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To my family

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INNA STARSKAIA: Early immune response in children developing type 1 diabetes
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ABSTRACT

Type 1 diabetes is a multifactorial autoimmune disorder. The pathogenesis of the disease is determined by the interplay of genetic and environmental factors, resulting in an aberrant immune response that leads to the destruction of insulin-secreting pancreatic beta cells. The appearance of islet-targeting autoantibodies, so called seroconversion, is currently the earliest biomarker for predicting the progression of type 1 diabetes before the clinical onset. One of the aims of this dissertation was to study early changes in immune responses that precede seroconversion in children who later progress to overt type 1 diabetes. In the first study we discovered numerous cell type-specific differential DNA methylation changes in circulating immune cells from children who later progressed to beta cell autoimmunity at the early stage of disease development, even prior to seroconversion. Another critical question addressed in this dissertation was the heterogeneity observed in the rate of disease progression to clinical disease as well as the post-onset beta cell decline. Recent studies reported on the existence of different disease pathways, or endotypes of type 1 diabetes behind one clinical phenotype. The second study revealed that the immune cell composition of peripheral blood mononuclear cells varied among children who later developed type 1 diabetes, when we compared children who had IAA, GADA or two autoantibodies detected as their first-appearing antibodies, supporting heterogeneity in the pathogenesis of the disease. Finally, in the third study we found that gene expression changes during the first year after clinical type 1 diabetes onset can predict the rate of disease progression. The predictive model allowed us to divide patients into rapid and slow progressors and was validated in an independent dataset of newly diagnosed type 1 diabetes patients. In conclusion, this dissertation presents findings on immune changes at the very early stage of type 1 diabetes development and support previous observations on heterogeneity of the disease.

KEYWORDS: Type 1 diabetes, early immune response, DNA methylation, autoantibodies, mass cytometry, heterogeneity, gene signature

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TIIVISTELMÄ

Tyypin 1 diabetes on autoimmuunitauti, jonka syntyyn vaikuttavat monet tekijät. Geenien ja ympäristötekijöiden vuorovaikutus johtaa poikkeavaan immuunivasteeseen ja insuliinia tuottavien haiman beetasolujen katoon. Tällä hetkellä diabetekselle tunnusomaisten autovasta-aineiden ilmaantuminen verenkiertoon, eli serokonversio, on varhaisin todettu merkki tautiprosessista. Yksi tämän väitöskirjatutkimuksen tavoitteista oli tutkia tyypin 1 diabetekseen immuunivasteiden varhaisia muutoksia lapsissa, jotka myöhemmin sairastuivat tyypin 1 diabetekseen. Ensimmäisessä tutkimuksessa havaitsimme taudin kehityksen varhaisessa vaiheessa, jo ennen serokonversiota, DNA-metylaation muutoksia immuunijärjestelmän soluissa lapsilla, joille myöhemmin kehittyi autovasta-aineita. Tavoitteena oli myös tutkia heterogeenisyyttä tyypin 1 diabeteksen taudinkehityksessä ja beetasolujen vähenemisessä taudin puhkeamisen jälkeen. Viimeaikaiset tutkimukset ovat osoittaneet useita tautimekanismeja tai taudin endotyyppejä, jotka johtavat tyypin 1 diabetekseen. Väitöskirjan toisessa osatutkimuksessa havaitsimme erilaisia veren immuunisolujen profiileja lapsilla, joille myöhemmin kehittyi tyypin 1 diabetes, kun vertasimme keskenään lapsia, joilta oli ensimmäisenä vasta-aineena mitattu IAA, GADA tai kaksi autovasta-ainetta. Nämä tulokset tukevat tyypin 1 diabeteksen tautimekanismien heterogeenisyyttä. Kolmannessa tutkimuksessa havaitsimme tyypin 1 diabeteksen etenemiseen liittyvien geenien ilmenemisen muutosten ennustavan taudin etenemisen nopeutta potilailla, joilla tauti on puhjennut hiljattain. Ennustavan mallin avulla potilaille voitiin ennakoida taudin nopeaa tai hidasta etenemistä. Tulosten varmistaiseksi käytimme riippumatonta tulosaineistoa hiljan diagnosoiduista tyypin 1 diabetespotilaista. Tämän väitöskirjan tulokset osoittavat joukon immuunivasteen muutoksia tyypin 1 diabeteksen kehityksen varhaisessa vaiheessa ja tukevat aiempia havaintoja taudin heterogeenisyydestä.

AVAINSANAT: Tyypin 1 diabetes, varhainen immuunivaste, heterogeenisyys

Table of Contents

Abbreviations

Aab	Autoantibodies
AUC	Area under the curve
C	Cytosine
CBV	Coxsackie B viruses
CGIs	CpG islands
CpG	C followed G, p stands for phosphate
CyTOF	Time-of-flight mass cytometry
DAISY	Diabetes Autoimmunity Study in the Young
DCs	Dendritic cells
DIPP	Type 1 Diabetes Prediction and Prevention study
DMC	Differentially methylated cytosine
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
FDA	United States Food and Drug Administration
FDR	False discovery rate
FCS	Flow cytometry standard
G	Guanine
GAD	Glutamic acid decarboxylase
GAD65	Glutamic acid decarboxylase 65-kilodalton isoform
GADA	Autoantibodies to glutamic acid decarboxylase
GO	Gene Ontology
GWAS	Genome-wide association study
HbA1c	Hemoglobin A1C
HLA	Human leukocyte antigen
IA-2	Insulinoma-associated antigen 2
IAA	Autoantibodies to insulin
ICA	Islet-cell antibodies
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit–related protein
MAIT	Mucosal-associated invariant T cell
meQTL	Methylation quantitative trait locus
MMTT	Mixed meal tolerance test

NK	Natural killer
NKT	Natural killer T cells
OGTT	Oral glucose tolerance test
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RRBS	reduced representation bisulfite sequencing
Tfh	Follicular helper T cells
TOF	Time-of-flight
Treg	Regulatory T cell
ZnT8	Zinc transporter 8

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Starskaia I, Laajala E, Grönroos T, Härkönen T, Junttila S, Kattelus R, Kallionpää H, Laiho A, Suni V, Tillmann V, Lund R, Elo LL, Lähdesmäki H, Knip M, Kalim UU, Lahesmaa R. Early DNA methylation changes in children developing beta cell autoimmunity at a young age. *Diabetologia*, 2022; 65:844-860.

II Starskaia I*, Valta M*, Pietilä S*, Suomi T*, Kalim UU, Pahkuri S, Rydgren E, Rasool O, Hyöty H, Knip M, Veijola R, Ilonen J, Toppari J, Lempainen J, Elo LL, Lahesmaa R. Distinct cellular immune responses in children en route to type 1 diabetes with different first-appearing autoantibodies. *Nat Commun,* 2024; 15: 3810. *Equal contribution.

III Suomi T*, Starskaia I*, Kalim UU*, Rasool O, Jaakkola MK, Grönroos T, Välikangas T, Brorsson C, Mazzoni G, Bruggraber S, Overbergh L, Dunger D, Peakman M, Chmura P, Brunak S, Schulte AM, Mathieu C, Knip M, Lahesmaa R, Elo LL, INNODIA Consortium. Gene expression signature predicts rate of type 1 diabetes progression. *EBioMedicine,* 2023; 92:104625. *Equal contribution.

1 Introduction

Type 1 diabetes is an autoimmune disease in which the immune system attacks and destroys the beta cells in the pancreas that make insulin. This is one of the most common chronic conditions in children. Within the past few decades, there has been an increase in the incidence rate of type 1 diabetes worldwide. Numerous factors, including genetic predisposition and environmental triggers have been implicated in pathogenesis of the disease.

As type 1 diabetes-associated autoantibodies resulting from autoimmune reaction, finding new means to detect the onset of the aberrant immune response preceding seroconversion would enable prediction and timely intervention to prevent the disease. We investigated early epigenetic changes linked to type 1 diabetes that occur before diagnosis and before the appearance of autoantibodies in the prospective DIABIMMUNE cohort (Study I). This study provided novel insights into cell type–specific differential epigenetic regulation of genes that may contribute to disease pathogenesis at the very early stages of disease development.

Next, there is a growing body of evidence indicating considerable heterogeneity in the rate of progression to clinical type 1 diabetes as well as in the decline of insulin secretion following disease onset. This variability among individuals presents a significant challenge to understand the pathogenesis of the disease and develop effective treatments. We analysed circulating immune cells in children (IAA-first, GADA-first, ≥2 Aab groups) who later develop type 1 diabetes and their carefully matched controls, as part of the Type 1 Diabetes Prediction and Prevention study (DIPP) (Study II). The results of the study support previous observations on heterogeneity of type 1 diabetes and indicate that immune response differs in children, depending on the type of autoantibodies that appear first. The findings may reflect different pathways, and pathogenesis, and possibly endotypes underlying the disease development.

Finally, we demonstrated that changes in gene expression in newly diagnosed type 1 diabetes patients from the "First 100" INNODIA cohort, between baseline and 12 months, can predict the decline in C-peptide at 24 months (Study III). Identification of a gene signature predictive of rate of progression of newly

diagnosed type 1 diabetes individuals would enable stratification of patients for therapeutic interventions.

2 Review of the Literature

2.1 Type 1 Diabetes

Type 1 diabetes is characterized by insulin deficiency and subsequent hyperglycemia due to the autoimmune destruction of insulin-producing pancreatic beta cells. The disease has severe long-term complications, including diabetes-related retinopathy, neuropathy, foot ulcers, cardiovascular and kidney diseases and others. Type 1 diabetes causes burden to the families and the health care system and requires daily insulin injections and monitoring of blood sugar. The incidence of the disease increases globally and Finland reported the highest rate of 52,2 per 100.000 person-years under the age of 15 in the time period 2015-2018 (Parviainen et al., 2020).

Despite the substantial investment of time and resources over the past three decades aimed at identifying disease-modifying therapies to halt or slow down the progression of type 1 diabetes, exogenous insulin replacement remains the primary treatment option. In December 2022, Teplizumab, an anti-CD3 monoclonal antibody, received approval from the United States Food and Drug Administration, marking a significant milestone as the first therapy aimed to delay the onset of overt type 1 diabetes in individuals at risk. This therapy has since been approved in several countries, including the United States (Evans-Molina & Oram, 2023). The unique status of type 1 diabetes as an outlier among autoimmune diseases in relation to existing therapies highlights the complexities and challenges associated with developing effective therapies to prevent or treat the disease.

2.1.1 Pathogenesis of type 1 diabetes: natural history

The progression of type 1 diabetes is generally characterized by two primary phases: the pre-symptomatic and the symptomatic phases. **Figure 1** illustrates stages of type 1 diabetes development.

The differentiation of the disease into stages is currently based on measurable markers of autoimmunity, such as presence autoantibodies in the serum, the status of glucose homeostasis and the manifestation of clinical symptoms. Stage 1 of the disease is defined by the presence of two or more type 1 diabetes-associated islet autoantibodies and normal glucose tolerance. The identification of islet-cell

antibodies (ICA) was reported for the first time in 1974 and supported the hypothesis of autoimmune nature of the disease (Bottazzo et al., 1974). Since then, several beta cell molecules were identified as target antigens in type 1 diabetes, including insulin, glutamic acid decarboxylase, tyrosine phosphatase-like insulinoma-associated antigen 2 (IA-2), islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP), zinc transporter 8 (ZnT8), chromogranin, and many others (Roep & Peakman, 2012). Currently, the detection of islet-targeting autoantibodies in serum is the earliest indicator used to predict type 1 diabetes progression.

Progression to Stage 2 is characterized by the continued presence of islet autoantibodies, and accompanied by the emergence of dysglycemia resulting from loss of functional beta cells. Dysglycemia is defined by several clinical parameters, including impaired fasting glucose level, oral glucose tolerance test (OGTT) and haemoglobin A1C (HbA1c) level. Finally, the condition advances to Stage 3, which is typically associated with the manifestation of clinical symptoms, including fatigue, polyuria, diabetic ketoacidosis and others; and requires exogenous insulin therapy (Atkinson et al., 2014; Eisenbarth, 1986; Insel et al., 2015).

