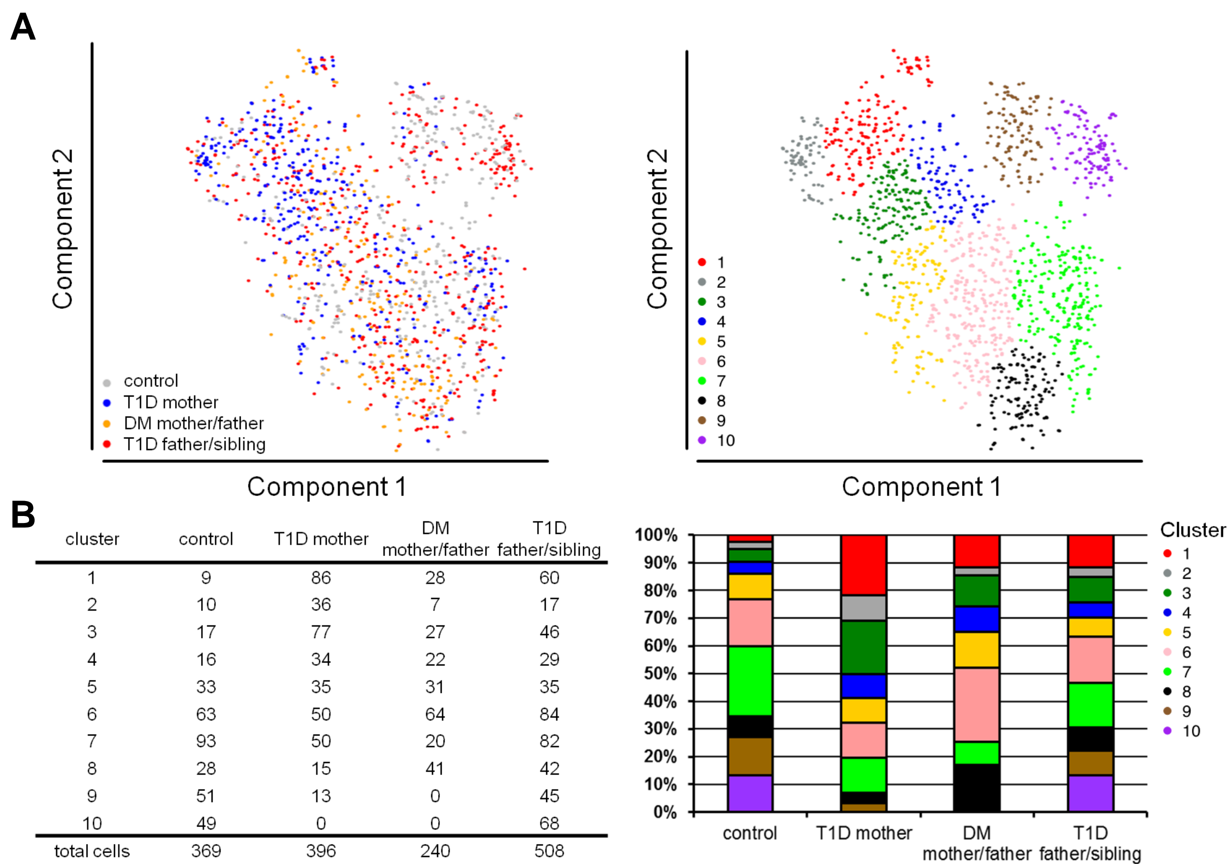


cluster	KLH	glycated KLH	insulin	glycated insulin	pro-insulin	glycated proinsulin
1	49	15	35	32	35	17
2	50	2	5	4	4	5
3	56	4	21	23	37	26
4	25	2	15	22	19	18
5	29	1	17	18	43	26
6	66	0	34	31	70	60
7	92	0	52	17	49	35
8	17	3	25	24	33	24
9	33	10	2	22	23	19
10	33	12	0	15	38	19
total cells	450	49	206	208	351	249

Figure 21: Distribution of identified gene expression clusters in dependency of antigen-reactivity of CD4$^+$ T cells at birth. a) Previously identified gene expression clusters were colored according to CD4$^+$ T cell reactivity against KLH (dark blue), glycated KLH (light blue), insulin (red), glycated insulin (orange), proinsulin (grey) and glycated proinsulin (black) and b) visualized as bar plots for comparison of cluster domination (y axis) among antigen responsiveness of CD4$^+$ T cells (x axis). As a reference, the clusters are also shown as a tSNE plot in the right panel of a).

I next examined the responses to the antigens in relation to parental diabetes status (Figure 22a). Antigen-specific CD4$^+$ T cells of neonates from healthy parents were the dominating cell fraction in previously described cluster 7 (93 cells, 25.2% of total cells per parental T1D group). Antigen-specific responses in neonates from healthy parents were further characterized by cells in cluster 6 (63 cells, 17.1%), 9 (51 cells, 13.8%) and 10 (49 cells, 13.3%). Compared to healthy controls, responding T cell signatures in the analyzed T1D risk groups were characterized by overall increased frequencies of cells in cluster 1 (T1D mother: 86 cells, 21.7%; DM mother and father: 28 cells, 11.7%; T1D father/sibling: 60 cells, 11.8%, all $p<0.0001$) and 3 (T1D mother: 77 cells, 19.4%, $p<0.0001$; DM mother and father: 27 cells, 11.3%, $p=0.0035$; T1D father/sibling:

46 cells, 9.1%, p=0.0118), as well as decreased cell frequencies in the *FOXP3*-dominated cluster 7 (T1D mother: 50 cells, 12.6%, p<0.0001; DM mother and father: 20 cells, 8.3%, p<0.0001; T1D father/sibling: 82 cells, 16.1%, p=0.0011). The antigen-response profile in neonates from mothers with T1D showed the highest frequency of cells in cluster 2 (36 cells, 9.1%) among all groups and was dominated by cells from cluster 1 and 3 which shared patterns of low gene expression *per se*. Responsive cells in children who had both a mother with diabetes and a T1D father were mainly found in cluster 6 (64 cells, 26.7%), while the pro-inflammatory clusters 9 and 10 were absent in these children. Responses in children with a father or sibling with T1D were mainly characterized by cells in cluster 6 (84 cells, 16.5%) and 7 (82 cells, 16.1%), but were also of the pro-inflammatory cluster 9 (45 cells, 8.9%) and 10 (68 cells, 13.4%; Figure 22b) phenotypes. Antigen-specific CD4$^+$ T cells were similar distributed in the previously identified clusters 4 and 5 among all parental T1D groups.



cluster	control	T1D mother	DM mother/father	T1D father/sibling
1	9	86	28	60
2	10	36	7	17
3	17	77	27	46
4	16	34	22	29
5	33	35	31	35
6	63	50	64	84
7	93	50	20	82
8	28	15	41	42
9	51	13	0	45
10	49	0	0	68
total cells	369	396	240	508

Figure 22: Distribution of identified gene expression clusters in dependency of parental T1D status at birth. a) Previously identified gene expression clusters were colored according to parental T1D status of the neonates with healthy parents (grey, 369 cells), with T1D mothers (blue, 396 cells), with GDM mothers and fathers with T1D (orange, 240 cells), and with a T1D father or sibling (red, 508 cells).

b) Bar plots for comparison of cluster domination (y axis) among four different parental T1D groups (x axis). As a reference, the clusters are also shown as a tSNE plot in the right panel of a).

I then compared gene expression of individual genes in proinsulin-responsive $CD4^+$ T cells from neonates of mothers with T1D to those obtained from children with another first degree relative suffering from T1D and to children with healthy parents. Proinsulin-responsive cells from children of mothers with T1D had a decreased expression of *CTLA4* ($p<0.0001$), C-C-chemokine receptors (*CCR3*, $p<0.0001$; *CCR5*, $p=0.0001$; and *CCR6*, $p=0.0008$), *AHR* ($p=0.0008$) and *RORA* ($p=0.0009$) as compared to cells from children with a father or sibling with T1D (Figure 23b); and decreased expression of *AHR* ($p<0.0001$), C-C-chemokine receptors (*CCR3*, $p<0.0001$; *CCR4*, $p=0.0005$; and *CCR5*, $p<0.0001$), *CD40* ($p<0.0001$), *CTLA4* ($p<0.0001$), *ICOS* ($p<0.0001$), *IL22* ($p=0.0001$) and *RORA* ($p=0.0007$) as compared to proinsulin-responsive cells from the control children (Figure 23c).

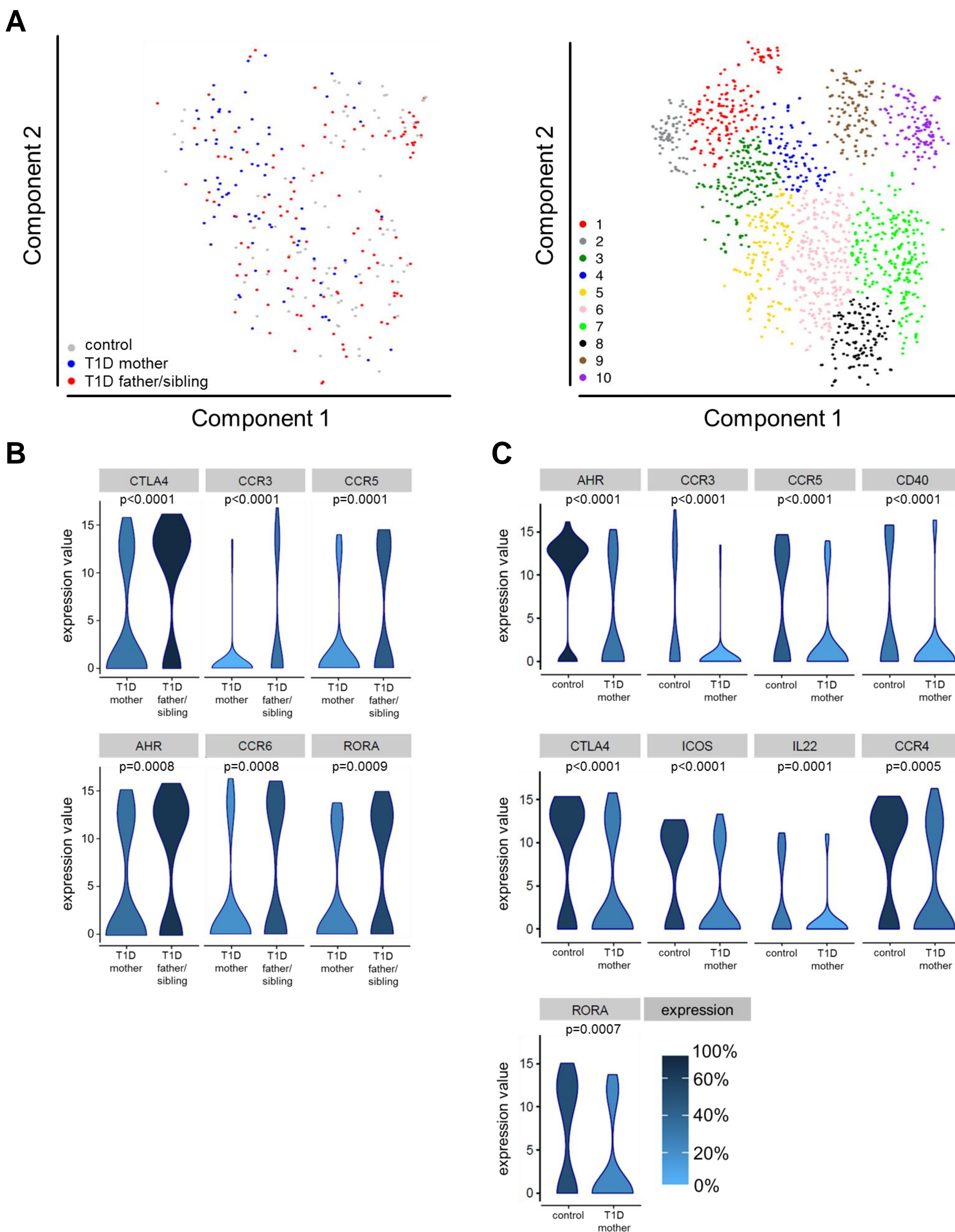


Figure 23: Cluster analysis of gene expression profiles in proinsulin-responsive CD4⁺ T cells at birth. a) 291 single cell sorted proinsulin-responsive CD4⁺ T cells of neonates with healthy parents (90 cells, grey), mothers (81 cells, blue) or fathers/sibling with T1D (120 cells, red; left) were tSNE-visualized

according cluster analysis of all antigen responses at birth (right). Violin plots of C_t values for genes showing significance with p<0.001 (Likelihood-ratio test) in proinsulin-specific $CD4^+$ T cells b) compared between neonates who have a mother and father or sibling with T1D or c) from healthy parents and mothers with T1D. Gene expression ranges from 0% (light blue) to 100% (dark blue).

