Autoimmunity-associated polymorphisms and their effects on the immunophenotype

Cohort generation and subject immunophenotyping in the Finnish Diabetes Prediction and Prevention study

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Abstract

Type 1 diabetes (T1D) has a multifactorial etiology wherein both genetic and environmental factors predispose and modulate an individual's risk of developing the disease. Hereditary factors are perceived to be the fundamental facilitators of the diabetic pathogenesis since they evidently predispose children to T1D via effects that modulate the immunological homeostasis, alter gene-environment interactions and enable the escape of self-reactive T and B cells targeting pancreatic self-antigens. Furthermore, environmental factors, such as viral infections, have been shown to modulate the risk of developing autoimmunity; with some factors exhibiting evident disease-associated effects, and others demonstrating weak or contradictory disease associations. This thesis project aimed to generate a cohort of 700 children enrolled in the Finnish Diabetes Prediction and Prevention study (DIPP). The cohort was genotyped for ten single nucleotide polymorphisms (SNP) associated with T1D and thought to modify the phenotype of immune cells. The generated data repository was thereafter used to perform a pilot immunophenotyping study of twelve matched pairs in order to demonstrate the feasibility of the study design and to study the effects of the rs231775 (A>G) polymorphism on CTLA-4 expression in conventional T cells and regulatory T cells (Treg). The thesis study's assembly of a novel DIPP data repository gave rise to a cohort of 694 genotyped, non-diabetic, autoantibody-negative, at-risk children with ≥ 2 stored peripheral blood monocyte samples taken between the ages of six months and five years. When studying CTLA-4 surface expression on PMA/Ionomycin stimulated $CD3^+$ T cells, amongst nine matched pairs (rs231775, GG vs. AA), we detected no differences in the frequency of CTLA-4^+ $CD4^+$ cells and $CD8^+$ cells. However, when staining CTLA-4 intracellularly in nonstimulated cells, the study of eleven matched pairs revealed a significant alteration in the frequency of CTLA-4^+ $CD3^+CD4^+CD25^+CD127^{Low}FOXP3^+$ Treg cells. Homozygosity for the risk-associated G-allele conferred a decreased frequency of CTLA-4^+ cells when examining total CTLA-4^+ Treg cells ($p = 0.0091$), naïve $CD45RA^+$ Treg cells ($p = 0.0136$), and with a borderline significance, memory $CD45RA^-$ Treg cells ($p = 0.0631$). The results maintain the assertion that the rs231775 SNP modifies the frequency of CTLA-4^+ Treg cells. Thus, further study is warranted to investigate whether the SNP affects the surface availability of CTLA-4 and weakens the immunoregulatory ability of Treg cells.