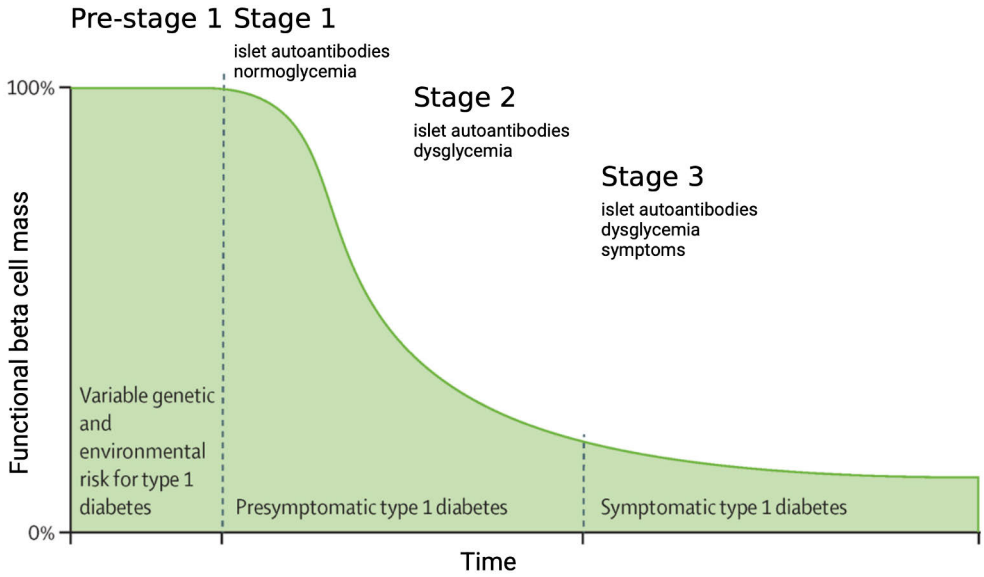

Figure 1. Natural history of type 1 diabetes. Modified from (Insel et al., 2015).

However, the appearance of Aab is preceded by an asymptomatic period, indicated at Figure 1 as Pre-stage 1. Pre-stage 1 has a particular interest, as the early identification of an ongoing pathological process could facilitate timely intervention and potentially avert the onset of the disease. This stage is characterized by genetic predisposition and an associated risk of disease development and potential

environmental factors that might trigger the disease. These aspects, including genetic and putative environmental factors, will be discussed in the following sections.

2.1.2 Genetics of type 1 diabetes

Twin studies revealed genetics significantly contribute to type 1 diabetes risk. The concordance rate for the disease is significantly higher among monozygotic compared to dizygotic twins, indicating a strong impact of genetic predisposition on type 1 diabetes risk (Kaprio et al., 1992). Specifically, the concordance rate among monozygotic twins was found to be approximately 50%, with variations reported in the literature from 30 to 65% (Kaprio et al., 1992; Redondo et al., 2001, 2008).

Further genetic studies revealed that the human leukocyte antigen (HLA) genes, including both class I (HLA-A, -B, and -C) and class II (HLA-DR, -DQ, and -DP) genes, have the strongest impact on type 1 diabetes development, accounting for up to 50% of the genetic risk associated with the disorder (Noble, 2015). Moreover, polymorphisms located outside the HLA region have been identified and well characterized, including variants in *INS*, *PTPN22, IFIH1*, *CTLA4*, *IL2RA*, *TYK2*, *UBASH3A*, *FUT2* genes (Shapiro et al., 2021). Next, genome-wide association studies (GWAS) have identified additional loci that harbour common variants associated with relatively small effects on type 1 diabetes risk development (Barrett et al., 2009; Burton et al., 2007; Chiou et al., 2021; Cooper et al., 2012; Smyth et al., 2006). Fine-mapping and functional analyses have confirmed the GWAS findings (Onengut-Gumuscu et al., 2015; Trynka et al., 2011). A recent comprehensive genetic study, which included a diverse cohort of 61,472 individuals, identified 78 significant regions outside the HLA locus that are linked to type 1 diabetes susceptibility (Robertson et al., 2021).

The presence of specific HLA class II haplotypes, including HLA-DRB1*04 (DR4), DQA1*0301-DQB1*0302 (DQ8), HLA-DRB1*0301 (DR3), and DQA1*0501-DQB1*0201 (DQ2), is associated with a significant increased predisposition for type 1 diabetes (Erlich et al., 2008). The most significant risk haplotypes associated with the disease were found to be DR3-DQA1*0501-DQB1*0201 and DR4-DQA1*0301-DQB1*0302. These alleles account for approximately 30%–50% of genetic risk for the disease genetic predisposition (Noble et al., 1996).

However, according to the DIPP study only about 10% of genetically at-risk children will eventually develop clinical disease, suggesting environmental factors, contributing to disease pathogenesis.

2.1.3 Putative environmental triggers

The role of environmental factors in the pathogenesis of type 1 diabetes is considered significant, particularly in light of the observed rapid increase in incidence rates that cannot be solely accounted for by genetic predisposition. Evidence indicates that children originating from regions with a low incidence of type 1 diabetes exhibit an increased risk of developing the disease when they are born or relocate to countries with a high incidence, such as Sweden, which follows Finland, the country with the highest incidence of type 1 diabetes globally (Hjern et al., 2012; Söderström et al., 2012). Furthermore, the prevalence of individuals with type 1 diabetes possessing high-risk genotypes in Finland has decreased from 25.3% to 18.2% over a period of 50 years (Hermann et al., 2003). In parallel, the incidence rate of type 1 diabetes surged by 57% between 1965 and 1984 (Tuomilehto et al., 1991). These observations suggest a significant impact of environmental factors on the rising incidence of the disease, thereby encouraging further investigations to explore their specific roles.

Among environmental triggers potentially contributing to type 1 diabetes, viral infections, diet, microbiome and toxins have been studied (Rewers & Ludvigsson, 2016). Enterovirus infection have been recognized as important factor in the pathogenesis of type 1 diabetes. Specifically, persistent Coxsackie B viruses (CBV) have long been linked with the development of islet inflammation and autoimmunity (Krogvold et al., 2015; Richardson & Morgan, 2018; Vehik et al., 2019). While the exact mechanisms of CBV-induced beta cell damage remain unclear, the presence of viral proteins in pancreatic beta cells of most individuals with type 1 diabetes suggests direct infection may lead to cell damage and the onset of islet autoimmunity (Richardson et al., 2009). Alternatively, molecular mimicry between viral and beta cell proteins could also contribute to the disease development (Marttila et al., 2001). The association between CBV and type 1 diabetes promoted the development of human CBV vaccines to prevent autoimmunity triggered by the viral infection (Jartti et al., 2024).

Next, several dietary factors, including breastfeeding, cow's milk, cereals, vitamin D, and omega-3 fatty acids have been studied in the context of type 1 diabetes risk development (Rewers & Ludvigsson, 2016). The relationship between the consumption of cow's milk during later childhood and its potential association with disease remains controversial. Some studies have indicated a correlation between cow's milk consumption and an increased risk of islet autoimmunity and type 1 diabetes (Lamb et al., 2015; Virtanen et al., 2000, 2012). Conversely, other research found a reduced risk of diabetes associated with cow's milk intake (Rosenbauer et al., 2008). Polyunsaturated fatty acids were shown to exhibit an inverse relationship with diabetes risk by some studies. Finnish children with lower levels of linoleic acid (an omega-6 fatty acid) had a higher risk of islet autoimmunity (Virtanen et al., 2010), while a US study suggested that higher omega-3 intake in

childhood was associated with a reduced risk of islet autoimmunity in at-risk children (Norris et al., 2007). Further, some studies found a link between low levels of vitamin D and type 1 diabetes development in newly diagnosed type 1 diabetes patients (Bener et al., 2009; Pozzilli et al., 2005). However, no significant association was found between insufficient vitamin D levels and an increased risk of type 1 diabetes in another study (Reinert-Hartwall et al., 2014).

The inconsistent findings of dietary risk associations with type 1 diabetes may be due to complex interplay of environmental modifiers, as well as genetic background and need further investigations.

2.1.4 Epigenetics of type 1 diabetes

Environmental influences are reflected in epigenetics, consequently affecting gene expression levels. Changes in epigenetic status can be detected by assays measuring DNA methylation and histone modifications. DNA methylation constitutes one of the most thoroughly studied and important epigenetic modifications, playing a role in the regulation of gene expression (Bird, 2002). Specifically, DNA methylation modulates gene expression by recruiting proteins associated with gene repression or by inhibiting binding of transcription factors to DNA. DNA methylation involves the addition of a methyl group to the cytosine base, mainly occurring at CpG dinucleotides and is generally associated with the silencing of gene expression (Weber et al., 2007). Although CpG dinucleotides are relatively sparse throughout the human genome, they are notably enriched in gene promoters and exons, with approximately 72% of human gene promoters exhibiting a higher frequency of CpG sites (Saxonov et al., 2006). These CpG sites within gene promoters often cluster in regions known as CpG islands (CGIs), which consist of several hundred to a few thousand base pairs and have a high enrichment of CpGs (Husquin et al., 2018). DNA methylation was found to be involved in the regulation of gene expression and cell differentiation processes (Li et al., 2021). Furthermore, the significance of epigenetic regulation of gene expression has been increasingly recognized in relation to various autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, and type 1 diabetes (Li et al., 2021).

Cis-acting methylation quantitative trait loci (meQTL) effects were identified in a substantial proportion of genome-wide association study (GWAS) loci associated with type 1 diabetes, indicating that DNA methylation may play a role in modulating the genetic risk for this disease (Kindt et al., 2018). An examination of the methylation status of susceptibility genes for type 1 diabetes, in relation to genetic polymorphisms (meQTLs), revealed a significant difference in the methylation of CpG sites within the MHC genes between individuals carrying the type 1 diabetes risk haplotypes DR3-DQ2 and DR4-DQ8. This differential methylation was

correlated with decreased mRNA and protein expression in dendritic cells (DCs) of children possessing the HLA-DR3-DQ2 haplotype (Kindt et al., 2018).

Several studies have observed a correlation between DNA methylation in circulating immune cells and type 1 diabetes (Belot et al., 2017; Disanto et al., 2013; Paul et al., 2016; Rakyan et al., 2011). Paul et al. carried out an observational study of DNA methylation patterns in monozygotic twins discordant for type 1 diabetes, using Illumina 450K array platform alongside whole genome bisulfite sequencing (Paul et al., 2016). This study focused on purified immune cell populations that are recognized as critical players in the disease pathogenesis, including CD4+ T cells, CD19+ B cells, and CD14+CD16– monocytes derived from peripheral blood mononuclear cells (PBMC). Their analysis identified thousands of differentially variable CpG sites in type 1 diabetes twins compared to their healthy co-twins. The differentially variable GpG sites predominantly occur in individuals with disease and represent substantial variations in DNA methylation levels, exceeding >10%. However, these variations are present in a smaller number of cases. The study found only one differentially methylated cytosine (DMC) within the CD4+ T cell population. This DMC was located in the intergenic region between the *DDIT4* and *DNAJB12* genes, distinguishing individuals with type 1 diabetes from their healthy co-twins (Paul et al., 2016).

However, most of these investigations have focused on individuals with overt type 1 diabetes. Only one recent study explored DNA methylation alterations in peripheral blood samples collected longitudinally prior to the clinical type 1 diabetes from children enrolled in the prospective Diabetes Autoimmunity Study in the Young (DAISY) cohort (Johnson et al., 2020). Using Human Methylation 450K Beadchip (450K) and EPIC platforms, they found two DMCs and 28 differentially methylated regions (DMR) between cases and controls. Notably, the differences were observed prior to both diagnosis and seroconversion, encouraging further studies on early epigenetic changes to improve risk prediction and prognosis.

2.1.5 Immune response in type 1 diabetes

The therapeutic application of anti-CD3 monoclonal antibodies to delay the progression of type 1 diabetes highlights the important role of CD4+ and CD8+ T cells in the pathogenesis of disease. Prior to discussing the role of T cells in the context of type 1 diabetes, it is essential to provide a brief introduction to T cells.

2.1.5.1 CD4+ and CD8+ T cell biology

T cell development is a highly regulated process that takes place in the thymus. T cells recognise antigen by a specific T cell receptor (TCR). There are two types of T cells: CD4+ helper T (Th) cells and CD8+ cytotoxic T cells.