3.4. Regulatory capacity of $CD4^+CD25^+CD127^{low}$ T cells in neonates of mothers with type 1 diabetes

As another possible related mechanism of protection conferred by maternal T1D during pregnancy, I examined whether there was evidence for increased regulation of proinsulin-responsive $CD4^+$ T cells by T_{reg}. I reasoned that an increased exposure to (pro)insulin in the periphery during fetal and neonatal life could lead to an increase in the number or function of (pro)insulin-specific T_{reg}. To investigate capacity of antigen-specific T_{reg}, I established a co-culture suppression assay using heparin blood samples from an adult control donor and tetanus toxoid as a model antigen (Figure 24). Suppression at a $T_{responder}$:T_{reg} ratio of 2:1 appeared suitable for the antigen-specific assay and was chosen for the proinsulin-specific responses.

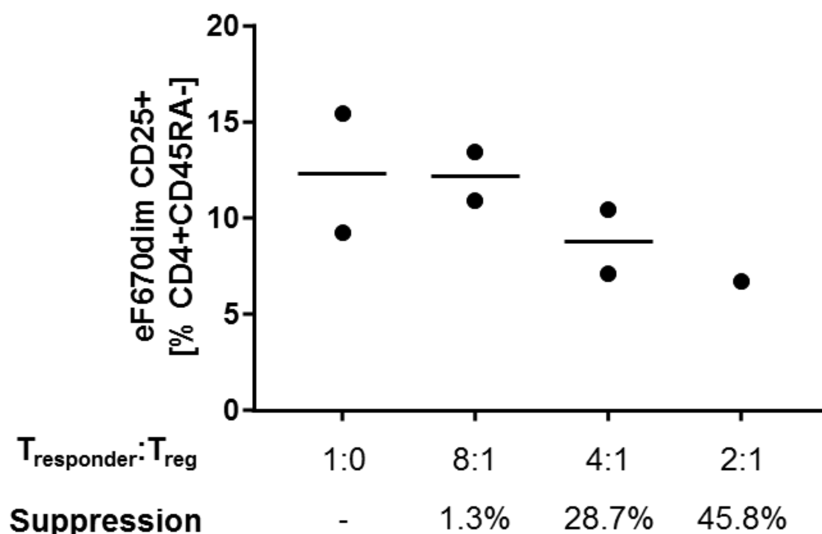


Figure 24: Representative suppression of tetanus toxoid-responsive, memory $CD4^+$ T cells in adult PBMCs. For establishment of $T_{responder}$:T_{reg} co-culture suppression assay in cord blood, responder $CD4^+$ T cells labeled with proliferation dye eFluor670 were incubated with three different ratios of $CD25^+CD127^{low}$ regulatory $CD4^+$ T cells (x axis) in presence of tetanus toxoid. T_{reg}-induced suppression of tetanus toxoid-specific proliferation of memory $CD4^+$ responder T cells (y axis) was calculated for each $T_{responder}$:T_{reg} ratio in cultures containing T_{reg} compared to those without T_{reg}.

I used frozen PBMC samples taken at age of 3 to 9 months from 7 children of mothers with T1D during pregnancy and 6 children who have a father (n=5) or sibling (n=1) with T1D and purified $CD25^+$ cells by MACS. $CD127^{low}CD25^+$ regulatory $CD4^+$ T cells were isolated from CD25-enriched PBMCs and I used the $CD25^+$ T cell depleted $CD4^+$ T cell fraction as responder cells for the proinsulin-responsive suppression assay. I also used $CD8^+$ T cells isolated by MACS as a responder cell population for a measure of general suppression capacity in the samples following stimulation with the polyclonal anti-CD3/CD28 stimulus.

Consistent with my hypothesis, regulatory $CD4^+$ T cells of children of mothers with T1D gestation showed increased regulatory capacity in a proinsulin-specific manner compared to children who have father or sibling with T1D (Figure 25a; $T_{responder}$:T_{reg} ratio 2:1; p=0.0016). In contrast, testing of this $T_{responder}$:T_{reg} ratio in presence of antigen-unspecific, polyclonal stimulus showed no differences between both groups (Figure 25b).

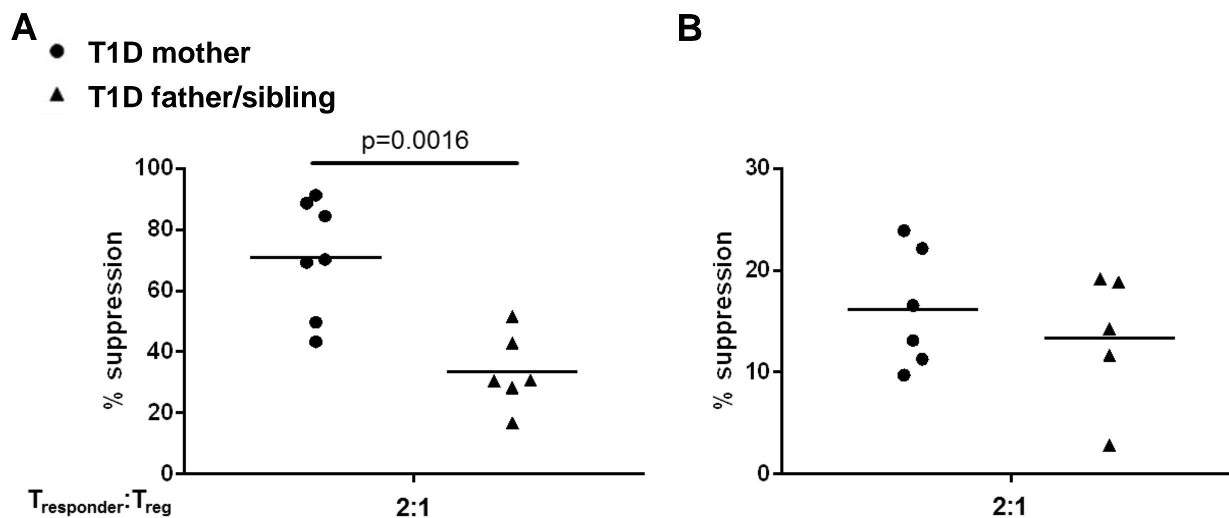


Figure 25: Regulatory capacity of $CD4^+CD25^+CD127^{low}$ T cells in neonates of mothers or fathers with T1D. Co-culture experiments of FACS-purified $CD4^+CD25^+CD127^{low}$ T_{reg} with a) MACS-depleted $CD8^-$ T cells in presence of proinsulin or b) $CD8^+$ cells in presence of anti-CD3/CD28 Dynabeads in neonates at age 3 to 9 months of T1D mothers (circles; n=6-7) or with a fathers or sibling with T1D (triangles; n=5-6). Suppression of proliferating memory $CD4^+$ responder T cells was calculated in cultures containing T_{reg} cells compared to those without T_{reg} (y axis) and for each stimulus, 2:1 $T_{responder}$:T_{reg} ratio was tested. Indicated in each scatter plot is the mean and p-values were obtained using the two-sided, unpaired Student's t test.

3.5. HLA-DR expression as an additional mechanism of regulation of antigen presentation in type 1 diabetes

Since alteration in the expression of markers important for peptide presentation and co-stimulation were shown at birth (Heninger et al., 2013), I investigated impaired function of APCs as a possible cellular mechanism to activate and prime naïve $CD4^+$ T cells at birth. For this purpose, I have analyzed surface expression indicated by median fluorescence intensities of markers important for adhesion (CD11c), co-stimulation (CD83 and CD86) and antigen presentation (HLA-DR) on cord blood-derived $CD14^+$ monocytes. In total 15 cord blood samples of children with healthy parents, 3 cord bloods of children from mothers with GDM, 17 cord bloods of children from mothers with T1D and 15 cord blood samples of children who have a fathers or sibling with T1D have been analyzed. No differences were observed for adhesion (CD11c) or co-stimulatory surface molecules (CD83 and CD86), but $CD14^+$ monocytes obtained from cord bloods of children of mother with T1D showed a tendency of decreased expression of HLA-DR compared to cord bloods of children with a father or sibling with T1D ($p=0.039$; Figure 26a).

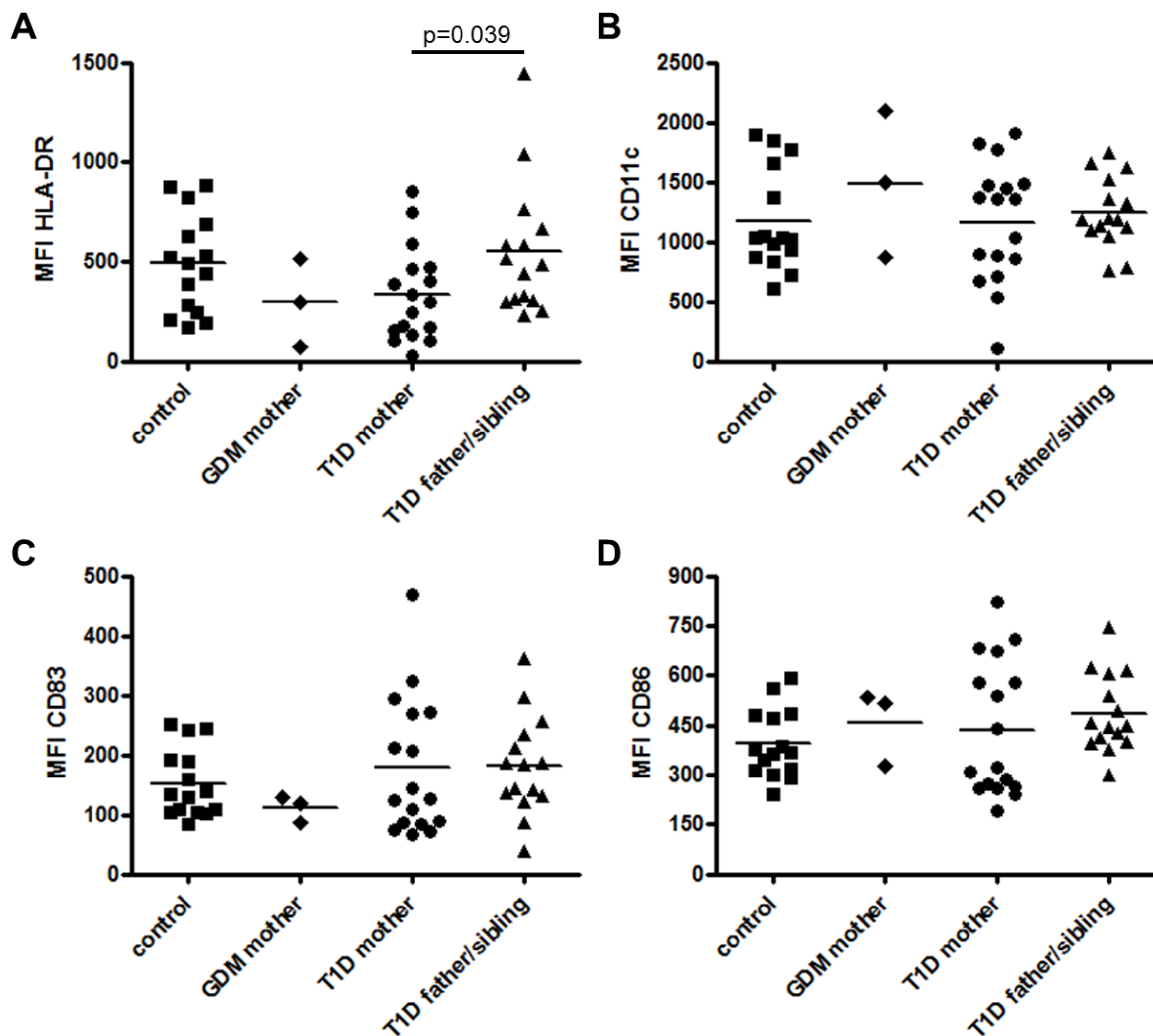


Figure 26: Characterization of cord blood-derived $CD14^+$ monocytes. MFI analysis of a) HLA-DR, b) CD11c c) CD83 and d) CD86 of $CD14^+$ monocytes of children from healthy parents (squares, n=15), mother with GDM (diamonds, n=3), with T1D (circles, n=17) or father/sibling with T1D (triangles, n=15). Indicated in each scatter plot is the mean and p-values were obtained using the two-sided, unpaired Student's t test.