Swedish abstract

Typ 1 diabetes (T1D) har en multifaktoriell etiologi där både genetiska och miljöbetingade faktorer predisponerar och omreglerar en individs benägenhet för att utveckla T1D. Ärftliga genetiska faktorer anses utgöra den främsta etiologiska orsaken bakom sjukdomsutvecklingen. Detta beror på att genetiska faktorer ger upphov till immunologiska riskeffekter som omreglerar en immunofenotyps cellulära homeostas, modifierar gen-miljöinteraktioner och möjliggör en okontrollerad expansion av autoreaktiva T- och B-celler; autoreaktiva celler som initierar en autoimmun attack mot de insulinproducerande β-cellerna. Avhandlingsprojektet var avsett att skapa en kohort på 700 barn värvade till den finländska prospektiva studien The Diabetes Prediction and Prevention Study (förkortning, DIPP). Kohorten genprofilerades för förekomsten av tio olika enbaspolymorfismer förknippade med en ökad risk för T1D-utveckling och som förväntas ha en modifierande påverkan på immunofenotypen. Datamaterialet användes därefter för att utföra en immunofenotypprofilering hos tolv fenotypparade individer. Profileringens målsättning var att granska studiedesignens utformning och studera CTLA-4 (+A49G) rs231775-polymorfismens förmodade modifierande verkan på proteinuttrycket av CTLA-4. Avhandlingsprojektet skapade en datasammanställning som inkluderar 694 genetiskt profilerade, autoantikropps-negativa, icke-diabetiska barn vilka är genetiskt predisponerade för T1D till den kliniska DIPP-studien. Proteinuttrycket av CTLA-4 studerades först i PMA/jonomycin-stimulerade $CD3^{+}$ T-celler bland nio fenotypparade individer (GG gentemot AA). Dock kunde inga skillnader hittas i frekvensen av CTLA-4-positiva $CD4^{+}$-hjälpar T-celler eller $CD8^{+}$-cytotoxiska T-celler efter extracellulär färgning för CTLA-4. CTLA-4 polymorfismen (+A49G) observerades utöva en signifikant verkan på frekvensen av CTLA-4-positiva $CD3^{+}CD4^{+}CD25^{+}CD127^{Low}FOXP3^{+}$-Treg-celler när man färgade CTLA-4 intracellulärt i ostimulerade celler hos elva fenotypparade individer. Homozygositet för den riskassocierade G-allelen medförde en lägre frekvens av CTLA-4-positiva Treg-celler ($p = 0{,}0091$), naiva $CD45RA^{+}$-Treg-celler ($p = 0{,}0136$), och som ett gränsfall, minnes $CD45RA^{-}$-Treg-celler ($p = 0{,}0631$). De framtagna resultaten förstärker därmed teorin att rs231775-polymorfismen sänker nivån av lagrat CTLA-4 i Treg-celler. Därav uppmuntras fortsatta studier av polymorfismens verkan på proteinets ytuttryck för att fastställa ifall polymorfismen förändrar T-cellernas immunregulatoriska förmåga.

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List of abbreviations

AAb	Autoantibody
APC	Antigen-presenting cell
BACH2	BTB domain and CNC homolog 2
BCR	B-cell receptor
CD	Crohn's disease
CeD	Celiac disease
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DIPP	The Diabetes Prediction and Prevention study
eQTL	Expression quantitative trait locus
fDC	Follicular dendritic cell
FMO	Fluorescence minus one
FPDR	The Finnish Pediatric Diabetes Register
FPIR	First-phase insulin response
FUT2	Fucosyltransferase 2
GADA	Glutamic acid decarboxylase antibody
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
IA	Islet autoimmunity
IA-2A	Protein tyrosine phosphatase-like antigen 2 antibody
IAA	Insulin autoantibody
IBD	Inflammatory bowel disease
ICA	Islet cell antigens
IFIH1	Interferon induced with helicase C domain 1
IFN	Interferon
IL-1β	Interleukin-1β
IL2RA	Interleukin 2 receptor chain alpha
iNKT	Innate natural killer T lymphocyte
IR	Incidence rate
ISG	Interferon-stimulating genes
JIA	Juvenile idiopathic arthritis
LADA	Latent autoimmune diabetes in adults
LD	Linkage disequilibrium
LNK	Lymphocyte adaptor protein
LYP	Lymphoid tyrosine phosphatase
MAF	Minor allele frequency
MAIT	Mucosal associated invariant T cell
mDC	Medullary dendritic cells
MG	Myasthenia gravis
MHC	Major histocompatibility complex

MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cells
NK	Natural killer cell
pAPC	Professional antigen presenting cell
PBMC	Peripheral blood monocyte cell
Ps	Psoriasis
PTPN22	Protein tyrosine phosphatase non-receptor type 22
PTPN2	Protein tyrosine phosphatase non-receptor type 2
pTreg	Peripheral regulatory T cell
RA	Rheumatoid arthritis
SLE	Systematic lupus erythematosus
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
T1DGC	Type 1 Diabetes Genetics Consortium
Tc	Cytotoxic T cell
Tconv	Conventional T cell
TCR	T-cell receptor
TEDDY	The Environmental Determinants of Diabetes in the Young
Teff	Effector T cell
TEMRA	Terminally differentiated effector memory T cell
Tfh	Follicular helper T cell
TGF-β	Transforming growth factor β
Th	Helper T cell
Tph	Peripheral helper T cell
TSA	Tissue-specific antigen
Treg	Regulatory T cell
tTreg	Thymic regulatory T cell
UBASH3A	Ubiquitin-associated SH3A protein
UC	Ulcerative colitis
VI	Vitiligo
VNTR	Variable number tandem repeats
ZnT8A	Zinc transporter 8 antibody

1. Introduction

Type 1 diabetes (T1D) is the most prevalent metabolic disease in children worldwide, and it has an incidence rate (IR) that has been increasing since the beginning of the twentieth century. Imperatively, the progressive increase in IR is of uppermost concern, and the high prevalence of diabetic disease poses a great challenge to the medical community's efforts to improve global health. Autoimmune diabetes, promoted by self-reactive T and B cells, drives the progressive destruction of insulin-producing pancreatic β-cells, causing at first dysglycemia, and later on, a total dependence on exogenous insulin injections to allow for the continued survival of the child. The factors contributing to the etiology of T1D can be divided into two distinct classifications; firstly, into genetic factors predisposing children to disease via strong hereditary risk effects with partially unknown causality, and secondly, into environmental factors which are associated with disease and able to modify certain aspects of the pathogenesis. The disease etiology is, therefore, inherently multifactorial, with both genetic and environmental factors predisposing and modulating an individual's risk of developing T1D.

The care of T1D patients still lacks treatment options that would eliminate the need for exogenous insulin treatment. Nevertheless, the creation of either preventative or interventional treatment options requires an understanding of why the disease develops, and on what grounds. The clinical pathophysiology of T1D after islet-autoimmunity (IA) initiation is well understood, albeit the field of medicine still lacks an explanation for how and why IA is initiated. There is, hence, a significant gap in the present understanding of the disease etiology. However, the nescience forms a path that the immunogenetics community might be able to illuminate since the researchers aim to understand how the diabetic autoimmune condition develops in individuals genetically predisposed to T1D. Nevertheless, further revelations of the disease etiology can, in time to come, provide a better understanding of the immunological involvement and hopefully enable one to intervene in the early pathogenesis to stop the disease from reaching fulminancy.

Multiple population-based, longitudinal prospective studies have been conducted since the 1980s (Ilonen et al., 1988; LaPorte et al., 1990) with the goal of detecting the genetic and environmental factors which predispose individuals to T1D or increase the susceptibility to the disease. These studies have been able to detect an array of immunogenetic factors which predispose individuals to disease and are associated with pathophysiological characteristics, such as disease progression rate and morbidity. Crucially, the main goal of the prospective studies has been to predict, prevent and intervene in the autoimmune condition in hope of either delaying or terminating the disease development.

This thesis is set to generate a 700-subject cohort of healthy, autoantibody-negative (AAb), at-risk children who have been enrolled in the Finnish prospective Diabetes Prediction and Prevention Study (DIPP). The cohort will be genotyped for ten functional single nucleotide polymorphisms (SNP), whereafter the dataset can be used to quantify the gene variants' poorly characterized downstream effects on immune cell functions and on peripheral blood monocyte (PBMC) population frequency parameters.

2. Literature review

2.1 Relevance through incidence, the epidemiology of T1D

T1D exhibits high levels of familial aggregation, and there are reported twin concordance ratios ranging from 30 to 50%. One study reported a twin concordance rate of 65% at the age of 60 in twins with a proband that has T1D (Redondo et al., 2008). Furthermore, a Finnish study reported a probandwise concordance rate of 42.9% in monozygotic twins, a rate which is considerably higher than the 7.4% observed in dizygotic twins (Hyttinen et al., 2003). The phenomenon of familial aggregation demonstrates that there is a significant genetic component to the etiology, although, environmental factors can concurrently modulate the susceptibility to autoimmunity or possibly even function as a determinator of the final outcome. It is now estimated that approximately 88% of the disease-phenotypic variance is acquired from genetic risk factors such as human leukocyte antigen (HLA) alleles or polymorphisms in other loci that are important for immune cell function or affect the β-cell phenotype (Hyttinen et al., 2003).