CD4+ T helper cells represent a diverse population of T cells that are crucial to the regulation of immune responses. T cells circulate between secondary lymphoid tissues, where they are become activated by antigen-presenting cells (APCs). The activation of CD4+ T cells occurs through the interaction of TCR with peptide-MHC class II complexes presented by APCs, costimulatory signals and cytokine-mediated signalling pathways. Following activation, CD4+ T cells differentiate into various subsets, each characterized by distinct profiles of surface markers, cytokine production, and function. At present, several subsets of T helper cells have been identified, including Th1, Th2, Th17, Tregs, Tfh, Th9, Th22, and CD4+ cytotoxic T cells. **The Figure 2** provides an overview of five major Th subsets. IFN-γ and IL-12 are critical drivers of Th1 cell differentiation, they induce the expression of the master transcription factor (TF) T-bet through the activation of STAT1 and STAT4, respectively (Szabo et al., 2000). Th1 cells are involved in immune responses against viruses and intracellular bacteria by producing IFN-γ. Th2 cells are differentiated via the signaling provided by cytokines IL-2 and IL-4, which leads to the expression of the key transcription regulator GATA-3. This cell subset regulates immune response

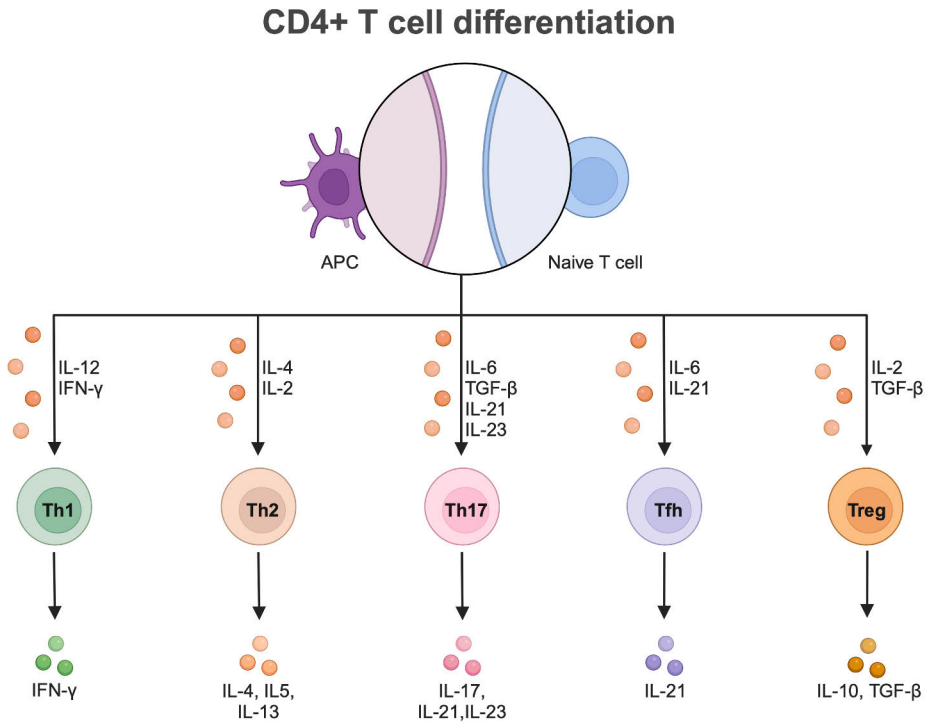

Figure 2. CD4+ T cell differentiation. Created with BioRender.com.

to extracellular pathogens, such as helminths by secreting IL-4, IL-5, and IL-13 cytokines (Szabo et al., 2000). Th2 cells also contributed to some inflammatory conditions, like allergy and asthma. The differentiation of Th17 cells occurs in the presence of IL-6 and TGF-β, leading to the expression of RORγt. Th17 cells are responsible for host protection against extracellular pathogens and fungi, and also contribute to pathogenesis of autoimmune diseases (Korn et al., 2009). IL-6 and IL-21 promote the differentiation of Tfh cells differentiation, these cells promote B cell proliferation, maturation, and antibody production (Crotty, 2011). Finally, Treg cells can be differentiated following TCR stimulation in the presence of TGF-β and IL-2, inducing expression of Foxp3, master regulator of this subset. The major function of Tregs is to maintain immune homeostasis and tolerance by preventing detrimental immune responses.

CD8+ T cells similar to CD4+ helper T cells, express a T-cell receptor. However, CD8+ T cells recognize peptide antigens presented by MHC class I molecules, which are expressed on the surface of all nucleated cells. The role of CD8+ T cells is critical in the immune defense against intracellular pathogens, such as viruses and bacteria, as well as in the surveillance of tumors. Upon recognition of their specific antigen and subsequent activation, CD8+ T cells kill infected or malignant cells using different mechanisms, including secretion of TNF and IFN-γ cytokines, releasing cytotoxic granules, such as granzymes and perforins.

2.1.5.2 CD4+ and CD8+ T cell in type 1 diabetes

CD4+ and CD8+ T cells with memory and effector phenotypes, were found even in the pancreases of non-diabetic individuals, and they comprised up to 80% of the total pancreatic lymphocyte population. While B cells and natural killer (NK) cells were also detected within the pancreatic environment, their prevalence was significantly lower than that of T cells (Radenkovic et al., 2017). Further, the frequencies of autoimmune CD8+ T cells were found to be elevated in the pancreases of individuals with type 1 diabetes compared to non-diabetic controls (Culina et al., 2018).

Indeed, autoreactive CD8+ T cells are identified as the primary effectors responsible for the destruction of beta cells. This involvement of CD8+ T cells has been observed by several studies (Coppieters et al., 2012; Culina et al., 2018; Pinkse et al., 2005; Wang et al., 2019). Coppieters et al. demonstrated the infiltration of pancreatic islets in patients with type 1 diabetes by autoreactive CD8+ T cells that are specific to islet-related epitopes, including GAD65, IA-2, and insulin. (Coppieters et al., 2012). Additionally, CD4+ T cells isolated from individuals with type 1 diabetes have been shown to recognize proinsulin epitopes presented by HLA-DQ8 and HLA-DQ8 transdimers (Pathiraja et al., 2015).

CD4+ T cells have an indispensable role in type 1 diabetes pathogenesis and many studies focused on their role in their contribution to the disease. Specifically, T helper 17 (Th17) and follicular helper T (Tfh) immune responses have been recently highlighted in the pathogenesis of type 1 diabetes (Walker & von Herrath, 2016).

A study showed that circulating CD4+ T cells from individuals recently diagnosed with type 1 diabetes secreted IL-17 in response to beta cell autoantigens (Arif et al., 2011). Furthermore, there was an increased expression of IL-17E and IL-17F in circulating lymphocytes of type 1 diabetes patients, compared to controls, which was also shown in gene expression levels (Kumar et al., 2014). Next, the activity of Tregs to suppress proliferation of effector T cells in recent-onset patients with type 1 diabetes was diminished in comparison to their matched non-diabetic control subjects (Lindley et al., 2005). Additionally, Tfh cells were shown to be involved in type 1 diabetes pathogenesis by many studies. CD4+T cells with memory phenotype from type 1 diabetes individuals exhibited increased expression of Tfh markers at mRNA level, such as *CXCR5*, *ICOS*, *PDCD1*, *IL21* when compared to control samples (Kenefeck et al., 2015). Further, the increased frequency of IL-21 producing memory CD4+ T cells was detected in type 1 diabetes patients compared to healthy controls (Ferreira et al., 2015; Xu et al., 2013).

Apart from T cells, some studies have also focused on the potential role of NK cells and neutrophils in pathogenesis of type 1 diabetes. While NK cells may also play a role in the cytotoxic destruction of beta cells, their role in disease development remains controversial, as they might exhibit both protective and destructive functions (Perricone et al., 2008). Further, studies demonstrated accumulation of neutrophils in the pancreas in patients with clinical disease and islet autoantibody-positive at-risk subjects (Valle et al., 2013; Vecchio et al., 2018), suggesting infiltrating neutrophils may directly contribute to beta cell damage.

2.1.6 Early immune response in type 1 diabetes

At present, the appearance of islet-targeting autoantibodies in the serum is the earliest indicator used to predict type 1 diabetes progression before its symptomatic onset. The appearance of Aab results from preceding autoimmune reaction, where the presentation of autoantigens to T cells and subsequent Aab production by B cells have already occurred. For that reason, the discovery of early changes within immune cell subsets (either frequency or functional status) is important to improve prediction and monitoring of type 1 diabetes.

Previous transcriptomic studies indicate that early changes, even before seroconversion, can be detected in children who later develop type 1 diabetes (Ferreira et al., 2014; Kallionpaa et al., 2014). In recent study from our group CD4+

T cells, CD8+ T cells and CD4–CD8– cells were purified from PBMC samples. We analysed gene expression in these purified cell fractions of samples longitudinally collected from seven children that developed beta cell autoimmunity and their matched controls. The results revealed early changes in the transcriptome of immune cells before the appearance of type 1 diabetes-associated Aab in children who later developed beta-cell autoimmunity (Kallionpää et al., 2019). Notably, we found the increased expression of *IL32* gene in PBMC samples of cases compared to controls (Kallionpää et al., 2019). This gene encodes a pro-inflammatory cytokine that induces TNF, IL-6, and IL-1b expression. IL-32 has previously been associated to several autoimmune disease, including type 1 diabetes and rheumatoid arthritis (de Albuquerque et al., 2021).

Previous research has indicted differences in circulating immune cells among individuals progressing to type 1 diabetes. Specifically, a study reported a higher level on apoptosis in CD4+CD25high T cells among both at-risk individuals who were Aab-positive and newly diagnosed type 1 diabetes patients in comparison to control individuals (Glisic-Milosavljevic et al., 2007). This finding suggests that the functional capacity of regulatory T cells (Tregs), which are critical for modulating pathogenic T cell activity, may play a significant role in the pathogenesis of the disease.

Additionally, increased frequency of activated Tfh cells was observed in children tested positive for multiple Aab and at the late stage of islet autoimmunity progression compared to control groups (Viisanen et al., 2017). Furthermore, a study showed T cells with CXCR5–PD-1high phenotype were associated with progression of type 1 diabetes (Ekman et al., 2019). The study found that the frequency of circulating CXCR5–PD-1high cells was significantly elevated in recent-onset type 1 diabetes and Aab-positive children who later developed disease. These cells exhibited a B cell-activating potential and have previously been implicated in the pathogenesis of rheumatoid arthritis (Rao et al., 2017).

However, these studies were carried using samples from children who had already developed autoantibodies, not before seroconversion. Discovery of immune response changes at the early stages of disease onset, before seroconversion, could provide new insights into molecular mechanisms leading to type 1 diabetes.

2.2 Heterogeneity in Type 1 Diabetes

Accumulating evidence indicates considerable heterogeneity in the rate of progression to clinical type 1 diabetes and in the post-onset insulin secretion decline (Battaglia et al., 2020), which represent a challenge for designing effective therapies.

2.2.1 Heterogeneity prior to clinical type 1 diabetes

A hypothesis has been proposed suggesting that the risk of developing type 1 diabetes correlates with the specific type of Aab that first appears during early life. The age at which the Aabs manifest, as well as their sequential order may be associated with distinct genetic backgrounds or environmental determinants, or potentially a combination of both (Krischer et al., 2015).

Among various Aabs, IAA and GADA are the most frequently identified as the first to appear, exhibiting notable differences in their respective ages of seroconversion. Specifically, IAA typically emerges during early childhood, between the ages of 1 and 2 years, and is associated with a rapid progression of the disease; however, its prevalence declines thereafter. In contrast, GADA tends to peak between the ages of 4 and 5 years, without a significant decline throughout childhood and exhibiting a moderate rate of disease progression (Krischer et al., 2015).

Further, cohort studies revealed the correlations between the type of Aab and specific genotypes, suggesting that genetic factors may influence the specificity of the first appearing autoantibody. Notably, the *INS* gene risk allele was found to be associated with the appearance of IAA as the initial autoantibody (Hermann et al., 2005; Ilonen et al., 2013). Additionally, IAA was linked to HLA DR4-DQ8 haplotype, whereas GADA was associated with HLA DR3-DQ2 haplotype (Krischer et al., 2015; Krischer, Lynch, et al., 2017). The analysis of the prospective TEDDY (The Environmental Determinants of Diabetes in the Young) cohort further indicated that different first appearing autoantibodies were associated with distinct non-HLA genomic regions. Specifically, the *INS* and *TTC34/PRDM16* regions were found to be linked to the presence of IAA as the first appearing Aab, whereas the *RBFOX1* region was associated with the initial appearance of GADA (Sharma et al., 2018).

The presence of IA-2A as a first appearing Aab has been correlated with a more rapid progression to type 1 diabetes when compared to cases where IAA or GADA were the first detected Aab (Ilonen et al., 2013). Furthermore, IA-2A has been identified as a significant predictor of type 1 diabetes onset in young children (Jacobsen et al., 2019),

Next, the appearance of a second Aab and progression to multiple Aab positivity was found to confer a significantly increased risk of progressing to clinical disease when compared to individuals who exhibit positivity for only a single Aab (Vehik et al., 2020; A. G. Ziegler et al., 2013). Furthermore, an earlier age at seroconversion and a shorter time until the emergence of the second autoantibody elevated the risk of developing the disease (Vehik et al., 2020). Interestingly, among various Aab, the appearance of IA-2A as a secondary autoantibody was linked to an increased risk of progression to type 1 diabetes, particularly in comparison to GADA or IAA (Vehik et al., 2020).