To link altered HLA-DR expression on protein levels to distribution of HLA risk alleles contributing to T1D, 27 of the 50 previously described cord blood samples have been genotyped for their HLA-DR3-DQ2 or HLA-DR4-DQ8 haplotype. Median fluorescence intensity expressed in dependency of haplotypes showed increased HLA-DR surface protein expression on $CD14^+$

monocytes of neonates with a heterozygous HLA-DQ8 risk genotype compared to neonates with a heterozygous HLA-DQ2 genotype (Figure 27; p=0.043).

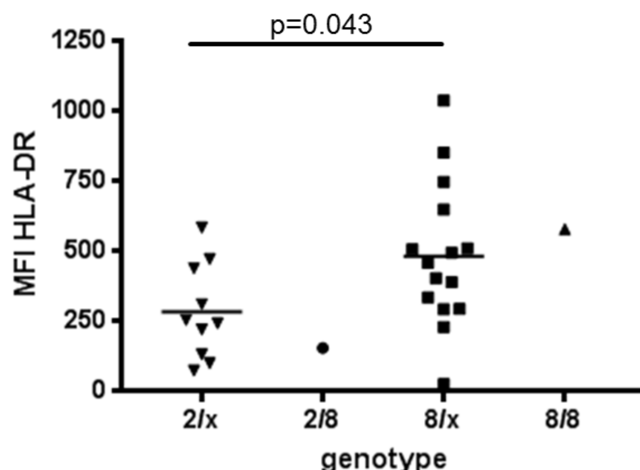


Figure 27: Correlation of HLA-DR surface expression on cord blood-derived CD14$^+$ monocytes and HLA risk alleles at birth. MFI analysis of HLA-DR expression on 27 cord bloods with respective HLA-DQ risk alleles (x axis). Indicated in each scatter plot is the mean and p-values were obtained using the two-sided, unpaired Student's t test.

3.6. Phenotype of antigen-specific CD4$^+$ T cells at onset of type 1 diabetes

I had established assays and expertise in looking at T cell responses and phenotypes in early life. I had observed some interesting gene expression phenotypes of proinsulin-responsive cells from cord blood and, therefore, became interested in testing whether CD4$^+$ T cells responses in patients had any resemblance to these. I therefore progressed to measuring the profiles of CD4$^+$ T cells in patients with T1D and compared them to controls. As some of my genes in cord blood included cytokines and because cytokine production defines T cell phenotypes, I decided to establish multi-dimensional cytokine protein FACS assays for phenotype characterization of antigen-responsive CD4$^+$ T cells.

3.6.1. Phenotyping of peripheral $CD4^+$ T cells at onset of type 1 diabetes

In a first approach, I took advantage of data of my collaborators Dr. Upadhyaya and Denise Kühn of the Bonifacio Lab at the CRTD. This already existing data consist of intracellular cytokine phenotyping of peripheral $CD4^+$ T cells at onset of T1D, which I analyzed using a Boolean combinatory approach. The data provided total peripheral blood $CD4^+$ T cell cytokine profiles. In an antigen-unspecific approach, peripheral $CD4^+$ T cells in patients at onset of T1D and matched control children were short-term stimulated by polyclonal activation with PMA and ionomycin and the frequency of cytokine producing cells were quantified for IL-2, IL-4, IL-17A, IL-21, IL-22, GM-CSF, IFNγ and tumor necrosis factor α (TNFα; Figure 28).

The most abundant cytokine producing cells were $IL\text{-}2^+$ (median, 35.7%; IQR, 32.6% to 40.5%) and $TNF\alpha^+$ cells (median, 19.5%; IQR, 15.5% to 21.8%). GM-CSF production in $CD4^+$ T cells (median, 5.2%; IQR, 3.7% to 6.9%) was also observed and was as abundant as IFNγ production (median, 5.0%; IQR, 3.2% to 6.5%). In contrast, $CD4^+$ T cells producing IL-4 (median, 1.4%; IQR, 1.0% to 2.0%), IL-17A (median, 0.5%; IQR, 0.3% to 0.8%), IL-21 (median, 0.4%; IQR, 0.3% to 0.6%), or IL-22 (median, 0.6%; IQR, 0.4% to 0.8%) were relatively infrequent (Figure 28a). Combinatory Boolean gating was used to identify, that GM-CSF production by $CD4^+$ T cells was usually concomitant with IL-2 and/or TNFα production, but was also seen with other effector cytokines such as IFNγ (Figure 28b). Nevertheless, no differences were observed in single or combinatory cytokine profiles of $CD4^+$ T cells or in cytokine production expressed as mean fluorescence intensity of cytokine positive cells between patients at onset of T1D and age-matched control children (Figure 28c).

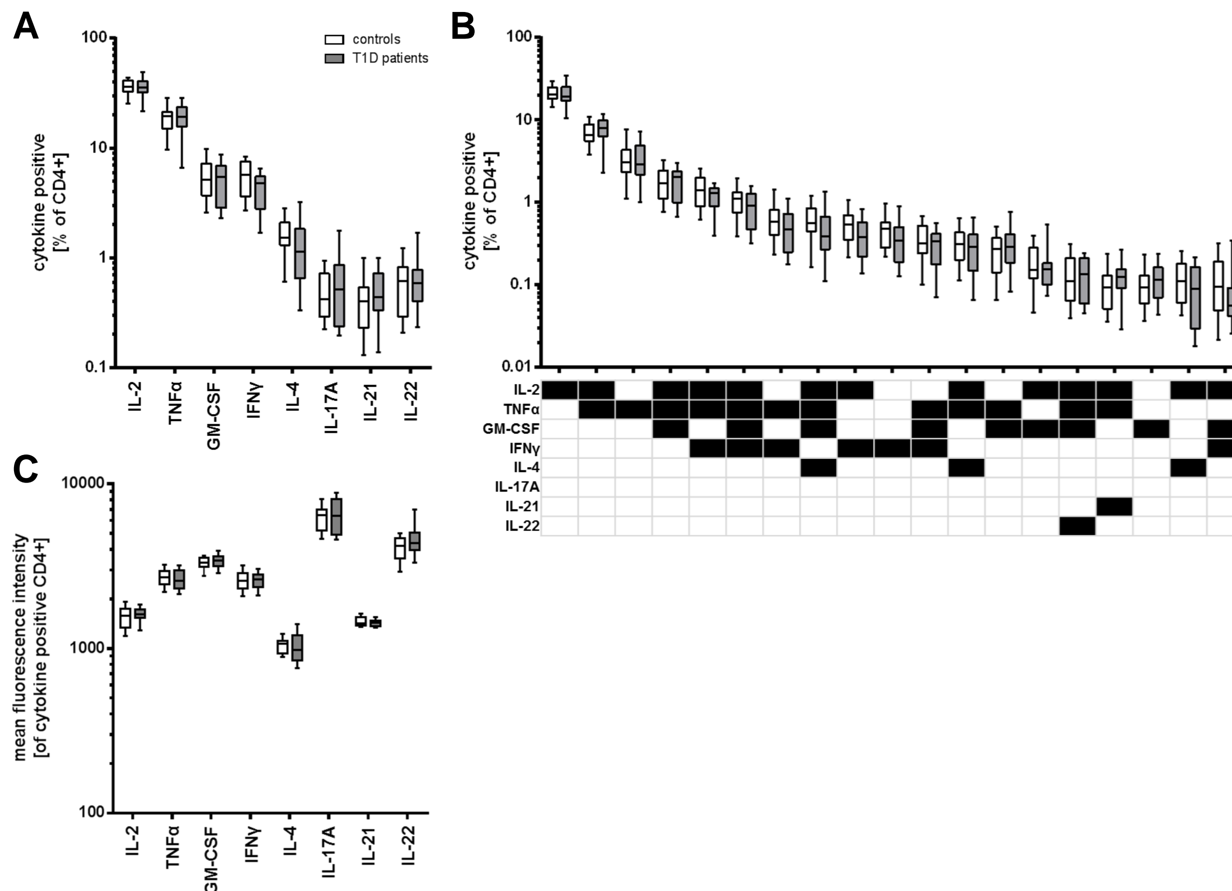


Figure 28: Cytokine profiles of CD4$^+$ T cells. a) Frequency of cytokine producing CD4$^+$ T cells determined by intracellular cytokine staining following short-term stimulation with PMA and ionomycin in islet autoantibody-negative controls (open, n=23) and patients at onset of T1D (shaded; n=18). b) Boolean analysis was used to identify the most dominant cytokine profiles characterized by exclusive or combinatory production of respective cytokines. c) Mean fluorescence intensity was used to reflect expression levels in cytokine positive CD4$^+$ T cells. Indicated in each boxplot are the median and the 10th-90th percentile of data. No differences were observed between groups using the non-parametric Mann-Whitney U test.

Results from a parallel surface staining of the same sample set on CD4$^+$ T cell maturation marker CD45RA showed similar distribution of peripheral CD4$^+$ T cells with a memory phenotype between patients at onset of T1D and matched control children (CD45RA$^-$; Figure 29a). Subsequent expression of previously identified cytokine production of GM-CSF, IFNγ, IL-4, IL-17A, IL-21 and IL-22 as a proportion of the total CD4$^+$CD45RA$^-$ T cell population showed no differences between patients at onset of T1D and matched control children (Figure 29b).

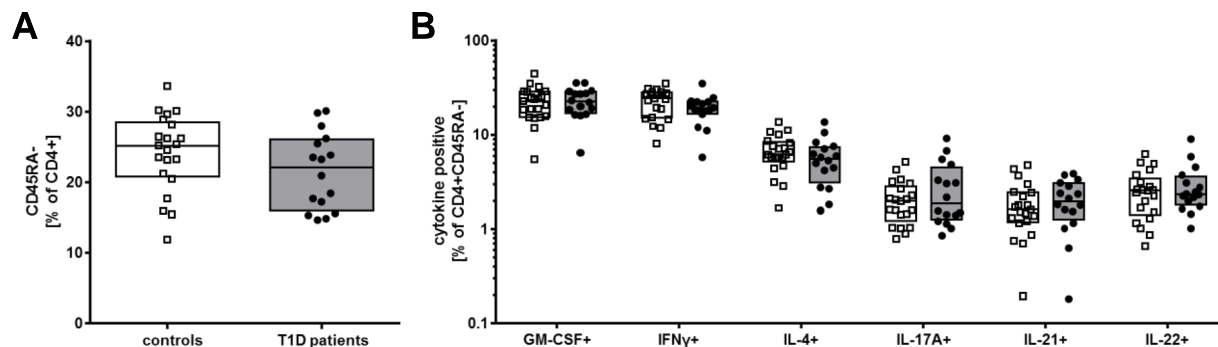


Figure 29: Memory phenotype of peripheral CD4$^+$ T cells in children at onset of T1D. a) Frequency of CD45RA$^-$ cells amongst peripheral CD4$^+$ T cells (y axis) in patients with T1D (T1D patients, circles, n=16) and matched control subjects (controls, open squares, n=21). b) The frequency of cytokine positive CD4$^+$CD45RA$^-$ T cells (y axis) for cytokines GM-CSF, IFNγ, IL-4, IL-17A, IL-21 and IL-22. Indicated in each boxplot are the median and interquartile ranges for the control subjects (open, n=16) and the T1D patients (shaded, n=21). The data of b) were interpolated from the cytokine positive frequencies in total CD4$^+$ T cells shown in Figure 28a and the frequency of CD45RA$^-$ cells within the CD4$^+$ T cells shown in Figure 29a. No differences were observed between groups using the non-parametric Mann-Whitney U test.

3.6.2. Phenotyping of GAD65- and proinsulin-responsive, memory CD4$^+$ T cells on protein levels

I established an antigen-specific, multi-parameter cytokine profiling approach in memory CD4$^+$ T cells. It has been repeatedly demonstrated that CD4$^+$ T cells of T1D patients showed increased proliferation against beta cell autoantigens (Mannering et al., 2004; Tree et al., 2004; Monti et al., 2007). I extended classical, previously described proliferation assays to a detailed description of cytokine production in autoantigen-responsive, memory CD4$^+$ T cells. For this purpose, I defined responsiveness of CD4$^+$CD45RA$^-$ T cells in the presence of proinsulin, GAD65, insulin and tetanus toxoid as a decline in proliferation dye intensity and included simultaneous measurement of 6 inflammatory cytokines in responder T cells. To characterize cytokine signatures, I included IFNγ and IL-10 as effector cytokines as their production by CD4$^+$ T cells has been shown to be impaired in T1D patients (Arif et al., 2004), as well as IL-17A and IL-21 as markers of already described phenotypes of beta cell autoantigen targeting CD4$^+$ Th17 (Honkanen et al., 2010) and circulating follicular helper T cells (Ferreira et al., 2015). Further, I added IL-22 to my flow cytometry panel to confirm already described enhanced expression of *IL22* mRNA in circulating, memory CD4$^+$ T cells in T1D patients on protein level (Honkanen et

al., 2010) and GM-CSF, which production by CD4$^+$ T cells has been linked to both Th1 and Th17 phenotypes and different autoimmune models in mice (Codarri et al., 2011).

Consistent with previous studies, I could show, that patients at onset of T1D had increased proliferative capacity in CD4$^+$CD45RA$^-$ T cells in presence of proinsulin (p=0.0002), GAD65 (p<0.0001) and insulin (p=0.033; Figure 30a). In the presence of tetanus toxoid no differences between both groups were observed in the proliferative response of memory CD4$^+$ T cells (Figure 30b).

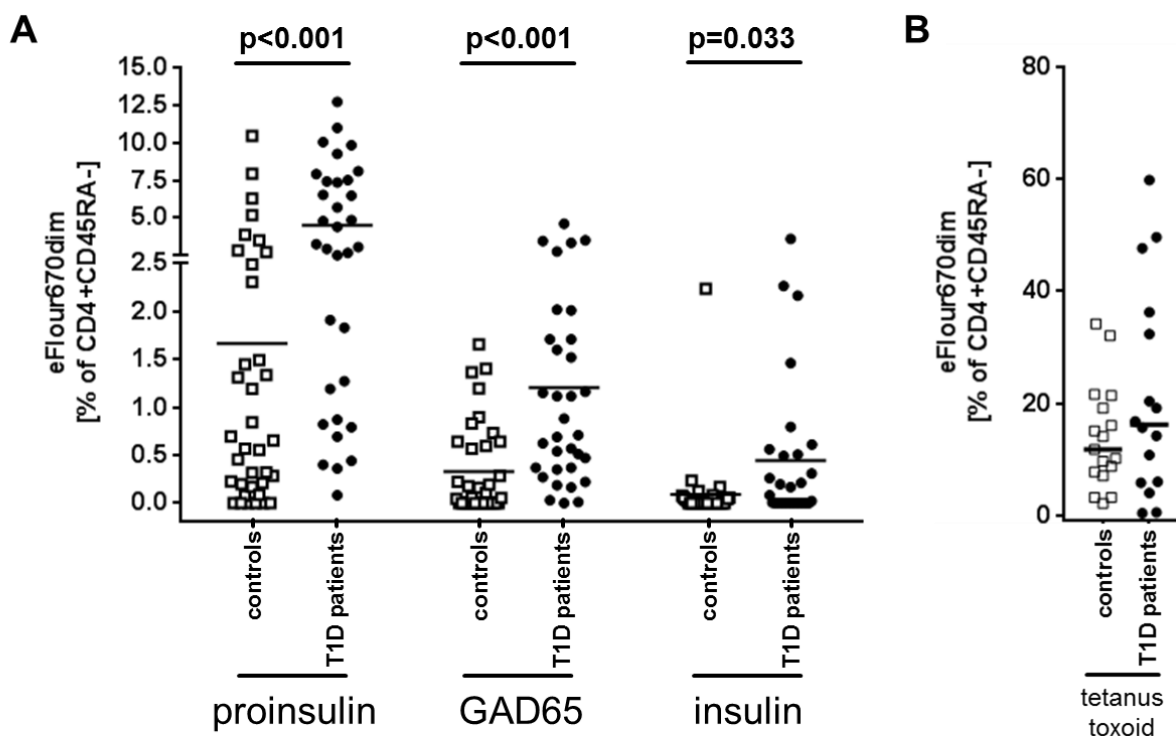


Figure 30: Background-subtracted proliferation of memory CD4$^+$ T cells in patients at onset of T1D. Antigen-responsive CD4$^+$CD45RA$^-$ T cells in islet autoantibody-negative children (open squares, n=36), and patients with T1D (circles, n=33) were identified via flow cytometry as a decrease in proliferation dye eFluor670 intensity. Frequencies of responsive CD4$^+$CD45RA$^-$ T cells in the presence of a) diabetes-related antigens proinsulin, GAD65 and insulin and b) tetanus toxoid are expressed as the proportion of dye-diluted CD4$^+$CD45RA$^-$ T cells within total CD4$^+$CD45RA$^-$ T cells after subtraction of values from medium alone control wells (y axis). Indicated in each scatter plot is the mean and p-values were obtained using the two-sided, unpaired Student's t test.

Following determination of responsiveness as decline in proliferation dye intensity, I could show that the cytokine production in proinsulin-, GAD65-, and tetanus toxoid-responsive $CD4^+CD45RA^-$ T cells of all subjects was dominated by GM-CSF, IFNγ and IL-21 (Figure 31). Production of IL-17A or IL-22 was significantly decreased in presence of all antigens (Wilcoxon matched-pairs signed rank test; $p<0.001$ for each antigen) and IL-10 producing antigen-responsive $CD4^+CD45RA^-$ T cells were almost absent in all samples tested.

Compared to healthy control children, T1D patients had increased frequencies of proinsulin-responsive $CD4^+CD45RA^-$ T cells producing GM-CSF ($p=0.002$), IFNγ ($p=0.004$), IL-17A ($p=0.008$), and IL-22 ($p=0.007$), which remained significant following consideration of multiple testing (n=6 cytokines; Figure 31a). In addition, I observed increased frequencies of proinsulin-responsive $CD4^+CD45RA^-$ T cells producing IL-21 ($p=0.011$) in patients at onset of T1D, which was no longer significant following consideration of multiple testing. In presence of GAD65, patients at onset of T1D showed increased frequencies of IL-21-producing $CD4^+CD45RA^-$ T cells ($p=0.039$; Figure 31b), which was no longer significant after considering multiple testing. Among the cytokines I tested, no differences were observed in cytokine positive, tetanus toxoid-responsive $CD4^+CD45RA^-$ T cells between patients at onset of T1D and control children (Figure 31c).

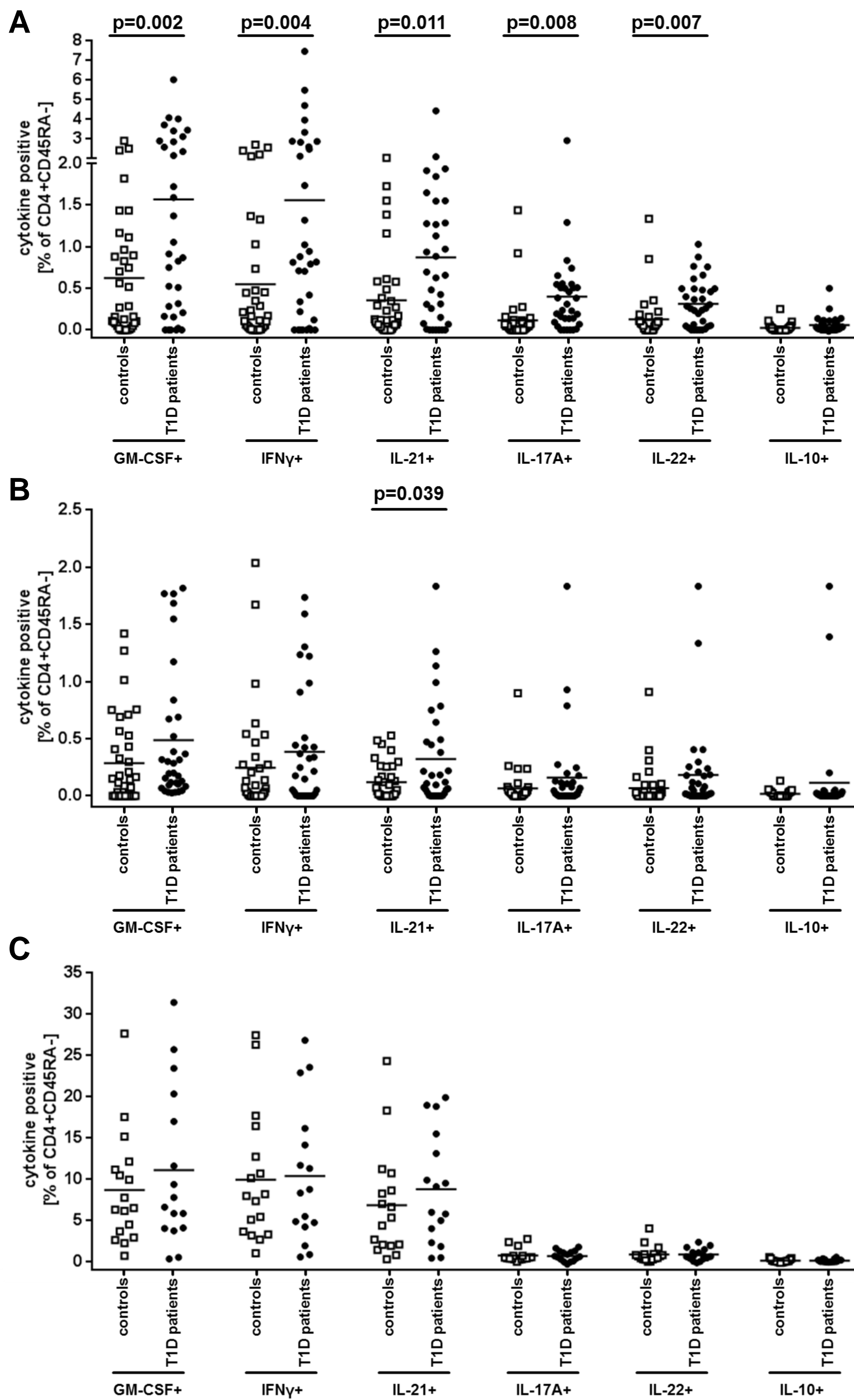

A
p=0.002
p=0.004
p=0.011
p=0.008
p=0.007
cytokine positive
[% of CD4+CD45RA-]
controls
T1D patients
GM-CSF+
IFNγ+
IL-21+
IL-17A+
IL-22+
IL-10+
B
p=0.039
C

Figure 31: Cytokine production in antigen-responsive, memory CD4$^+$ T cells. Flow cytometry identification of cytokine production in CD4$^+$CD45RA$^-$ T cells responsive to a) proinsulin, b) GAD65 and c) tetanus toxoid in islet autoantibody-negative control children (open squares, n=36), and patients with T1D (circles, n=33). Cytokine production in proliferating cells is expressed as the proportion of total CD4$^+$ CD45RA$^-$ T cells, characterized by positivity for the respective cytokine and proliferation dye dilution after subtraction of values from medium alone control wells (y axis). Indicated in each scatter plot is the mean and p-values were obtained using the two-sided, unpaired Student's t test.

Correlation analysis between proinsulin- and GAD65-responsive CD4$^+$CD45RA$^-$ T cells showed only a very weak relationship for GM-CSF (r^2=0.231; p<0.001) and IFNγ producing CD4$^+$CD45RA$^-$ cells (r^2=0.111; p=0.005) in presence of both beta cell autoantigens (Figure 32).