Additionally, a significant association was identified between longitudinal autoantibody profiles and rates of disease progression. A recent prospective study involving 1,845 at-risk children who tested positive for at least one autoantibody (IAA, GADA, or IA-2A) revealed distinct longitudinal autoantibody profiles correlated with varying progression rates to clinical disease (Ghalwash et al., 2024). Children exhibiting an early persistent profile characterized by the presence of IAA, GADA, and IA-2A demonstrated the highest rate of progression to clinical disease, with a 5-year risk estimated at 69.9% and a median age of onset at 4.3 years. This group was followed by those with persistent IAA and GADA, as well as GADA and IA-2A profiles, which exhibited 5-year progression risks of 39.1% and 30.9%, respectively, with later median ages at diagnosis of 9.2 years and 8.2 years. Furthermore, individuals with persistent GADA alone showed a 5-year progression risk of 10.5%. In contrast, those with single and transient autoantibody presence, predominantly IAA and GADA, exhibited a markedly lower 5-year risk of disease progression at only 1.6% (Ghalwash et al., 2024).

Additionally, studies indicated that the loss of IAA in individuals who tested positive for all three autoantibodies (IAA, GADA, and IA-2A) was associated with a reduced 10-year progression risk of 23%, compared to a 76% risk in those who retained positivity for IAA. Thus, the loss of IAA appears to be linked to a delay in disease progression (Endesfelder et al., 2016; Ghalwash et al., 2024).

2.2.2 Heterogeneity after type 1 diabetes diagnosis

Numerous studies demonstrated the presence of functioning beta cells at the time of diagnosis in individuals with type 1 diabetes, with some patients exhibiting a substantial preservation of beta cell mass even after the diagnosis (Campbell-Thompson et al., 2016; Keenan et al., 2010; Lam et al., 2017; Oram et al., 2014). The measurement of C-peptide secretion is currently used as a reliable biomarker for assessing residual beta cell function in individuals diagnosed with the disease (Palmer et al., 2004). C-peptide is a peptide connecting A-chain and B-chain of proinsulin. During the synthesis of insulin, C-peptide is cleaved from proinsulin and secreted into circulation simultaneously with insulin in equimolar amounts. Notably, some patients may experience a phase of partial remission following the initiation of insulin therapy, during which they still respond to low levels of insulin to maintain euglycemia. This phenomenon is commonly referred to as the "honeymoon phase," and may last for a duration of several weeks to over a year (Abdul-Rasoul et al., 2006; Böber et al., 2001; Harsunen et al., 2023; Muhammad et al., 1999).

Many studies identified various factors that affect the rate of beta cell decline, including the age at diagnosis, HLA type, and body mass index (BMI). Specifically, numerous investigations demonstrated the age at the disease onset associated with

the rate of decline in insulin secretion, with younger individuals exhibiting a more rapid decrease (Barker et al., 2014; Dufort et al., 2019; Harsunen et al., 2023; Krischer, Liu, et al., 2017; Petrone et al., 2005; A.-G. Ziegler & Bonifacio, 2012). Next, research by Petrone et al., showed a connection between HLA genotype and residual beta cell function, revealing that high-risk allele DRB1*03-DQB1*0201/DRB1*04-DQB1*0302 were associated with lower C-peptide levels at the time of diagnosis (Petrone et al., 2005). However, several recent studies did not find any significant correlations between C-peptide loss after the disease onset and HLA class II genotypes (Dufort et al., 2019; Mortensen et al., 2009). Additionally, higher body weight was linked to rapid disease progression during one follow-up year after the diagnosis of type 1 diabetes in patients aged 10-18 years (Lauria et al., 2015). Interestingly, a study showed that in children diagnosed with type 1 diabetes, the presence of ZnT8A at the time of diagnosis was associated with reduced C-peptide levels two years later (Juusola et al., 2016), suggesting that ZnT8A positivity may indicate a more aggressive disease phenotype in children with early-onset type 1 diabetes. Further research on exploring additional factors contributing to the variation among individuals in the rate of type 1 diabetes progression is needed.

The heterogeneity in beta cell function preservation might reflect distinct immunological processes contributing to the disease severity. Recent studies showed associations between type 1 diabetes, age, and immune responses. Arif et al., identified distinct immune responses among patients with type 1 diabetes, focusing on peripheral immune responses and pancreatic histopathology (Arif et al., 2014). The authors characterized two distinct immunological profiles based on the infiltration of immune cells within the pancreatic tissue. The first profile, termed hyper-immune CD20Hi, was characterized by a high infiltration rate and frequency of CD20+ B cells, and correlated with a lower mean age at disease onset. While, the second profile, called pauci-immune CD20Lo, had lower infiltration and frequency of CD20+ B cells (Arif et al., 2014).

This finding was further confirmed by subsequent research conducted by Leete et al., which explored the immune cell composition within the pancreatic islets of younger versus older children diagnosed with type 1 diabetes (Leete et al., 2016). The results indicated that children diagnosed at the age of 7 years or younger displayed a CD20Hi profile, which was associated with a more aggressive disease. In contrast, those diagnosed between the ages of 7 and 13 years exhibited a CD20Lo pattern, corresponding to a less aggressive disease progression and the presence of a greater number of residual beta cells. This study highlighted the significant relationship between age and the immunological composition within the pancreas in type 1 diabetes patients (Leete et al., 2016).

Additionally, a study demonstrated that circulating autoreactive effector memory CD8+ T cells expressing CD57 positively correlated with change in C-peptide levels

in recently diagnosed type 1 diabetes patients (Yeo et al., 2018), potentially reflecting underlying pathophysiological processes in the pancreas. These CD8+ T cells exhibited enhanced effector function, characterised by higher granzyme B levels and reduced CD28 expression compared to their CD57– counterparts. Notably, the association between CD8+ T cells and C-peptide loss was particularly pronounced in children under 12 years of age. Recent research focusing on gene expression profiling in new-onset type 1 diabetes patients revealed that immune cell type gene signature correlates with disease outcome (Dufort et al., 2019). The study found that patients with elevated levels of B cell gene expression and reduced neutrophil gene expression levels had more rapid C-peptide decline.

In conclusion, the heterogeneity represents a challenge for designing clinal trials, especially those therapies which aimed at preserving beta cell function. Investigating the nature of this heterogeneity in beta cell function could provide insights into different pathophysiological mechanisms and lay foundation in patient stratification for designing therapies.

2.3 Methods used in the study for DNA methylation and PBMC immune profiling

Here, I would like to highlight and provide some technical details and principles of methods which were used for studying DNA methylation in circulating immune cells (Study I) and comprehensive immune profiling of PBMC samples applied in Study II.

2.3.1 Reduced representation bisulphite sequencing

Reduced representation bisulfite sequencing (RRBS) is an approach that integrates restriction enzyme digestion with bisulfite sequencing to analyze DNA methylation patterns. Specifically, the restriction enzyme used in this technique, typically MspI, cleaves DNA at all CCGG sites, irrespective of the methylation status of the cytosine within the CpG dinucleotide. This selective cleavage enriches CpG-dense sites of the genome, reducing the number of nucleotides to be sequenced to 1% of the genome (Meissner, 2005). Following this, bisulfite treatment converts unmethylated cytosine residues into uracil, while methylated cytosines remain intact (Frommer et al., 1992). Typically, RRBS is capable of capturing approximately 2.5 to 3 million CpG sites, covering 5% to 10% of the total CpG sites present within the human genome. Consequently, RRBS represents a cost-effective alternative to whole-genome bisulfite sequencing, which is generally more expensive. Furthermore, RRBS demonstrates a superior capacity for targeting a larger number of CpG sites

compared to the widely used Illumina 450K DNA methylation microarray platform, which is restricted to approximately 450,000 sites.

Briefly, the RRBS protocol includes several steps. First, the protocol involves the digestion of genomic DNA using the MspI restriction enzyme, which cleaves the DNA into fragments of varying lengths. Following this enzymatic digestion, the protocol proceeds to library preparation, which entails several key processes: end repair, which is essential for completing the 3' terminus of the DNA strands; A-tailing, which involves the addition of a single adenosine nucleotide to facilitate the ligation of methylated sequence adapters; and, finally, the ligation of these sequencing adapters. Next, a size selection of the resulting DNA fragments is performed by gel electrophoresis to separate fragments based on their size, and purification by gel excision. This is followed by bisulfite conversion, in which unmethylated cytosine is deaminated into uracil, which allows differentiation of methylated from unmethylated cytosines. The bisulfite-converted DNA is then amplified using PCR, followed by a purification step prior to sequencing. Finally, the sequencing of the prepared library is performed utilizing next-generation sequencing technologies.

2.3.2 Immune profiling by mass cytometry

The immune system consists of highly diverse specialized immune cell populations. To be able to understand better the immune responses, particularly in the context of autoimmune diseases, the circulating immune system cell populations and their functional status should be analysed simultaneously in a patient sample. Conventional flow cytometry utilizes fluorescently labelled antibodies; however, the overlapping emission spectra of various fluorophores can complicate the accurate measurement of multiple markers. This challenge is particularly observed in heterogeneous samples, such as PBMC or whole blood.

Recent technological advances have resulted in the development of a single-cell proteomic analysis technique – time-of-flight mass cytometry (CyTOF). The technology utilizes antibodies conjugated to rare-earth metal isotopes with unique mass, that are detected by inductively coupled plasma mass spectrometer. This method allows simultaneous identification of up to 50 markers, including surface and intracellular proteins in individual cells of a sample. Furthermore, conventional fluorophores used for flow cytometry are sensitive to fixation, freezing and storage, but metal-conjugated antibodies allow flexibility in handling and analyzing samples, specifically of large cohort studies. Thus, the additional advantage of this technology to conventional flow cytometry also includes the possibility of cryopreservation and long-term storage of already-stained samples before acquiring the samples at a mass cytometer, thereby mitigating the risk of potential samples loss due to instrument

malfunctioning. This also allows to acquire the samples in a single batch which will reduce sample-to-sample variation and improve data consistency.

The procedure of mass cytometry analysis includes several steps and process is illustrated at **Figure 2**. Single-cell suspensions are stained with heavy metal-labelled antibodies and subsequently introduced into a mass cytometer. The cell suspension is further passed into nebulizer, which turns the suspension into small droplets, which subsequently exposed to an inductively coupled plasma. Plasma breaks down the droplet contents into a cloud of elemental ions. The ion clouds pass through a quadrupole mass filter to remove biologically abundant, low-mass ion species, like carbon and hydrogen, and enrich for the remaining ions, which are conjugated to their respective probes. Finally, ions get separated by their mass-to-charge ratio in a TOF mass spectrometer and converted to electrical signals and eventually data matrix (Bandura et al., 2009; Bjornson et al., 2013).

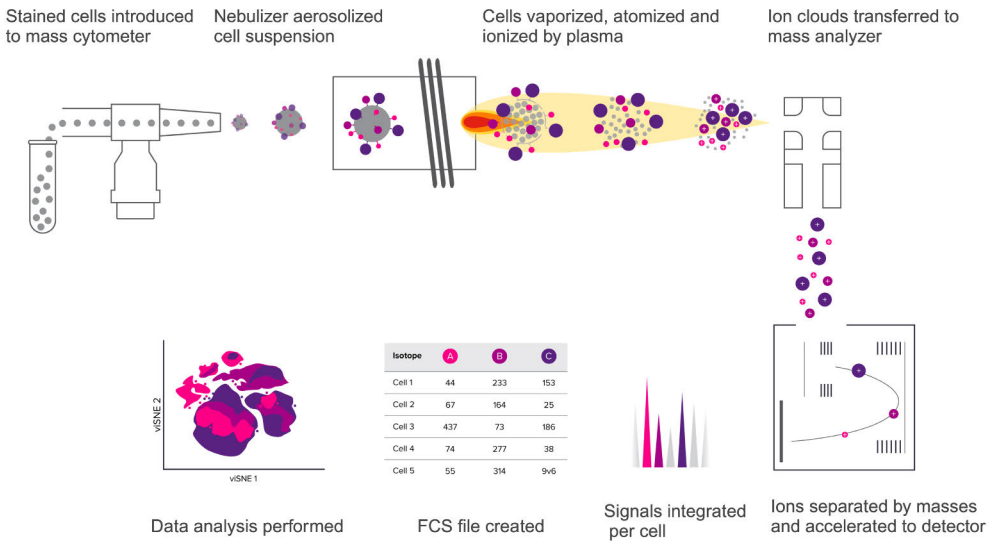

Figure 3. Mass cytometry workflow. Modified from fluidigm.com/Immuneprofile, 04/2020 and (Bandura et al., 2009). FCS, flow cytometry standard. Created with BioRender.com.