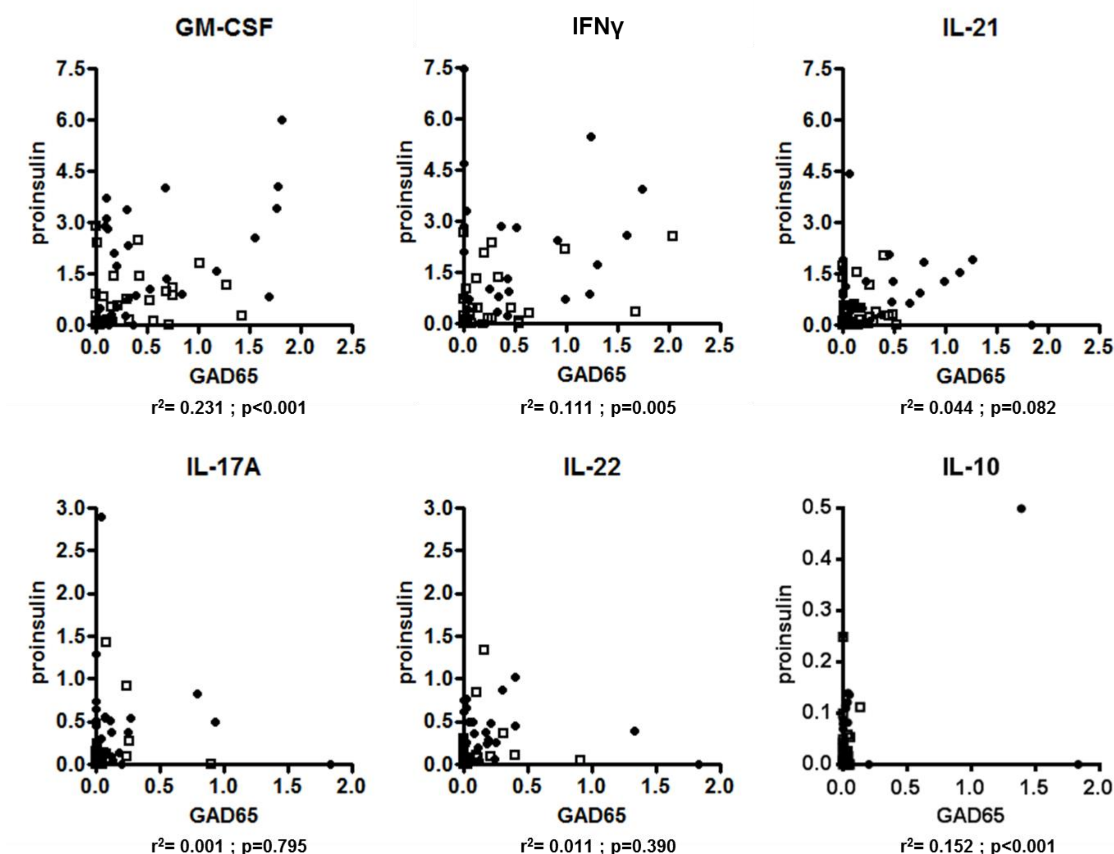


Figure 32: Correlation of cytokine production in proinsulin- and GAD65-responsive CD4$^+$CD45RA$^-$ T cells. Linear regression of cytokine producing, proinsulin- and GAD65-responsive CD4$^+$CD45RA$^-$ T cells in islet autoantibody-negative children (open squares, n=36) and patients with T1D (circles, n=33). Linear regression of all data points for each cytokine tested is indicating by the coefficient of determination (r^2); p-values were calculated from using Pearson correlation and are not corrected for multiple testing (n=6 cytokines).

When cytokine production was expressed as mean fluorescence intensity of proinsulin-, GAD65- or tetanus toxoid-responsive, cytokine positive $CD4^{+}CD45RA^{-}$ T cells, no differences were observed between patients at onset of T1D and matched control children following consideration of multiple testing (n=6 cytokines; Figure 33).

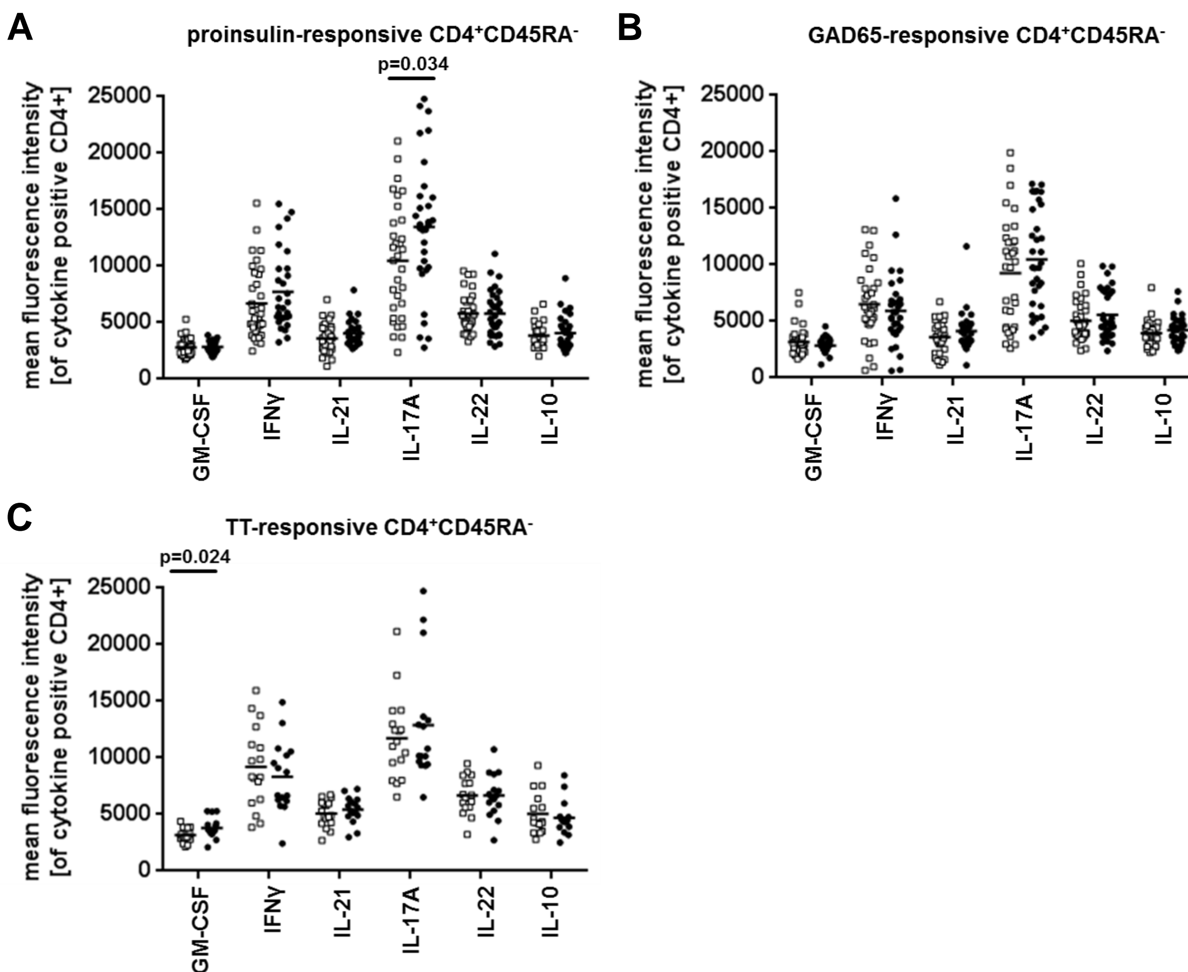


Figure 33: Mean fluorescence intensity of antigen-specific, cytokine positive $CD4^{+}CD45RA^{-}$ T cells. Mean fluorescence intensity of cytokine positive, antigen-responsive $CD4^{+}CD45RA^{-}$ T cells in the presence of a) proinsulin, b) GAD65 and c) tetanus toxoid (TT) in islet autoantibody-negative children (open squares, n=36) and patients with T1D (circles, n=33). Indicated in each scatter plot is the mean and p-values were obtained using the two-sided, unpaired Student's t test. Significant p-values <0.05 were no longer significant following consideration of multiple testing (n=6 cytokines).

Since I observed increased frequencies of GM-CSF, IFNγ, IL-17A, IL-21, and IL-22 secreting $CD4^+CD45RA^-$ T cells to proinsulin in patients at onset of T1D, I used a boolean combinatory approach to examine which multi-parameter cytokine profiles were increased in proinsulin- and GAD65-responsive $CD4^+CD45RA^-$ T cells in T1D patients. For this purpose, a mean response cut off above 0.05% of cytokine positive, antigen-responsive $CD4^+CD45RA^-$ T cells was set, identifying 7 combinations of proinsulin-responsive $CD4^+CD45RA^-$ T cells and 4 cytokine combinations of GAD65-responsive $CD4^+CD45RA^-$ T cells (Figure 34). In the presence of both beta cell autoantigens, response profiles containing GM-CSF$^+$, IFNγ$^+$, and IL-21$^+$ $CD4^+CD45RA^-$ T cells were the most abundant. In response to proinsulin, 4 of the 7 profiles were increased in T1D patients compared to matched control children, and of these, GM-CSF$^+$ only (p=0.006) and IFNγ$^+$ only (p=0.0035) responses remained significant after consideration of multiple testing (n=7). The GM-CSF$^+$ only response was also the most abundant and increased (p=0.037) in patients for GAD65-responsive $CD4^+CD45RA^-$ T cells, but was no longer significant after considering multiple testing (n=4).

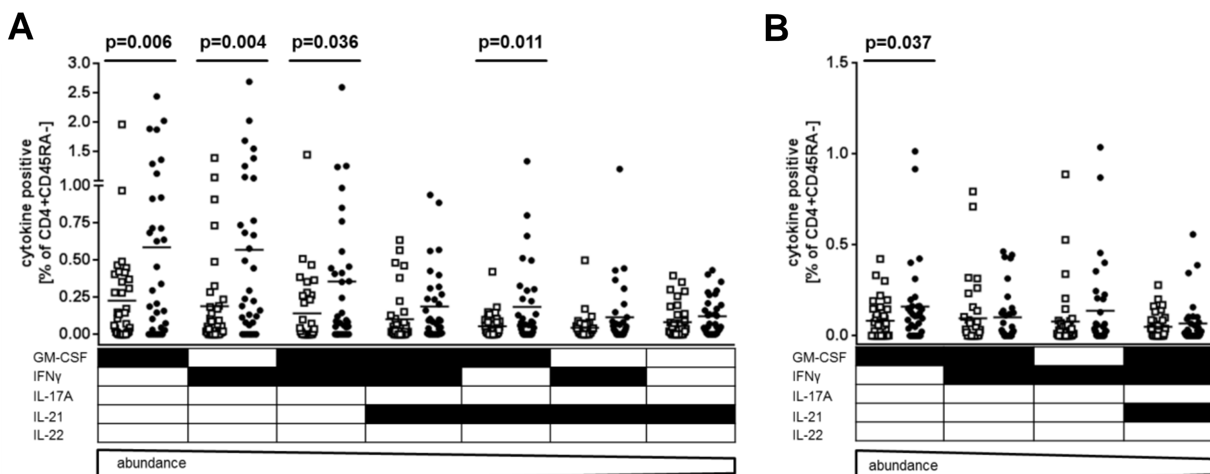


Figure 34: Multi-parameter cytokine profiling in beta cell antigen-responsive $CD4^+CD45RA^-$ T cells. A Boolean combinatory approach was used to identify the most dominant cytokine profiles of a) proinsulin- or b) GAD65-responsive $CD4^+CD45RA^-$ T cells characterized by exclusive or combinatory production of respective cytokines. The frequency of particular cytokine combination in antigen-responsive $CD4^+CD45RA^-$ T cells after subtraction of frequencies in the medium alone control wells is shown on the y axis for islet autoantibody-negative children (open squares, n=36) and patients with T1D (circles, n=33). Abundance order was determined from the median rank of the cytokine profile of responsive cells for each of the samples tested and above a mean response cut off at 0.05% in the patients. The combinations from most abundant (left) to least abundant (right) are shown for proinsulin- (7

combinations) and GAD65-responsive $CD4^{+}CD45RA^{-}$ T cells (4 combinations). Indicated in each scatter plot is the mean and p-values were obtained using two-sided, unpaired Student's t test.

In order to further characterize distribution of the 7 most abundant cytokine signatures in proinsulin-responsive $CD4^{+}CD45RA^{-}$ T cells, a multidimensional cluster analysis was performed of the total 69 samples analyzed (Figure 35). Four potential clusters were identified by tSNE analysis. Cluster 1 (green; n=18 samples) and cluster 2 (red; n=21 samples) contained samples with rare or no cytokine positive responsive cells. Of interest, the majority (27 of 39; 69.2%) of samples in these clusters were from control individuals. In contrast, clusters 3 and 4 were dominated by samples from patients with T1D (21 of 30; 70%; Figure 35b) and featured multiple cytokine profiles in their proinsulin responses. The $IFN\gamma^{+}$ only phenotype was the dominated feature in the patient samples in cluster 3, whereas cluster 4 included $GM\text{-}CSF^{+}$ only and $GM\text{-}CSF^{+}IFN\gamma^{+}$ combinatory phenotypes (Figure 35c).

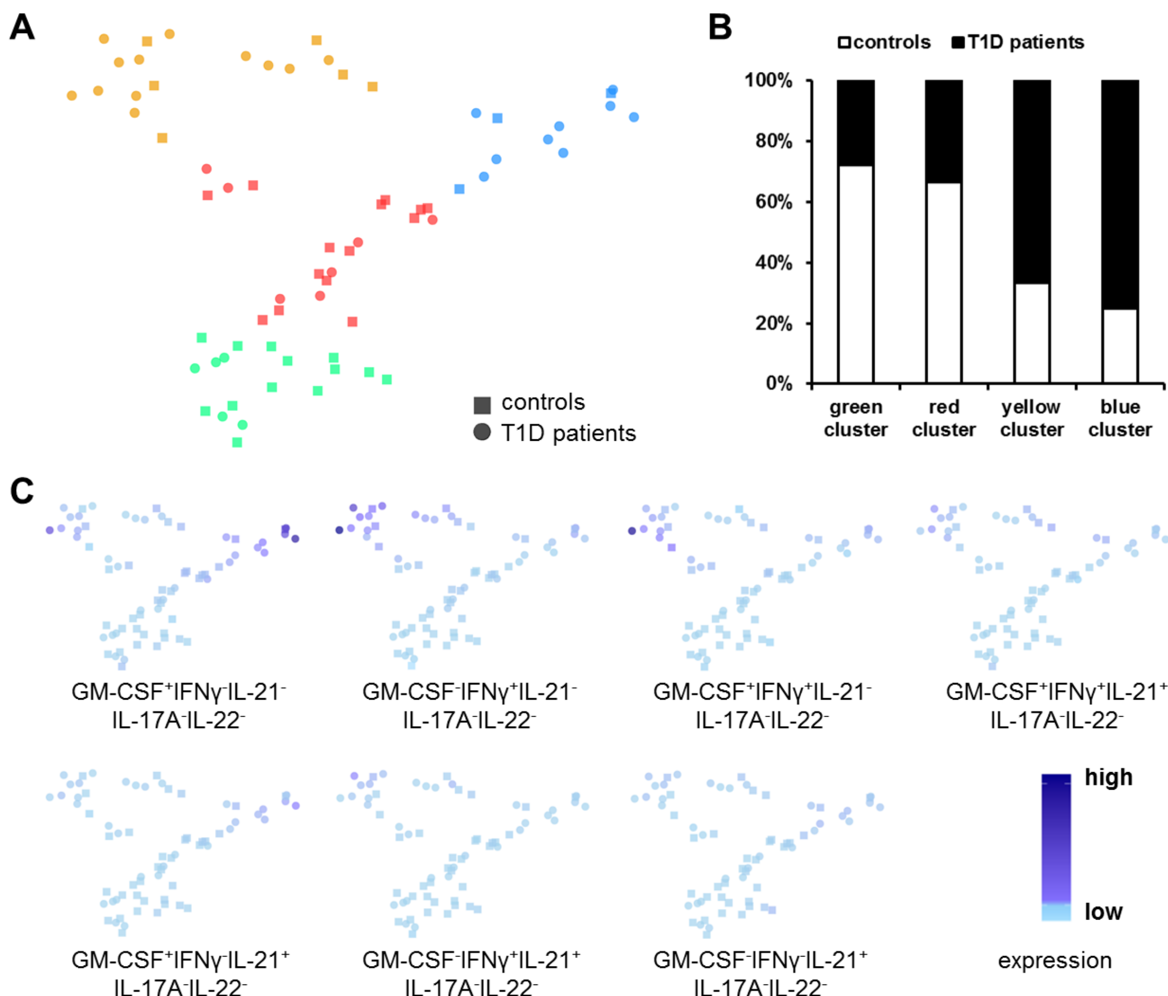


Figure 35: tSNE analysis of proinsulin-responsive $CD4^+CD45RA^-$ T cell cytokine profiles. Previously identified 7 most abundant cytokine signatures in proinsulin-responsive $CD4^+CD45RA^-$ T cells were analyzed using tSNE analysis. a) tSNE visualized four major clusters, defined as clusters 1 (green, 18 samples), cluster 2 (red, 21 samples), cluster 3 (yellow, 18 samples), and cluster 4 (blue, 12 samples) in samples of healthy control children (squares) and patients with T1D (circles). b) Distribution of islet autoantibody-negative control children (green cluster, n=13; red cluster, n=14; yellow cluster, n=6; blue cluster, n=3) and of patients with T1D (green cluster, n=5; red cluster, n=7; yellow cluster, n=12; blue cluster, n=9) among different clusters. c) Expression intensities (light blue, lowest; dark blue, highest frequency) of each of the 7 most abundant cytokine signatures observed in proinsulin-responsive, memory $CD4^+$ T cells.

3.6.3. Phenotyping of proinsulin-responsive CD4⁺ T cells on gene expression levels

In order to validate previously identified cytokine signatures of CD4⁺CD45RA⁻ T cells in presence of proinsulin on protein levels, I isolated proinsulin-responsive CD4⁺CD45RA⁻ T cells (n=104) from three different patients at onset of T1D for single cell gene expression analysis (Figure 36). Of these 104 isolated cells, 19 cells (18% of total) showed *GMCSF* expression, of which 10 cells co-expressed *IFNG* (n=9) or *IL21* (n=6; Figure 36a). Compared to residual cells without *GMCSF* expression (n=85), *GMCSF* positive cells were characterized by lower expression of *FOXP3* (p<0.0001), *CD27* (p=0.0061), *CD25* (p=0.0076), *CCR7* (p=0.011), and *CD137* (p=0.016), and increased expression of *CD154* (p=0.020; Figure 36b).

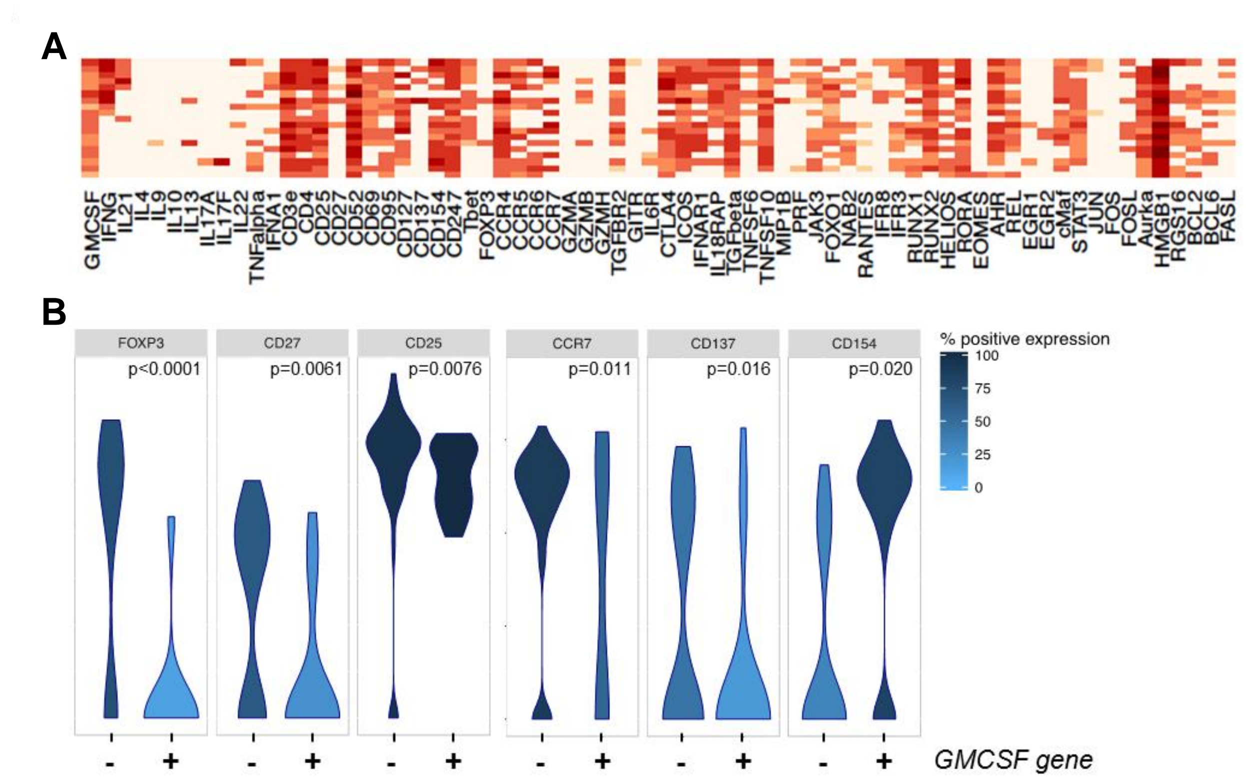


Figure 36: Gene transcription profiles of proinsulin-responsive CD4⁺CD45RA⁻ T cells. Multi-parameter gene expression in single cell sorted proinsulin-responsive CD4⁺CD45RA⁻ T cells from three patients with T1D. a) Of 104 processed cells in total, *GMCSF* gene expression was observed within 19 cells and co-expression of all genes tested is shown as a heatmap. b) Violin plots representing the distribution of C_t expression values for each significant gene (p<0.0005 via Likelihood-ratio test) in cells with (+; n=19) or without (-; n=85) *GMCSF* gene expression. The proportion of cells expressing indicated genes ranged from light blue (0%) to dark blue (100%).

4. Discussion

T1D is a T cell-mediated autoimmune disease resulting in the destruction of insulin-producing beta cells in the pancreas. The process starts early in life and understanding the events that modify T1D risk will be helpful in developing early intervention strategies (Puff et al., 2016; Ziegler et al., 2016). Of particular interest, offspring of mothers with T1D have about half the risk to develop islet autoantibodies compared to children who have a father or sibling with T1D (Bonifacio et al., 2008). In line with these observations, transplantation of pre-implantation stage mouse embryos of non-obese diabetic (NOD) mice into recipients without diabetic genetic predisposition and NOD mice showed a significant delay in insulitis in offspring of NOD mice (Kagohashi et al., 2005). I proposed that relative protection against T1D by maternal T1D during gestation is due to hyperglycemic conditions *in utero*. Elevated glucose levels induce increased production of (pro)insulin by the fetus, and this could favor central tolerance to these key autoantigens of childhood T1D. In my thesis, I designed and developed state of the art *in vitro* assays to investigate this possibility as a potential cellular mechanism contributing to the protection against islet autoimmunity by a maternal T1D environment during gestation.