In our Study II we used Maxpar Direct Immune Profiling Assay (Standard BioTools). The assay represents a validated panel of dry metal-conjugated antibodies for deep immune profiling of human whole blood or PBMC. It has a 31-marker antibody panel, which enables identification of 37 immune cell populations as shown in **Figure 3**. Importantly, the panel was developed by the manufacturer based on the recommendations of the Human Immunophenotyping Consortium (Maecker et al., 2012). The flexibility of panel design allows 9 additional markers to be included to the main panel. In total our panel included 40 markers listed in **Table 1**. We have

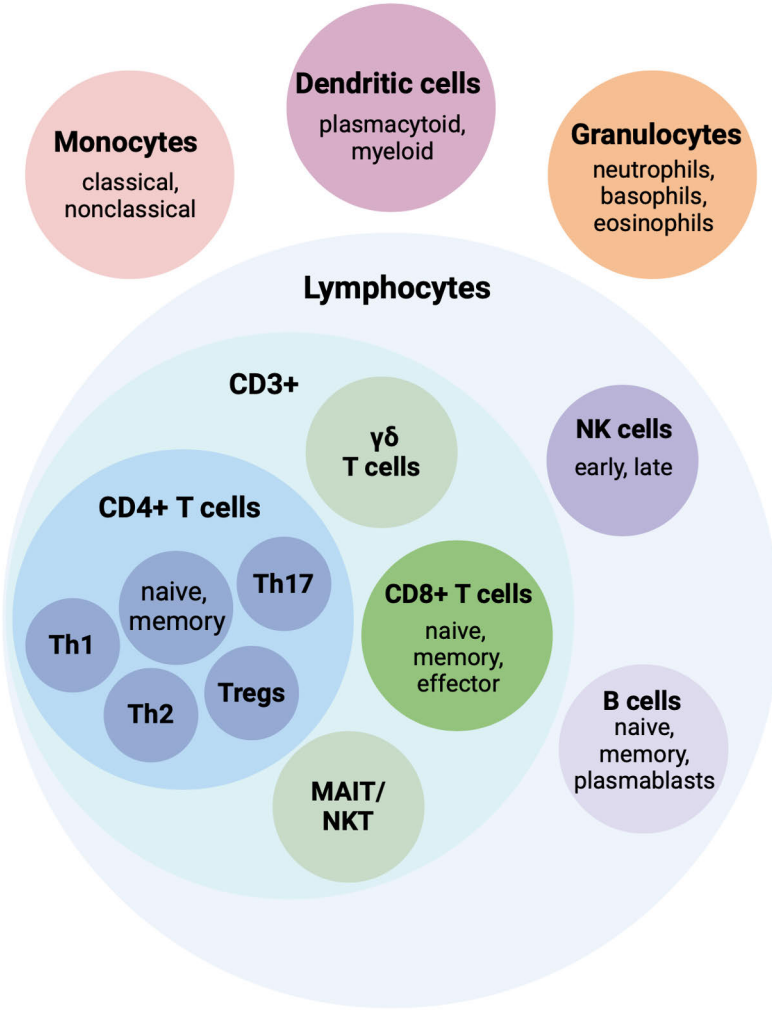

Figure 4. Circulating immune cell subsets identified by applying Maxpar Direct Immune Profiling Assay. Modified from fluidigm.com/Immuneprofile, 04/2020. Created with BioRender.com.

decided to use this opportunity to achieve a deeper insight into heterogeneity of regulatory T cells, particularly important for autoimmunity. Regulatory T cells maintain immune homeostasis by preventing detrimental immune responses and the break in immunological tolerance contribute to the development of autoimmune diseases (Grant et al., 2015). Tregs were shown to represent a heterogeneous mixture of subsets, which reflects different states of activation, maturation, and function (Mason et al., 2015). Accordingly, we have chosen to include in the panel additional markers that enable us to carry out deep characterization of regulatory T cell

properties. We included antibodies to co-receptors CTLA-4, PD-1, LAG-3 and TIGIT, markers of Treg suppressive function. Moreover, Tregs exert their suppressive function via several mechanisms. For example, ICOS and CD39 enable us to define Tregs producing immunosuppressive cytokines (IL-10 and TGFb) and Tregs capable of cAMP suppression, respectively (Mason et al., 2015).

Table 1. List of markers used in the Study II. Markers that are additional to the standard panel are denoted in italics.

Marker	Cell population	Marker	Cell population
Live/Dead	Discriminate dead cells	CD127	Treg
CD45	Leukocytes	CD25	Treg
CD66b	Granulocytes	CD294	Eosinophils, Basophils
CD3	T cells	CCR4	Treg, Th2, Th17
CD4	CD4+ T cells	CCR6	Th17
CD8	CD8+ T cells	CCR7	Naïve and memory T cells
CD11c	Monocytes, mDC	CXCR3	Th1
CD14	Monocytes	CXCR5	Tfh
CD16	Neutrophils	HLA-DR	mDC, T cells
CD19	B cells	TCR γδ	γδ T cells
CD20	B cells	CD161	MAIT/NKT cells
IgD	B cells	*LAG-3*	Treg
CD27	Plasmablasts; memory T cells	*CD69*	T cell
CD28	T cells	*CTLA-4*	Treg
CD38	Myeloid DC, plasmablasts	*CD15s*	Treg
CD45RA	Naïve T cells	*CD39*	Treg
CD45RO	Memory T cells	*PD-1*	Treg
CD56	NK cells	*ICOS*	Treg, Tfh
CD57	NK cells	*TIGIT*	T cells
CD123	Basophils, pDC	*CCR10*	T cells

3 Aims

The objective of this thesis is to study the early changes in immune responses in children progressing to type 1 diabetes, as well as those who have recently been diagnosed with the disease. The specific aims of the study are as follows:

I. Identify early epigenetic changes in children developing beta cell autoimmunity at a young age.

II. To study immune responses in children *en route* to type 1 diabetes with different first-appearing autoantibodies to provide insights into the heterogeneity of the disease.

III. Identify whole blood gene expression changes associated with disease progression in recent-onset type 1 diabetes patients during a follow-up year after the diagnosis.

4 Materials and Methods

The samples for the studies I and II were part of the two prospective cohorts: Pathogenesis of Type 1 Diabetes – Testing the Hygiene Hypothesis (DIABIMMUNE) and Type 1 Diabetes Prediction and Prevention Study (DIPP), respectively. The sample for the study III were part of the INNODIA study.

4.1 Material and methods of Study I

4.1.1 Cohort and study designs

The samples for study I were longitudinally collected as part of the DIABIMMUNE study. The DIABIMMUNE project aims at testing hygiene hypothesis and investigating its role in the development of autoimmune disease, particularly in type 1 diabetes. A total of 836 children possessing the HLA-DR-DQ risk allele were monitored and sampled at 3, 6, 12, 18, 24, and 36 months of age. Within this population, seven children developed islet-specific Aab, of which four subsequently progressed to clinical type 1 diabetes. To study DNA methylation changes associated with type 1 diabetes, we performed a longitudinal analysis of samples collected at multiple time points, specifically at 3, 6, 12, 18, 24, and 36 months of age, from seven matched case–control pairs. The selection of the pairs was based on specific matching criteria, including sex, place of birth, age and HLA risk class of individuals. The case individuals were identified as those who tested positive for at least two type 1 diabetes-specific autoantibodies at the age of 1–2 years, whereas the control individuals remained negative for autoantibodies throughout the follow-up period. The presence of autoantibodies targeting insulin (IAA), glutamic acid decarboxylase (GADA), islet antigen-2 (IA-2A), and zinc transporter 8 (ZnT8A) was measured using specific radiobinding assays on serum samples. Additionally, islet cell antibodies (ICAs) were assessed through immunofluorescence in subjects who tested positive for autoantibodies. The details of the study design are presented in **Figure 5**.

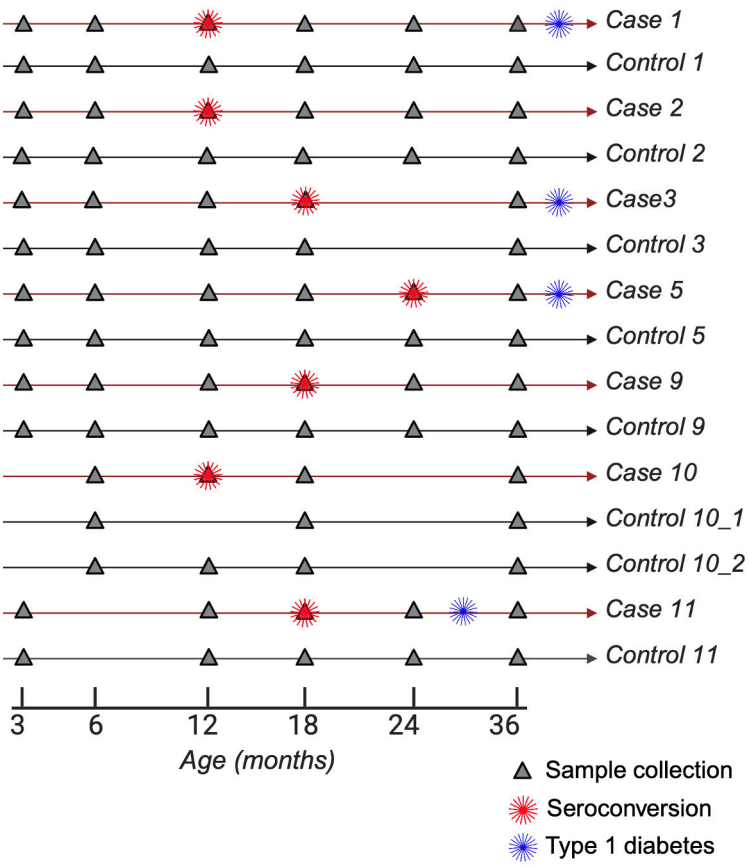

Figure 5. The cohort and design of the Study I. Reproduced from (Starskaia et al., 2022) with the permission from *Diabetologia*. Each individual case is represented by a red line positioned above the corresponding control individual, which is depicted as a black line. The time points for sample collection are indicated by triangles. The diagnosis of type 1 diabetes is denoted by blue asterisks, while seroconversion is marked by red asterisks. Created with BioRender.com.

4.1.2 Sample fractionation and DNA extraction

For the fractionation process, PBMC samples were rapidly thawed using a water bath at 37°C, followed by counting of cell numbers and assessment of cell viability. The average viability of the cells was determined to be 90%. Positive enrichment of CD4+ and CD8+ T cells was carried out using magnetic autoantibody-coupled beads. Next, DNA extraction from the isolated CD4+ T cells, CD8+ T cells, and remaining CD4−CD8− cell fractions was performed using the AllPrep Universal Kit (Qiagen), in accordance with the manufacturer protocol.

4.1.3 RRBS library preparation and sequencing

Library preparation was carried out using 100 ng of genomic DNA, following a protocol that was adapted from a gel-free multiplexed RRBS method (Boyle et al., 2012) and performed as described earlier (Laajala et al., 2022). The samples were normalized and subsequently pooled for automated cluster preparation utilizing an Illumina cBot station (Illumina). Sequencing was performed on an Illumina HiSeq 2500 instrument (Illumina) and TruSeq v3 sequencing chemistry (Illumina). Paired-end sequencing with 2 × 100-bp read length was used with a 6-bp index run. The technical quality of the HiSeq 2500 run was good, with the number of clusters as expected. Over 76% of all bases achieving an Illumina quality score of Q30 was required.

4.1.4 RRBS data analysis and data interpretation

Briefly, the data analysis workflow includes pre-processing, extraction of methylation counts, SNP removal, coverage filtering, and finally differential methylation analysis. The majority of the data analysis was carried out using R software versions 3.6.1 and 4.0.4. The annotation of DMCs to specific genomic regions was performed using the R package genomation, version 1.16.0, using the Genome Reference Consortium Human Build 37 (GRCh37/hg19).

Next, we used our previously published RNA sequencing data derived from the same samples to assess the correlations between methylation and gene expression. Spearman correlation coefficients were calculated for all samples within each cell fraction, examining the relationship between each DMC and all genes with transcription start sites (TSSs) located within a 250 kb window in both directions from the genomic location of the CpG site.

Gene Ontology terms were used for the pathway enrichment analysis. Only DMCs with Benjamini–Hochberg corrected p-value < 0.1, along with their corresponding nearest and correlating genes, were included in the analysis. GO terms were considered to be significantly enriched at FDR<0.05 (Fisher's exact test).