A major part of my thesis was focused on determining the responsiveness of $CD4^+$ T cells to beta cell autoantigens and to assess the phenotype of this response. I had to develop novel methods that were able to measure these cells, which are at low frequency and have a naïve $CD4^+$ T cell phenotype in cord blood. I introduced multiple proliferation dyes and multiple replicates so that I could be confident of the quantification of the responses I saw. I took advantage of the recently started ImmunDiabRisk study. With this study, I gained access to cord blood samples of neonates who have a first degree relative with T1D and of children from healthy parents. This enabled me to compare features of cord blood in children who had a mother with T1D, a father or sibling with T1D and children born to non-diabetic parents. I helped process almost 100 cord bloods. The IDR study was initiated at the beginning of my thesis and, therefore, no long-term follow up samples were available to correlate potential protective effects by maternal T1D during pregnancy to later autoimmune outcome and clinical disease manifestation. However, short-term follow up PBMC samples of children at the age of 3 to 9 months were available as an additional feature of the study.

My major finding was that children who had a father or sibling with T1D, but <u>*not*</u> children of mothers with T1D had increased frequencies of proinsulin- and insulin-responsive, naïve $CD4^+$

T cells at birth as compared to children without a sibling or parent with T1D. This is the first demonstration that there are increased frequencies of autoreactive T cells in children with a father or sibling with T1D. They are in line with a previous finding indicating that children with high risk HLA class II genotypes have proinsulin- and GAD-responsive $CD4^+$ T cells at birth (Heninger et al., 2013). Importantly for my hypothesis of how maternal T1D may protect the offspring against T1D, the frequency of these proinsulin-responsive cells was also significantly less in children of mothers with T1D than in children with a father or sibling with T1D. Precursor frequencies of KLH-specific $CD4^+$ T cells were not significantly affected by parental T1D, suggesting, that there is no *per se* immunomodulatory effect during maternal T1D gestation. These findings indicate that the maternal T1D environment during gestation directly reduces or prevents the acquisition of proinsulin- and insulin-responsive, naïve $CD4^+$ T cells. This fully supports my hypothesis that a maternal T1D environment is more efficient in deleting or regulating (pro)insulin-reactive $CD4^+$ T cells. I predict that is due to increased exposure of the fetus to (pro)insulin during gestation and of the infant in the first months of life. Increased serum concentrations of the prohormone and hormone are present in both periods, caused by the hyperglycemia in the mother (Stumpp et al., 2014). Sosenko and colleagues demonstrated that varied glucose concentrations led to an increased beta cell stimulus in neonates at birth (Sosenko et al., 1979). The resulting glucose-stimulated insulin secretion by the beta cells increases during gestation and is even enhanced by pulsatile hyperglycemia (Hay, 2006). It is of potential interest to determine whether hyperglycemia will also increase the expression of insulin in the thymus since an impaired expression of insulin in the thymus is one mechanism for increased susceptibility for T1D conferred by the *INS* gene (Vafiadis et al., 1997).

A particular strength of my findings was that I used an assay with up to 30 replicates. I reasoned that the precursor frequencies of the naïve, autoreactive T cells would be low in cord blood and that the robustness of my findings would depend upon consistency of the responses I saw. I had to generate dendritic cells since it was previously shown that cord blood monocytes were poor stimulators of naïve T cells (Heninger et al., 2013). I also included low dose IL-7 to maintain viability of the naïve cord blood $CD4^+$ T cells, and removed T_{reg} to avoid confounding effects of regulation. With 30 replicates of each condition, I was able to identify responders to antigen using the two-sided, unpaired Student's t test vs. no antigen controls. Thus, I was able to show that there were indeed significant responses to proinsulin and insulin in naïve cord blood $CD4^+$ T cells and also in a minority of control children and in children of mothers with T1D. These were clearly less frequent and less pronounced than in children who had a father or sibling with

T1D. The findings are consistent with the notion that negative selection in the thymus only partially deletes autoreactive T cells, resulting in detection of autoreactive $CD4^+$ T cells in periphery of healthy individuals (Walker and Abbas, 2002). My finding was also important as it allowed me to compare the profile of the responsive cells in the three settings.

I used single cell gene expression as the state of the art methodology to assess cell-specific phenotypes on an already established set of 48 genes by our collaborator Dr. Anne Eugster. Multi-parameter tSNE cluster analysis was applied on comprehensive data of 1513 sorted single cells in order to identify different T cell subpopulations with specific gene expression patterns (Eugster et al., 2013; Buettner et al., 2015). With this approach, I identified ten major clusters of cord blood-derived antigen-responsive, naïve $CD4^+$ T cells, which showed unique signatures such as increased C-C chemokine receptor expression (cluster 2 and 7), as well as regulatory (cluster 6, 7 and 8) or pro-inflammatory phenotypes (cluster 9 and 10). The majority of identified clusters were equally distributed in the presence of the different antigens used in the assay. Cells in regulatory clusters 6 and 7 were elevated in the responses to diabetogenic antigens insulin and proinsulin, but no other differences were observed between antigens, suggesting only minor evidence for diabetes antigen-specific phenotypes. Responsive $CD4^+$ T cells in children of mothers with T1D were generally less inflammatory. They lacked the strong pro-inflammatory phenotype of clusters 9 and 10. These pro-inflammatory profiles were unusual and included the expression of *CD40*, *TBET*, *IL22*, *INFG*, *IL9* and *CXCR5*. CD40-expressing $CD4^+$ T cells are potentially of interest since CD40 has been suggested to be a marker for autoreactive $CD4^+$ T cells in animal models of autoimmunity including autoimmune diabetes (Baker et al., 2008). IL-9 is associated with gut inflammatory diseases (Hufford and Kaplan, 2014), IL-22 is often co-expressed in Th17 cells, and TBET and IFNγ are hallmarks of Th1 cells. CXCR5 is expressed on T_{fh} and a subset of central memory T cells (Chevalier et al., 2011). These pro-inflammatory responses were only observed in antigen-responsive $CD4^+$ T cells from children with a father, sibling or no first degree relative with T1D. However, they were found in a minority of these children and in both KLH and proinsulin-responsive $CD4^+$ T cells.

The most striking difference between the responsive $CD4^+$ T cell profiles was a decreased expression of genes belonging to the C-C-chemokine receptor group in the children of mothers with T1D. C-C chemokines are characterized by chemoattractant properties to cells expressing CCRs and induce intracellular signaling upon receptor binding resulting in cell differentiation and proliferation. They are important for T cell homing to various target tissues such as lymph nodes, gut and skin. Of particular interest, various studies have linked chemokines to

autoimmune diseases such as rheumatoid arthritis and chronic obstructive pulmonary disease (reviewed in Raman et al., 2011) as well as autoimmune diabetes in mice and humans (Atkinson and Wilson, 2002). In the NOD mouse, it was shown that CCR5 expression in the pancreas was associated with an accelerated development of insulitis (Cameron et al., 2000). Lee and colleagues showed that resveratrol treatment inhibited CCR6-mediated migration of inflammatory Th17 cells and macrophages to pancreatic islets and prevented disease onset in NOD mice (Lee et al., 2011). However, human studies are still limited to the analysis of CCR expression of peripheral lymphocytes outside pancreatic sites of inflammation. Another feature of proinsulin-responsive $CD4^+$ T cells from children with T1D mothers was decreased expression of CTLA4. CTLA4 functions as a negative regulator of T cell response and is highly expressed in T_{reg} and fully activated $CD4^+$ T cells. Thus, it is tempting to conclude that the presentation of autoantigen to cord blood $CD4^+$ T cells from children who have a mother with T1D results in a self-limiting response with little upregulation of chemokine receptors, little expression of pro-inflammatory cytokines, and low expression of molecules that are normally required to down-regulate the response.

A second thesis that I proposed was that the increased concentrations of glucose seen by the fetus may result in glycation of proinsulin or insulin and that the glycated forms may be less immunogenic. I was unable to verify if there was more glycated hormone in children of mothers with T1D. I expected that hyperglycemia will be transient and that glycation is unlikely to be extensive. Nevertheless, I was able to look at responses to glycated forms of KLH, proinsulin and insulin. I saw no striking differences in the magnitude of the cord blood $CD4^+$ T cell response to the glycated and non-glycated forms and only minor differences in the gene expression profiles of the $CD4^+$ T cells responding to glycated as compared to non-glycated antigen. Thus, I conclude that glycation of proinsulin or insulin is unlikely to be relevant to the T1D protection to offspring conferred by maternal T1D.

My third thesis was that maternal T1D led to increased regulation of (pro)insulin-specific $CD4^+$ T cells. I was able to test this in a small number of children from whom I had follow up samples obtained in the first year of life. Co-cultures of regulatory and responder T cells are a commonly used *in vitro* tool to assess the suppressive capacity of antigen-specific T_{reg} (Collison and Vignali, 2011). I was able to show that the suppression of $CD4^+$ T cell responses to proinsulin was greater when $CD4^+$ effector T cells and T_{reg} were derived from children with a mother with T1D than when they were from children with another T1D first degree relative. The T_{reg} in each case were equally able to suppress polyclonally stimulated $CD8^+$ T cells suggesting that there

was not a general enhancement of T_{reg} function in children of mothers with T1D. The findings suggest that either there are more proinsulin-specific T_{reg} in the children of mothers with T1D than in children with another T1D first degree relative or that the responder $CD4^+$ T cells from children of mothers with T1D are more susceptible to regulation. I was unable to design an experiment that would distinguish these two possibilities since it was not possible to keep just the effector $CD4^+$ T cells or just the T_{reg} cells constant in the assay. A debate as to whether T_{reg} cells are defective or the effector T cells are resistant to regulation in T1D remains unresolved (Tree et al., 2006; Schneider et al., 2008; Buckner, 2010; Hull et al., 2017). My findings suggest that maternal T1D may provide some correction of potential defects in the offspring.

In addition to examining the $CD4^+$ T cells in offspring of mothers with T1D, I was able to generally quantify cord blood populations in relation to maternal T1D. I phenotyped all received cord blood samples by establishing standardized routine protocols for flow cytometry. Strikingly, the birth immune phenotype in children of mothers with type 1 or gestational diabetes showed increased frequencies of $CD34^+$ HSCs compared to cord blood compositions in neonates with another first degree T1D relative or without T1D family history. I also observed a decrease in the frequency of $CD14^+$ monocytes and in the surface expression of markers important for peptide presentation in cord blood monocytes from children of T1D mothers compared to those in children who have a father or sibling with T1D. The increased HSCs in the cord blood may be a direct result of exposure to the hyperglycemic conditions, which could increase stem cell turnover in these children. Supporting this hypothesis, Harris and colleagues showed that increased glucose concentration accelerated HSC hematopoiesis and enhanced cellular glucose uptake and metabolism, leading to increased HSC frequencies in the zebrafish (Harris et al., 2013). Beside the effect on HSC quantity in zebrafish, elevations in glucose levels enhanced the expression of *runx1*, which regulates the differentiation of HSCs into mature blood cells. Additionally, it has been shown in mice and humans, that glucose utilization affects nucleotide biosynthesis as well as lineage and erythroid commitment in HSCs (Oburoglu et al., 2014). To identify possible mechanisms induced by altered stem cell turnover in a maternal T1D environment during pregnancy, additional studies will be performed beyond my thesis. For this purpose, transcriptome analysis of isolated $CD34^+$ HSCs will be applied in already selected cord bloods of 5 children from mothers with T1D and 5 children of T1D fathers with our cooperation partner Dr. Anne Eugster at the CRTD. For extensive characterization of HSCs on gene and protein levels, my already existing phenotyping panel, including the $CD34^+$ HSC marker, will be extended to additional surface markers with respect to maturation status, lineage commitment

and activation (Majeti et al., 2007). Results will be compared to read-outs of $CD34^+$ HSCs from healthy donors, which will be *in vitro* challenged by different glucose concentrations or by serum samples from mothers with or without T1D.

To my knowledge, no data has been published referring to monocyte frequencies in human cord blood samples in relation to glucose concentrations. However, it has been shown, that increased *in vitro* glucose concentrations drive human THP-1 monocytes to elevated expression of Toll-like receptors (Dasu et al., 2008) and TNFα (Chacon et al., 2007), which are crucial for shaping inflammatory immune responses, as well as increased cyclooxygenase-2 enzyme expression, which has been implicated in the pathogenesis of several inflammatory diseases (Shanmugam et al., 2004). To address the question if hyperglycemic condition during maternal T1D environment induces altered gene expression in $CD14^+$ monocytes at birth, I will additionally include this immune cell subset to the planned transcriptome analysis of HSCs.

In addition to alterations in frequencies of $CD14^+$ monocytes at birth, I briefly examined the effect of maternal T1D environment on antigen presentation. Here, I showed that monocytes of children from mothers with T1D showed decreased protein surface levels of HLA-DR compared to children with another T1D first degree relative. In addition, I observed that HLA-DR levels were specifically elevated in children with heterozygous HLA-DQ8 risk genotypes for T1D. I applied median fluorescence intensity analysis as a commonly used tool in flow cytometry to characterize expression levels of intracellular and surface markers (Lekkou et al., 2004). I further examined HLA-DR expression at later age and in relation to the DR3-DQ2 and DR4-DQ8 genotype. I was able to show differential expression of HLA-DR on monocytes and dendritic cells in relation to HLA genotype and related these differences to DNA methylation (Kindt et al., 2017). These differences could potentially explain the variation in T1D risk associated with the DR3-DQ2/DR4-DQ8 and the DR4-DQ8/DR4-DQ8 genotypes (reviewed in Redondo et al., 2017). Positive correlation of increased HLA-DR, but not HLA-DQ expression to high risk genotypes at the MHC class II region has been observed in other autoimmune diseases such as autoimmune vitiligo (Cavalli et al., 2016). We postulated that the expression may affect the ability to delete autoreactive T cells, a hypothesis that is central to my thesis. It would be important to determine whether the expression differences are also present in activated APCs and in the thymus and whether hyperglycemia can affect the expression.

Since I had established a multi-parameter *in vitro* assay to in-depth describe characteristics of diabetogenic $CD4^+$ T cells, I applied this to patients at the onset of T1D. I modified the assay by

including the measurement of intracellular cytokines at the end of culture. In addition to previous studies (Mannering et al., 2004; Mannering et al., 2010), I combined proliferation dye based *in vitro* assays for detection of antigen-specific $CD4^+$ T cells and multi-parameter cytokine measurement to in-depth characterize the functionality of pathogenic Th subsets in T1D. In my combinatory *in vitro* approach, I used CD45RA as an exclusion marker for naïve $CD4^+$ T cells during my read-out in order to focus on cytokine production in antigen-experienced $CD4^+CD45RA^-$ T cell population. This provided me with the novel feature of measuring both responsiveness (proliferation and activation) and cytokine profiles of the responsive, memory $CD4^+$ T cells. With this assay, I showed that patients at onset of T1D had increased frequencies of memory $CD4^+$ T cell responses to (pro)insulin and GAD65 as compared to matched control children. Proinsulin- and GAD65-responsive $CD4^+CD45RA^-$ T cell cytokine profiles were dominated by the production of GM-CSF, IFNγ, and/or IL-21 and to a much lesser extent, IL-17A and IL-22. In T1D patients, both proinsulin- and GAD65-specific $CD4^+$ T cells consistently showed increased production of GM-CSF and a pro-inflammatory GM-$CSF^+IFN\gamma^-IL17A^-IL$-$21^-IL$-$22^-$ phenotype. One possible caveat of my findings is that the observed increased responsiveness against proinsulin and insulin observed in patients were partly induced by insulin injection as a crucial step at onset of T1D in the analyzed patients. A considerable proportion of the cells responding to our antigens of interest were negative for the cytokines I tested. With respect to cytokine detection of peripheral total $CD4^+$ T cells following polyclonal stimulation, it is possible, that a large proportion of antigen-specific $CD4^+$ T cells as well mainly produce IL-2 and TNFα, and these cytokines should be included in future panels. In addition, future panels should be extended to cytokines important for immune regulation during insulitis such as TGFβ (Tonkin and Haskins, 2009) and cell surface chemokine receptors belonging to CC- and CXC-receptor classes, as well as subset-specific master regulators belonging to the signal transducer and activator of transcription (STAT) class, which are commonly used markers to identify different T helper cell subsets (reviewed in Mackay, 2000; Vahedi et al., 2012).

Previous studies reported a pro-inflammatory Th1/Th17 adaptive T cell response in T1D characterized by increased levels of IFNγ, IL-21, IL-17A and IL-22 (Arif et al., 2004; Honkanen et al., 2010; Kenefeck et al., 2015; Walker and von Herrath, 2016). With my approach, I could show that antigen-specific $CD4^+$ T cells represent a cellular source of these T1D-relevant effector cytokines. In addition to previously described effector cytokines, I introduced GM-CSF as a novel candidate to shape the inflammatory environment in T1D. GM-CSF producing $CD4^+$ T cells have been recently described in other autoimmune diseases and were associated to

both Th1 and Th17 phenotypes (Codarri et al., 2011; Sheng et al., 2015; Shiomi et al., 2016). In synovial fluid $CD4^+$ T cells have been identified as a cellular source of GM-CSF to accelerate the differentiation of inflammatory dendritic cells in patients with rheumatoid arthritis (Rasouli et al., 2015). Ponomarev and colleagues showed in a mouse model of multiples sclerosis (MS), that GM-CSF produced by autoreactive $CD4^+$ T cells is crucial for the development of autoimmune inflammation in the central nervous system at onset of experimental autoimmune encephalomyelitis (Ponomarev et al., 2007). In line with these findings, GM-CSF produced by $CD4^+$ T cells could be suppressed by IFNβ therapy in MS patients (Rasouli et al., 2015). In mouse models it has been shown, that GM-CSF production as well as terminal differentiation of Th17 cells into mature effector cells is promoted by IL-23 (Codarri et al., 2011) and potentially by IL-1β (El-Behi et al., 2011). In contrast, IL-23 appears to be inhibitory for GM-CSF production in human T cells, while IL-12 may enhance production (Noster et al., 2014). STAT5 has been identified as a major transcription regulator in naïve $CD4^+$ T cells to differentiate into GM-CSF producing, effector T cells (Noster et al., 2014). Of particular interest, STAT5 has been repeatedly described to play an important role in IL-2 and IL-7 signaling, which are both reported to be relevant to autoimmune diabetes in the mouse (Grinberg-Bleyer et al., 2010; Penaranda et al., 2012) and humans (Hulme et al., 2012; Evangelou et al., 2014). My novel finding of a pro-inflammatory and non-regulatory phenotype of proinsulin-responsive, GM-CSF-producing $CD4^+$ T cells supports a role of GM-CSF in T1D. Arif and colleagues used antigen-specific ELISPOT *in vitro* assays to identify a bias towards antigen-specific Th1 phenotypes in T1D patients compared to a regulatory T cell phenotype in healthy controls (Arif et al., 2004). By comparing both *in vitro* assays, I could as well detect increased frequencies of IFNγ-secreting T cells in the presence of proinsulin, but due very low detection range, I was not able to confirm increased IL-10 production by $CD4^+$ T cells in healthy controls. Previous studies reported increased frequencies of IL-21-producing, peripheral $CD4^+CD45RA^-$ T cells following short-term polyclonal stimulation as well as increased frequencies of IL-17 production in Th17 cells by introducing CCR6 as an additional surface marker (Ferreira et al., 2015). Although, increased production of IL-21 as well as co-production of IL-17 and IFNγ by polyclonally stimulated $CD4^+$ T cells has been confirmed by other groups (Honkanen et al., 2010; Kenefeck et al., 2015), I could not reproduce these data. Nevertheless, my observation of an increase in $IL\text{-}21^+$ and $IL\text{-}17^+$ autoantigen-responsive $CD4^+$ T cells in patients, supports a role these cytokines in the disease.

In my thesis, I combined and refined state of the art *in vitro* assays in order to enhance detection and to in-depth characterize rare antigen-specific $CD4^+$ T cells in autoimmune

diabetes. Following successful adaption of these assays to cord blood samples, I identified hyperglycemic conditions during gestation as a possible cellular mechanism that contributes to the relative protection against T1D conferred to offspring by a maternal T1D environment during pregnancy. I propose that an improved ability to delete (pro)insulin-reactive $CD4^{+}$ T cells is the main mechanism of protection offered to children of mothers with T1D. I also propose that there are additional protective mechanisms that will influence immunity in these children. It is possible that high glucose concentrations directly act on HSC differentiation as seen in animal models and humans (Harris et al., 2013; Oburoglu et al., 2014). Similar direct effects on genetic modifications in progenitor cells have been connected to mevalonate pathway (Bekkering et al., 2018), diet (Christ et al., 2018) and lipid consumption in mice (Vannini et al., 2012). Moreover, it has been shown that administration of *Bacillus Calmette–Guérin* to the bone marrow in a mouse model of tuberculosis changed the transcription profile of HSCs leading to enhanced myelopoiesis which resulted in a trained, highly protective macrophage phenotype against pulmonary *Mycobacterium tuberculosis* infection (Kaufmann et al., 2018). Recently, Mitroulis and colleagues have shown in mouse models that progenitor commitment of HSCs towards increased myelopoiesis is induced in the presence of pro-inflammatory Th1 cytokines such as IL-1β and GM-CSF with adaption to glucose metabolism (Mitroulis et al., 2018). In order to characterize maturation of the immune system in neonates of mothers with T1D, planned transcriptomics as well as *in vitro* stimulation models of HSCs and myeloid cells may give insight to possible re-programming of these cells, which could affect the maturation of the immune system in T1D (Figure 37). I expect that the relationship between increased glucose levels and early priming of HSCs as well as effects on antigen-specific T cells will provide avenues to develop therapeutic strategies to prevent beta cell autoimmunity in genetically at risk children.

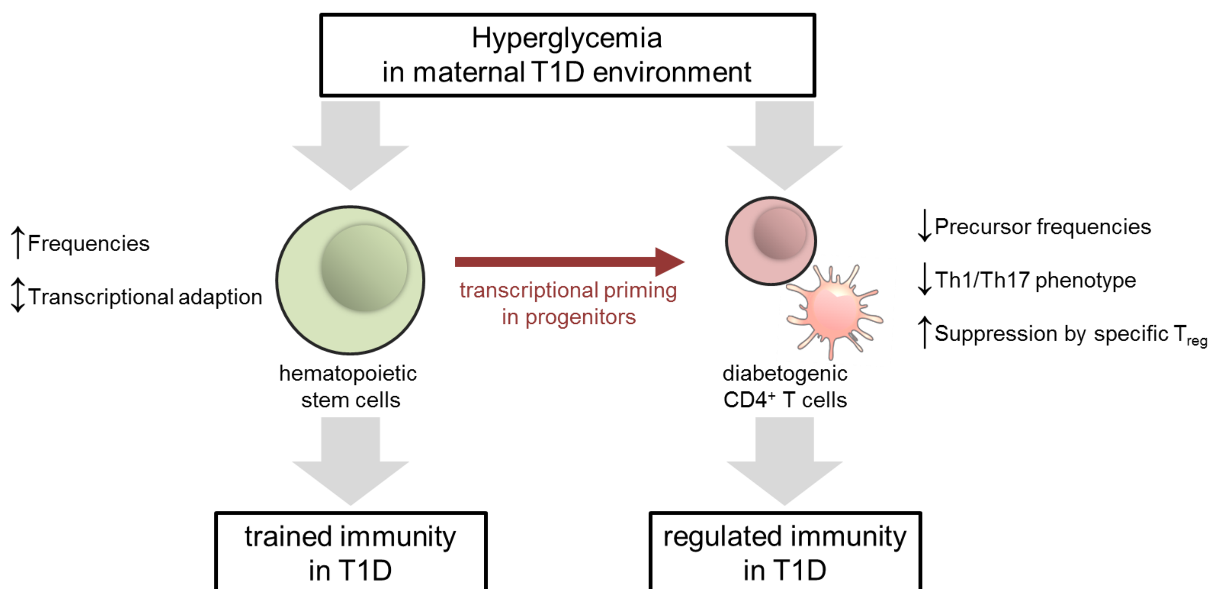


Figure 37: Potential mechanism of relative protection against T1D conferred by maternal T1D environment during gestation.

The work of my thesis also contributed to further understanding of $CD4^+$ T cell differentiation in T1D by identifying GM-CSF as a novel inflammatory cytokine, shaping the immune landscape of T1D. With respect to potential, future intervention studies these findings raise the possibility of therapeutically targeting GM-CSF production by pathogenic T cells in T1D.

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