4.1.5 Results validation

PyroMark Assay Design 2.0 software (Qiagen) was used for the design of assays. Target-specific primers included a primer pair with site-specific biotinylation for target amplification, as well as a pyrosequencing primer. 200 ng of DNA extracted from three selected case-control pairs were used for sample preparation. First, samples were sodium bisulfite treated using EZ DNA Methylation-Gold Kit (Zymo Research). Subsequently, the selected targets were amplified using the PyroMark PCR Kit (Qiagen). A biotin-labeled template strand was then used for

pyrosequencing reaction with a specific primer on the PyroMark Q24 system (Qiagen) and using PyroMark Q24 Advanced CpG Reagents (Qiagen).

4.2 Material and methods of Study II

4.2.1 Cohort and study designs

The samples for study II were longitudinally collected as part of the DIPP study. The DIPP study was initiated in 1994 in Finland and involved the screening of over 250.000 newborns for HLA-conferred genetic susceptibility to type 1 diabetes at Turku, Oulu and Tampere University Hospitals. Approximately 10% of the screened population carry risk haplotypes. Children possessing a genetic predisposition to type 1 diabetes are subsequently invited to participate in the follow-up study, during which they are closely monitored for the development of ICA and autoantibodies to insulin, GAD, IA-2A, and ZnT8 until the age of 15 or clinical disease onset. Seroconversion is defined as positivity confirmed at two consecutive study visits. About 5-10% of individuals who carry HLA risk haplotypes will eventually progress to clinical type 1 diabetes. The discovery cohort for the Study II consisted of 29 case-control pairs. The case subjects were selected based on the type of autoantibodies that appeared first: IAA, GADA, and ≥2 Aab groups. Eleven and nine of the selected cases had IAA or GADA as the first-appearing autoantibody, respectively. Nine children showed positivity for at least two autoantibodies at seroconversion. All cases progressed to type 1 diabetes. The seroconversion ages for the respective subgroups (IAA-first, GADA-first, ≥2 Aab first) were 2.1 years (±1.2), 2.8 years (±1.1), and 4.1 years (±2.8). The onset of the disease occurred at mean ages of 8.6 years (±2.7), 10.0 years (±3.4), and 9.9 years (±4.0), respectively. The control subjects were carefully selected by matching criteria for HLA, age and sex. To study changes in early immune response associated with type 1 diabetes, we performed a longitudinal analysis of samples collected at multiple time points (**Figure 6**). The samples were selected from specific time points, including around 1 year of age, 3–6 months before seroconversion, 6–12 months after seroconversion, and approximately 1 year before the type 1 diabetes diagnosis. The PBMC samples from the children in Study II were collected over a span of two decades.

A validation cohort consisted of 30 case-control pairs. Seventeen of the cases had progressed to type 1 diabetes, and 13 were positive for multiple type 1 diabetes-associated autoantibodies. Two samples were analyzed for each case: one sample 3–6 months before and another 6–12 months after seroconversion. The IAA-first, GADA-first and ≥2 Aab first groups had 10 pairs each. The autoantibody-negative controls were selected using the same criteria as in the discovery cohort.

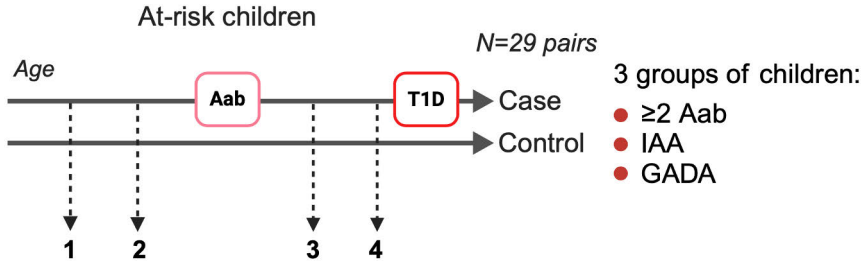

Figure 6. The schematics of Study II cohort. A case is represented by a line positioned above the corresponding control individual, which is also depicted as line. The time points for sample collection are indicated by dashed arrows. From each case and its matching control individuals we analysed three or four samples: one or two samples taken before seroconversion (indicated as time point 1 and 2) and one or two samples taken after seroconversion but before type 1 diabetes diagnosis (indicated as time points 3 and 4). The diagnosis of type 1 diabetes and seroconversion are marked on the case line. Created with BioRender.com. Aab, autoantibodies; T1D, type 1 diabetes.

4.2.2 Sample preparation, staining and acquisition

Cryopreserved PBMC samples were thawn in a water bath at 37°C, followed by a washing procedure with pre-warmed RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and a DNase-containing solution (Immunospot). For each sample, a total of 3×10^6 PBMCs were aliquoted for subsequent staining and were then washed with Maxpar Cell Staining Buffer (CSB) (Standard BioTools). Fc receptor blocking step was performed using the Human TruStain FcX Fc receptor blocking solution (BioLegend). The samples were stained with metal-conjugated antibodies for a duration of 30 minutes at room temperature. Following this, the cells underwent two washing steps with CSB. Staining was then carried out using the Maxpar® Direct™ Immune Profiling Assay (MDIPA) kit (Standard BioTools), in accordance with the manufacturer's instructions. After two additional washing steps, the cells were fixed using a freshly prepared solution of 1.6% formaldehyde for 10 minutes at room temperature. Finally, the samples were incubated overnight at 4°C with the Cell-ID intercalator solution (Standard BioTools) at a concentration of 125 nM. Prior to acquisition, the cells were washed twice with CSB and twice with Cell Acquisition Solution (Standard BioTools).

The samples were analyzed at Helios mass cytometer (Standard BioTools). A total of 300,000 to 600,000 events were collected for each sample using CyTOF Software version 6. For most of the samples, a total of 0.36, 0.37, and 0.40 million viable intact cells were analyzed for for ≥2 Aab, IAA, and GADA first groups, respectively. The minimum reliable cell count for each population was established at 100 viable intact cells.

To reduce technical variabilities and enhance data consistency, a series of methodological measures were considered. First, all samples were uniformly

handled, stained, and acquired at a single site, with all procedures performed by the same personnel and operator. Next, a master mix of additional antibodies was prepared for the entire study cohort, aliquoted, and stored at –80°C to ensure a consistent staining pattern. Furthermore, all samples corresponding to each case–control pair were processed concurrently, including thawing, staining, and acquisition within the same batch. The statistical model employed accounted for batch effects by incorporating the case–control pair as a random effect. Additionally, an internal control, specifically a PBMC sample sourced from a single donor, was utilized. This internal control sample was processed, stained, and acquired along with the study samples from each case–control pair.

4.2.3 Data analysis

Data cleanup was performed using the automated Maxpar Pathsetter™ software version 2.0 (Standard BioTools). Subsequent data analysis was carried out the R software, version 4.3.0. The data were pre-processed using the flowCore (v. 2.12.2) and transformed using arcsin with a cofactor of 5. The cell clustering was performed using the FlowSOM package (version 2.8.0), followed by metaclustering with the ConsensusClusterPlus package (version 1.64.0). First, we identified the main immune cell types of PBMC samples, followed by exploring the heterogeneity of CD4+ and CD8+ T cell populations. For the PBMC heterogeneity analysis, we used a combination of unsupervised clustering and supervised clustering (removing and merging of clusters and manual annotation of clusters based on known biology). To identify various cell types—including B cells, CD8+ T cells, γδ T cells, natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, NK cells, plasmacytoid dendritic cells (pDCs), CD4+ T cells, basophils, monocytes, and myeloid dendritic cells (mDCs)—we utilized a range of lineage-specific markers, including CD45, IL-3R/CD123, CD19, CD4, CD8a, CD11c, CD16, CD161, CD56, γδ TCR, CD294, CD14, CD3, CD20, CD66b, and HLA-DR. The unsupervised clustering yielded a total of 20 clusters; however, four clusters were excluded from further analysis due to their representation of either debris (lacking lineage markers expression) or doublets (exhibiting the expression of multiple lineage-specific markers, such as concurrent expression of CD3 and CD20). Additionally, the CD66b+ granulocyte cluster was removed, as it was uncertain whether it represented low-density neutrophils or contamination with conventional neutrophils during the sample preparation process. The remaining clusters were subsequently merged based on lineage marker expression, resulting in the identification of 10 main cell types. Following the identification of these cell types, we determined the cell type proportions and the mean marker intensities in each cell type.

To study the variations in cell type proportions between case and control groups, we applied linear mixed-effects modeling implemented in the lmerTest package (version 3.1-3). Age, sex, HLA-DR status (classified into three groups: carriers of DR3 haplotype, DR4 haplotype, and individuals possessing both haplotypes), and case-control status were designated as fixed effects, while processing batch and case-control pair were treated as random effects, following the model*:* Cell type proportion ~ Age + Sex + HLA + Age*CaseCtrl + (1|Batch) + (1|Pair). The significance of sample subsets (IAA-first, GADA-first, and ≥2 Aab first groups) was further evaluated using the model: Cell type proportion ~ Age + Sex + HLA + Age*Aab Group + (1|Batch) + (1|Pair). A likelihood-ratio test was used to compare the goodness of fit of the two models for each cell type. For each sample subset (IAA-first, GADA-first, and ≥2 Aab first groups), we statistically tested the differences in marker intensities between case and control groups using linear mixed-effects modelling. In this analysis, age, sex, HLA-DR3/4 status, and case-control status were treated as fixed effects, while case-control pair was considered a random effect. The model for each sample subset was expressed as: Marker intensity ~ Age + Sex + HLA + Age*CaseCtrl + (1|Pair). For the comprehensive analysis across all samples, the model used was: Marker intensity ~ Age + Sex + HLA + Age*CaseCtrl + (1|Batch) + (1|Pair). Furthermore, we assessed the heterogeneity of CD4+ and CD8+ T cells using functional markers, which involved secondary sub-clustering of the main clusters. The proportions of cell subtypes and mean marker intensities were determined similarly as above and subjected to statistical testing. False discovery rates (FDR) were adjusted using the Benjamini–Hochberg method against all marker levels in all cell type clusters. This correction was performed separately for cell type proportions and for the additional subsets of CD4+ and CD8+ T cells. Findings were considered to be significant at FDR < 0.05.

4.2.4 Results validation

Results were validated in an independent cohort of children. Two statistically significant findings, CD161 expression in NK cells and CD39 expression in memory Tregs were validated using flow cytometry technology. Upon thawing, PBMCs were washed with a buffer (PBS, 2%FBS, 0,1%NaN3 sodium azide). For the detection of CD161 in NK cells, PBMCs were incubated with a specific antibody cocktail for a duration of 30 minutes at 4°C, protected from light. The antibody cocktail included CD45 FITC (BioLegend), CD19 PE (eBioscience), CD3 PerCP-Cy5.5 (BD Biosciences), CD14 PE-Cy7 (BioLegend), CD56 BV421 (BD Biosciences), and CD161 APC (BioLegend). For the detection of CD39 in memory regulatory T cells Tregs, CD4+ T cells were isolated from the PBMCs using a human CD4 T cell isolation kit (Dynabeads, Thermo Fisher). The isolated CD4+ T cells were then

incubated with a separate antibody cocktail for 30 minutes at 4°C protected from light. This cocktail consisted of CD3 PerCP-Cy5.5 (BD Biosciences), CD4 BV421 (BD Biosciences), CD25 BB515 (BD Biosciences), CD127 PE-Cy7 (BioLegend), CD45RA BV786 (BioLegend), CD45RO PE (BD Biosciences), and CD39 APC (BioLegend). Following the surface staining with antibodies, the cells were incubated with Fixable Viability Dye eFluor 780 (eBioscience, Thermo Fisher Scientific). Data acquisition was performed using the BD LSRFortessa™ Cell Analyzer (BD Biosciences, USA) in using BD FACSDiva™ software. Data were analyzed with FCS Express 7 Flow software (De Novo Software).

4.3 Material and methods of Study III

4.3.1 Cohort and study designs

The samples for the study III were collected from the first 100 newly diagnosed patients as part of the INNODIA study, comprising 52 males and 48 females. The cohort was chosen based on clinical information, positivity at least for one type 1 diabetes associated autoantibodies (IAA, GADA, IA-2A, or ZnT8A), gender distribution, and sample availability. A final cohort consistent of 94 patients with an average age of 13.2 years and a disease duration of 3.9 weeks at baseline. Samples were collected within 6 weeks of diagnosis (n = 92) and 12 months post-diagnosis (n = 49). Measurements included C-peptide, glucose, HbA1c, and islet-related autoantibodies, and were analysed in certified hospital labs following harmonized protocols.

4.3.2 RNA sequencing and data pre-processing

Frozen whole-blood PAXgene samples were thawed at room temperature for a duration of 2 hours before the extraction of RNA. RNA extraction was carried out using PAXgene Blood miRNA Kit (PreAnalytix/Qiagen). Library preparation and sequencing were performed at the Finnish Functional Genomics Centre. The RNA sequencing libraries were prepared utilizing the TruSeq stranded mRNA HT kit and the associated protocol (Illumina). The quality and quantity of the amplified libraries were subsequently evaluated using the Advanced Analytical Fragment Analyzer (Agilent) and Qubit Fluorometric Quantitation, respectively. Finally, the pooled libraries were sequenced on an Illumina NovaSeq 6000 instrument, using a paired-end sequencing approach with a read length of 2 × 50 base pairs.

Prior to normalization, hemoglobin-related genes: HBA1, HBA2, and HBB, which accounted for approximately 11% of all reads, were filtered. The resulting filtered data were scaled in counts per million (CPM) using the Trimmed Mean of

the M-values (TMM) normalization factors and log2 transformed using the R package edgeR. Genes exhibiting an average CPM >1 were included into the analysis.

4.3.3 C-peptide/glucose ratio

The fasted C-peptide to glucose ratio was used as a measure for disease progression due to the unavailability of mixed meal tolerance test (MMTT) data for participants aged 5 years or younger, which would have significantly reduced the number of participants eligible for the analyses. Previous research has proposed that baseline C-peptide, adjusted for baseline glucose levels, serves as an appropriate surrogate of MMTT area under the curve (AUC) (Ruan et al., 2019).

4.3.4 Data analysis and interpretation

A linear mixed effects model was applied to gene expression data, treating visit, sex, and age as fixed effects, while individual, sequencing pool, and study site were random effects. The linear mixed effects modeling was carried out using the lmerTest R package, with Benjamini-Hochberg adjusted p-values for multiple testing. Models were tested with and without body mass index (BMI) as a covariate, however only 3% of the genes analyzed (450 out of 13,558) showed a significantly better fit with BMI (lower Akaike information criterion AIC and a Chi-squared test $p < 0.05$ between the models). The results were largely consistent regardless of BMI inclusion, leading to the decision to use the model without BMI to enhance the generalizability of the model. Additionally, while many comorbidities were identified, only a limited number of individuals exhibited shared comorbidities. We have decided not to include comorbidities into our model, as their inclusion leads to the model complexity and risk of overfitting.

Proteins from 187 differentially regulated genes identified between visits were analyzed using the STRING database (https://string-db.org/, accessed on September 10, 2021), incorporating both experimentally validated and predicted interactions with a combined confidence threshold of 0.4, representing a medium level of confidence. Pathway enrichment analysis was conducted separately for upregulated and downregulated genes using Gene Ontology platform (http://geneontology.org). Pathways were considered significantly enriched at FDR <0.05 (Fisher's exact test).

Cell-type proportions were estimated from RNA sequencing data using the EPIC computational deconvolution method (Racle et al., 2017). A signature matrix was developed from a publicly available human immune cell dataset (GEO accession number GSE60424) utilizing the CIBERSORTx online tool (Newman et al., 2019). This dataset includes TMM-normalized RNA-seq data from FACS-sorted whole-

blood samples, which includes neutrophils, monocytes, B cells, CD4+ T cells, CD8+ T cells, and NK cells across various diseases.

Uniform manifold approximation and projection (UMAP) (McInnes et al., 2018) was utilized to analyze gene ratios between baseline and 1-year follow-up visit, employing the uwot R package with the parameters set to a neighborhood size of 15 and a minimum distance threshold of 0.001. Associations between gene expression ratios and clinical variables were assessed using Pearson's correlation for continuous variables and point-biserial correlation for dichotomous variables, using only complete pairs of observations. Glycated hemoglobin (HbA1c) values exceeding 100 were excluded as outliers. Additionally, ranked gene set enrichment analyses were conducted against Hallmark sets from the Molecular Signature Database (MSigDB, version 6.2) using the R package fgsea, with a significance threshold set at p-value <0.01.

Individuals were classified as rapid or slow progressors based on changes in their fasted C-peptide/glucose ratio from baseline to a 2-year follow-up. Hierarchical clustering using Euclidean distances and complete linkage divided participants into three groups: rapid progressors (ratio decrease <−30), slow progressors (ratio increase >5), and the rest as intermediates. Gene expression ratios were analyzed between baseline and a 1-year follow-up, with differences between rapid and slow progressors assessed using a reproducibility optimized test statistic (Suomi et al., 2017). A paired test was utilized, with 1000 bootstrap and permutation samplings, and a top list size of 10,000. The score for each individual was determined based on 16 differentially expressed genes ($p < 0.01$), calculated as the difference between the mean expression levels of downregulated and upregulated genes, following a similar methodology as in previous study (Seyednasrollah et al., 2016).

4.3.5 Validation

To validate our model, we used published data from the whole blood transcriptome analyses of newly diagnosed type 1 diabetes patients (Dufort et al., 2019). Validation data were obtained from Gene Expression Omnibus (accession number GSE124400). Gene expression ratios between baseline and 1-year measurements were calculated for 57 patients with transcriptomics data from comparable timepoints (at diagnosis and 1-year follow-up visits). A score for each individual was derived by subtracting the mean ratios of upregulated genes from the mean ratios of downregulated signature genes, based on 16 signature genes identified in the discovery cohort. Changes in AUC C-peptide levels from a 2-hour mixed-meal tolerance test were assessed between baseline and 2-year follow-up visits using the first and last available measurements. Participants were grouped into six clusters through hierarchical clustering based on Euclidean distances and Ward's linkage.

The two clusters showing the largest decrease in C-peptide levels over time were classified as rapid progressors, while those with the smallest decrease were classified as slow progressors.

5 Results

5.1 Early DNA methylation changes in children developing beta cell autoimmunity (Study I)

To investigate the early epigenetic changes associated with type 1 diabetes prior to diagnosis and the onset of autoantibodies, we employed RRBS to analyze DNA methylation in purified CD4+ T cells, CD8+ T cells, and CD4−CD8− cell fractions. A differential methylation analysis of all longitudinal samples (**Figure 5**), specifically those collected prior to the clinical onset of type 1 diabetes and seroconversion, was performed by applying linear mixed effects modelling. The analysis identified 225, 114, and 87 differentially methylated CpGs in CD4+ T cells, CD8+ T cells, and CD4−CD8− cell fractions, respectively, with a false discovery rate (FDR) < 0.1 and a mean methylation difference > 0.1. In CD4+ T cells, we found hypomethylated DMCs at the intergenic region between *TRAF3* and *RCOR1* (FDR=1.24×10^{-5}), and promoter of *ARRDC2* (FDR=1.94×10^{-5}), while hypermethylated DMCs were observed at the promoters of *RGPD8* (FDR=1.25×10^{-7}) and *FOXR1* (FDR=3.71×10^{-6}) in cases compared to controls. In CD8+ T cells, the most significant DMC was identified within the intergenic region located between the pseudogene *LOC390705* and the non-coding RNA *LOC113002582* (FDR=1.27×10^{-5}) and was hypomethylated in cases compared to controls. In the CD4−CD8− cell fraction, significant differences in methylation levels were detected in proximity to the protein-coding genes *WDR7* (hypermethylated in cases vs controls, FDR=7.62×10^{-12}) and *EXOC5* (hypomethylated in cases vs controls, FDR=1.25×10^{-7}). The study also combined individual DMCs into differentially methylated regions (DMRs), identifying 79, 56, and 45 DMRs across the cell types. Notably, most DMRs were cell type-specific, only three DMRs showed consistent methylation changes across all fractions. The majority of DMRs were located in intergenic regions (47% in CD4+ T cells, 39% in CD8+ T cells, and 44% in CD4−CD8− cells), with a smaller proportion in introns and promoters.

Furthermore, we performed differential methylation analysis in the samples collected prior to seroconversion, which is designated as pre-seroconversion analysis. The analysis revealed significant DNA methylation differences before the

detection of islet autoantibodies, identifying 249, 144, and 143 DMCs in CD4+, CD8+, and CD4−CD8− cell fractions, respectively. A majority of DMRs were specific to the pre-seroconversion phase. Significant DMCs included one at the *ARRDC2* gene promoter (hypomethylated in cases vs controls, FDR=1.64×10^{-16}) in CD4+ T cells and another in the *PCBP3* intron (hypomethylated in cases vs controls, FDR=4.78×10^{-16}) in CD8+ T cells. Interestingly, *ARRDC2* has been previously linked to early-onset Crohn's disease (Zeissig et al., 2015).

Additionally, we examined the relationships between DNA methylation and gene expression by utilizing RNA sequencing data derived from the same samples. The analysis identified DMCs with a significant correlation (Spearman rank coefficient >|0.5|) to gene expression, particularly in CD4+ and CD8+ T cells. Notable findings include two DMCs in CD4+ T cells associated with the *DGKQ* gene expression showing positive correlation (Spearman $\rho = 0.619$), while four DMCs near the *PCBP3* gene exhibited an inverse correlation with gene expression (Spearman $\rho = -0.591$).

Finally, we validated the RRBS results through pyrosequencing from the same participants (N= three case-control pairs) within the same cohort. The methylation direction was consistent between RRBS and pyrosequencing for eight out of ten targets. Significant positive correlations were found between RRBS and pyrosequencing at specific CpG sites located in the promoter of *ARRDC2*, intron of *PCBP3*, and in the intergenic region near *TRAF3*, all within CD4+ T cells. In CD8+ T cells, a DMC near *IL32* was validated, showing hypomethylation in the promoter region in type 1 diabetes compared to controls.

5.2 Distinct immune responses in children progressing to type 1 diabetes with different first-appearing autoantibodies (Study II)

In this study we analyzed the composition of PBMCs in children who later developed type 1 diabetes using a 40-marker mass cytometry panel. The heterogeneity of PBMC was assessed in two steps. First, we identified main immune cell types of PBMC samples, and further explored the granularity of CD4+ and CD8+ T cells. To examine changes in cell type proportions and marker expression in PBMC samples collected longitudinally at multiple timepoints (**Figure 6**), we applied linear mixed effect modelling. We found significant differences in cell type proportions in the ≥2 Aab first and GADA-first groups, but not in the IAA-first group. Notably, NK cells were more abundant in the ≥2 Aab first (FDR=0.04) and GADA-first (FDR=0.046) groups, while the ≥2 Aab first group had fewer CD4+ (FDR=0.005) and CD8+ (FDR=0.0005) T cells compared to controls. Conversely, the GADA-first group showed an increase in CD8+ T cells (FDR=0.007) in cases vs controls. When we did

combined analysis of all samples, no significant differences in cell type proportions were found between children with type 1 diabetes and controls, except for a decrease in CD8+ T cells (FDR=0.008) linked to disease progression.

We further analysed the expression of functional proteins in the identified subsets. In children from the ≥2 Aab first group, there was a significant upregulation of CD161 expression in both NK (FDR=0.034) and CD4+ T cells (FDR=0.00006) compared to controls, particularly noticeable at early disease stages before seroconversion. CD161 was also upregulated in CD4+ T cell subsets, including CD4+CCR4+ and CD45RA+CCR7+ with the latter representing a majority of the CD4+ T cells and exhibiting a naive phenotype. CD161 expression levels in NK and CD4+ T cells did not differ between cases and controls in the IAA- and GADA-first groups or when analyzed across all samples.

Further, TIGIT expression was found to be downregulated in the CD57+ subset of CD4+ T cells (FDR=0.006) in children from the ≥2 Aab first group compared to controls. This subset, which may represent cytotoxic T cells, comprised up to 0.52% of the total CD4+ T cell population. In contrast, no significant differences in TIGIT expression were observed in the IAA- or GADA-first groups. On the other hand, CD39 levels were elevated in both CD4+ and CD8+ T cell (FDR=0.036) subsets among IAA-first children. Specifically, increased CD39 expression was noted in the CD25+CD127– memory Treg phenotype (FDR=0.007) and HLA-DR+ICOS+ effector memory T cells (FDR=0.005). No differences in CD39 expression were detected in the ≥2 Aab or GADA-first groups across all samples.

Finally, we carried out validation of selected targets in an independent cohort of children using flow cytometry. The selection of validation targets was based on expression levels and statistical significance: CD161 in NK cells and CD39 in Treg cells. The results confirmed that CD161 expression in NK cells was significantly higher in children with ≥2 Aab at initial seroconversion (p=0.0018, n=40) compared to controls, with a notable difference observed at the early stage of disease development prior to seroconversion. However, we did not validate the results of CD39 differential expression (n=28).

5.3 Gene expression changes predicts rate of type 1 diabetes progression (Study III)

In this study we investigated gene expression changes in whole blood during the first year after type 1 diabetes diagnosis through RNA sequencing of 141 samples. The analysis identified 187 differentially expressed genes between the baseline and 1-year follow-up. Gene Ontology enrichment analysis showed significant pathways related to immune response to bacteria and oxidative phosphorylation among the downregulated genes, while no enrichment was found for upregulated genes. A

protein interaction network analysis revealed four functional modules, highlighting that genes associated with defense against bacteria and oxygen transport were downregulated over time.

We next explored the correlations between gene expression and various autoantibodies (IAA, GADA, IA-2A, and ZnT8A) in patients during a baseline visit. Significant associations were found between gene expression changes and ZnT8A autoantibody positivity. A total of 421 genes exhibited a point-biserial correlation with ZnT8A autoantibody positivity, (FDR < 0.05, |r| > 0.4). Among these identified genes, *IL6R*, *RBPJ*, *SKAP2*, *SIRPG*, and *UBASH3A* are located in proximity to SNPs associated with type 1 diabetes. Additionally, we studied whether gene expression changes correlated with clinical measures of disease progression, specifically the change in C-peptide/glucose ratio at two-year follow-up visit. The analysis revealed 392 genes associated with C-peptide/glucose ratio ($p < 0.05$, $|r| > 0.4$). Noteworthy genes positively associated with C-peptide changes included *CXCR4* and *BTN3A2*, both relevant to autoimmune diabetes.

We next sought whether changes in gene expression during the first year after diagnosis can predict the rate of disease progression in patients with type 1 diabetes over a two-year period. Patients were categorized into three groups based on changes in their fasted C-peptide/glucose ratio: slow progressors, rapid progressors, and an intermediate group. A comparison of gene expression data from the baseline and one-year follow-up revealed 16 signature genes that could differentiate rapid and slow progressors, including oxidative phosphorylation (*COX7B*, *COX7C*, *ATP5MPL*), RNA processing and translation (*SNRPE*, *LSM3*, *SAPNP*) and neutrophil-specific genes *C4BPA*, *NCF1*, *PRRG4*. Notably, these gene expression changes exhibited opposite profiles between the two groups of fast and rapid progressors. We next validated our model using independent published dataset, representing the cohort of new-onset type 1 diabetes patients (N=57). The difference in the AUC C-peptide decline between the predicted groups was statistically significant (Wilcoxon rank sum test p=0.0077).

Finally, neutrophils were identified as the cell type most strongly linked to disease progression. Neutrophil abundance positively correlated with the progression of the disease, while B cell proportions were inversely correlated with neutrophil levels. This is consistent with previous studies indicating that higher B cell proportions are associated with faster disease progression.

6 Discussion

The Study I identifies DNA methylation changes in children who develop type 1 diabetes prior to clinical diagnosis and seroconversion. By analysing purified cell fractions from PBMCs, specifically CD4+, CD8+, and CD4–CD8– subsets, we identified numerous immune cell subset-specific DNA methylation modifications. Notably, these changes were detected in genes previously associated with type 1 diabetes, including *IL32*, *TRAF3*, and *DGKQ*, as well as in novel candidate genes such as *ARRDC2* and *PCBP3*. Notably, in this study we found hypomethylation at the promoter region of *IL32* gene in CD8+ T cells of cases compared to controls. Furthermore, methylation-expression analysis indicated a weak inverse correlation between *IL32* mRNA expression and methylation status. These findings suggest that epigenetic modifications at the *IL32* promoter may lead to the increased expression of *IL32* mRNA level. These observations confirm the findings of the transcriptome analysis, which revealed significant upregulation of *IL32* in cases compared to controls (Kallionpää et al., 2019).

Although the small sample size (n=7) limits the study, matched controls were used to mitigate confounding factors, and multiple time points were analyzed for each individual. A key strength of the study was the correlation between DNA methylation and gene expression in the same samples, highlighting genes potentially influenced by differential methylation.

While previous studies did not find significant methylation differences associated with type 1 diabetes progression at birth (Laajala et al., 2022; Paul et al., 2016), our study indicates DNA methylation changes can be detected at early stage of disease development, even prior to seroconversion. These results are consistent with the study by Johnson et al., which identified pre-seroconversion DNA methylation changes that could predict disease progression (Johnson et al., 2020). However, there was no overlap with findings from this study, possibly due to methodological differences. The study conducted by Johnson et al. focused on the analysis of whole peripheral blood samples, wherein neutrophils represent the most abundant cell type, comprising up to 70% of the total cellular population. In contrast, our study specifically examined epigenetic changes in the CD4+, CD8+ T cell, and CD4–CD8– cells subsets of PBMC samples. Furthermore, the aforementioned study

employed the Illumina 450K DNA methylation microarray platform, which is limited to approximately 450,000 methylation sites. In contrast in our study, we used RRBS, enabling identification of 1.95, 2.46, and 1.79 million CpG sites in the CD4+, CD8+ T cell, and CD4–CD8– cells subsets, respectively. Nonetheless, an important finding shared by both studies is the presence of detectable epigenetic changes in circulating immune cells at the very early stages of disease development. The findings indicate a complementary relationship between the two studies, contributing to a more comprehensive understanding of the epigenetic landscape associated with disease progression.

Overall, the results of the Study I provide new insights into cell type-specific DNA methylation changes associated with type 1 diabetes, establishing a foundation for future research aimed at identifying early methylation signatures for disease prediction and management and providing insights into disease pathogenesis.

Next, previous studies reported a considerable heterogeneity in the development and rate of progression to clinical type 1 diabetes, which hampers the understanding of disease pathogenesis and developing therapies.

It has been hypothesized that the molecular mechanisms and disease pathways are distinct in different individuals. The immune cell composition in the pancreatic islets differs between young and older children with type 1 diabetes. Younger children (diagnosed at age 7 or younger) exhibit more inflammatory lesions with T and B cells, while older children (diagnosed at age 12 and older) show minimal B cell presence. Disease progression was described to be more aggressive in young children compared to older ones (Leete et al., 2016, 2020). Additionally, studies reported that there are differences in the progression to clinical disease in children who develop either IAA (seen predominantly in young children) or GADA as the first autoantibodies (Krischer et al., 2021). Ability to define, detect and understand patient populations with distinct underlying pathogenesis i.e., endotypes, would be a breakthrough towards personalized treatment.

In the Study II we tested the hypothesis that there are distinct characteristics of early immune response in children who later develop type 1 diabetes based on the type of autoantibody that appears first. Such differences were indeed detected comparing children whose first autoantibodies were IAA or GADA or who already had ≥2 autoantibodies in their first sample. The analysis revealed type 1 diabetes associated changes in cellular composition and expression of proteins in circulating immune cells depending on the type of autoantibodies that appear first. Importantly, when the results from all study participants were combined, the analysis revealed no such differences between those who later developed type 1 diabetes and controls. Nevertheless, interpreting these findings in the context of disease pathogenesis presents challenges, as the relationship between changes in circulating immune cells and the immune responses occurring within the pancreatic tissue remains unclear.

To enhance the understanding of these results, further investigations are needed, including comprehensive immune profiling of blood samples, pancreas-draining lymph nodes, and pancreatic tissue itself.

The results of the study support previous observations on heterogeneity of type 1 diabetes and indicate that immune response differs between children depending on the type of autoantibodies that is detected first. The findings may reflect different pathways and pathogenesis, possibly endotypes underlying the disease development.

The findings of this study may be useful for developing markers for early detection and prediction of type 1 diabetes. The application of omics technologies shows promise in the discovery of novel prognostic biomarkers that can enhance the prediction of this autoimmune condition. Notably, T cells, which play a crucial role in the pathogenesis of type 1 diabetes, have emerged as particularly promising candidates for further studies. Several antigen-nonspecific candidate T-cell biomarkers have been proposed (Ahmed et al., 2019). Among these, an increased frequency of circulating memory Tfh cells was observed in individuals at risk and in those recently diagnosed with type 1 diabetes (Ferreira et al., 2015; Viisanen et al., 2017). Additionally, another potential biomarker identified is the elevated proportion of IL-17-producing naïve and memory CD4+ and CD8+ T cells in patients with type 1 diabetes (Honkanen et al., 2010; Marwaha et al., 2010). Interestingly, in our study, we observed a significant increase in the expression of CD161+ CD4+ T cells, exhibiting both naïve and effector phenotypes, in children with two or more autoantibodies compared to control subjects. CD161 is recognized as a marker associated with Th17 cells. Our findings support the observation on the increased expression of Th17 cell signatures in type 1 diabetes and suggest that these differences may manifest prior to the clinical onset of the disease. Should these results be validated in additional cohorts, they may serve as prognostic biomarkers for type 1 diabetes progression.

In conclusion, the results of Study II encourage further investigations and highlight the importance of analyzing the factors underlying the heterogeneity to open the possibilities to intervene earlier in a pathway-specific manner in the disease process to delay or prevent its progression in children.

The Study III investigated gene expression changes in the peripheral blood of newly diagnosed type 1 diabetes patients during the first-year post-diagnosis and their correlations with the C-peptide/glucose ratio. We found significant changes in gene expression during one year follow-up after the disease onset. The genes were primarily associated with immune response to bacteria, oxidative phosphorylation, and RNA processing. Notably, the immune response to bacteria, particularly the neutrophil-mediated response, was one of the most significantly downregulated processes as the disease progressed. Interestingly, prior research observed a correlation between type 1 diabetes and an elevated risk of infectious diseases, with

patients having a higher prevalence of bacterial infections compared to non-diabetic individuals (Bertoni et al., 2001; Shah & Hux, 2003; Simonsen et al., 2015). This phenomenon may be attributed to a reduced immune response to bacterial infections with the disease progression. Alternatively, the observed downregulation of immune responses to bacteria during 1-year follow-up year after the diagnosis could be interpreted as a normalization of immune activity, which may have been elevated at the onset of type 1 diabetes. However, this interpretation remains speculative, as the study design did not include a control group of non-diabetic individuals for comparative analysis.

Interestingly, we found decrease in neutrophil proportions significantly associated with rapid C-peptide loss. Previous studies indicated that newly diagnosed patients with type 1 diabetes exhibit lower neutrophil levels, as well as autoantibody-positive individuals when compared to non-diabetic controls (Harsunen et al., 2013; Valle et al., 2013). Additionally, we identified a strong inverse correlation between estimated B cell proportions and neutrophil levels, supporting previous findings that higher B cell proportions are linked to faster disease progression (Dufort et al., 2019).

Finally, we identified 16-gene signature that could predict the rate of insulin secretion decline, and validated the prediction model in the independent dataset (Dufort et al., 2019).

Overall, the study found gene expression changes in recent-onset type 1 diabetes patients and identified a predictive gene signature for disease progression. A predictive signature may be useful in patient stratification, leading to more homogeneous groups of patients for clinical trials and therapies. Additionally, it may also enable earlier interventions, potentially slowing disease progression and improve monitoring of disease progression.

7 Summary/Conclusions

This thesis focused on studying early changes of immune response of circulating immune cells from children who later progressed to type 1 diabetes. Additionally, it aimed at exploring immune profiles of children with distinct autoantibodies profiles. To address these questions, we used state-of-the-art methods and clinical samples from different cohorts. Our findings:

We identified DNA methylation changes in purified CD4+, CD8+, CD4–CD8– cell subsets of PBMC samples from children who later develop beta cell autoimmunity and type 1 diabetes and their matched controls. The results of the study provide novel insights into cell type-specific differential epigenetic regulation of genes that may play a role in the pathogenesis of type 1 diabetes (Study I).

We found differences in immune cell composition of PBMC samples from children who later develop disease depending on the type of autoantibodies that appear first (IAA-first, GADA-first, ≥2 autoantibodies first groups). The analysis also revealed type 1 diabetes associated changes in expression of proteins important for immune cell functions in circulating immune cells. The results of the study support previous observations on heterogeneity of type 1 diabetes and indicate that immune response differs between children depending on the type of autoantibodies that is detected first (Study II).

Finally, we identified transcriptome changes of circulating immune cells from newly diagnosed type 1 diabetes patients during one follow-up year after the diagnosis. Using clinical measures and gene expression changes, we identified a 16-gene signature that predicted the rate of disease progression (Study III).

In conclusion, this thesis contributes to the current body of knowledge on the early immune response changes associated with the type 1 diabetes. By employing cutting-edge methodologies, we have identified epigenetic changes that may play important roles in the pathogenesis of the disease. Additionally, our findings support the heterogeneous nature of type 1 diabetes. The results encourage further studies and highlight the importance of analyzing the factors underlying the heterogeneity to open the possibilities to intervene earlier in a pathway-specific manner in the disease process to delay or prevent its progression in children.

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