HLA-based risk factors constitute approximately 40–50% of the total genetic risk effect, that is, when comparing low-penetrance and low-effect risk factors, such as non-HLA SNPs, with HLA-based risk factors (Noble & Valdes, 2011). Is should be noted that only 10–15% of all individuals genetically predisposed to T1D eventually develop the disease (Knip et al., 2005). Furthermore, there has been an increase in individuals belonging to a neutral or intermediate HLA-based risk group who later on develop T1D (Fourlanos et al., 2008; Hermann, Knip, et al., 2003; Vehik et al., 2008). The increase in intermediate-risk individuals who develop T1D is believed to be caused by an increased prevalence of environmental risk factors or a change in their penetrance. Continuous changes in living conditions and in diets have globally resulted in a major generational change, a change that may have decreased the cumulative penetrance of traditional HLA-based genetic risk factors' effects on disease susceptibility. To conclude, gradual alterations in the prevalence and penetrance of traditional environmental factors are thought to underlie the increase in global IR and the observed changes in pathophysiological aspects, such as changes in the time point of disease initiation and in the rate of disease progression (Ilonen et al., 2019).

The epidemiological characteristics of T1D are distinctive since they in theory establish a pediatric metabolic disease that has an outcome which is lethal without medical intervention. However, with recent advancements in global T1D awareness and in T1D healthcare, increasingly fewer children are succumbing to metabolic ketoacidosis in the industrialized world. According to the 9th edition of the International Diabetes Federation (IDF) Atlas (2019), the estimated per annum global incidence of T1D in under 15-year-olds is 98,200. Moreover, the corresponding global prevalence of existing cases is estimated to be at 600,900 (Patterson, Karuranga, et al., 2019). A major distinction in global prevalence-incidence statistics is that the high-prevalence, high-incidence countries are situated in Europe and North America, while the low-incidence countries are found in Asia, Africa, and South America. When considering children under the age of 15, the IR of T1D in low-prevalence regions, such as Asia and South America, ranges from 1–3/100,000; as compared to an IR of 10–20/100,000 in South European countries and 40–60/100,000 in Nordic countries (Norris et al., 2020). The global peak incidence of T1D can be found amongst 10–14-year-olds. However, those who develop IA and clinical T1D at a younger age often

exhibit a more rapid disease progression with a more morbid dysglycemia (Norris et al., 2020). Notably, high-incidence countries have lower peak-incidence ages as compared to low-incidence countries; a difference that has been connected to a region's child mortality rate (Patterson, Karuranga, et al., 2019). Thus, when creating an overview of global T1D trends, the IDF has a need to extrapolate for incomplete data and also compensate for higher mortality rates in economically developing countries (Patterson, Karuranga, et al., 2019). Moreover, since developing countries have higher levels of child mortality due to poorer social security and less available treatment options, it has been difficult to estimate the incidence and prevalence of T1D in economically developing countries; especially when many regions have not gathered extensive epidemiological data during the past century.

Statistical analysis of 22 European countries' T1D data, including national data collected between 1989–2013, revealed a 3.4% (95% CI = 2.8-3.9%) per annum increase in IR (Patterson, Harjutsalo, et al., 2019). The report also found that there is an even increase in male and female IR amongst 0- to 4-year-olds (3.7% and 3.7% per annum, respectively) and amongst 5- to 9-year-olds (3.4% and 3.7% per annum, respectively). However, the IR was slightly higher amongst boys than girls when studying 10- to 14-year-olds (3.3% and 2.6% per annum, respectively). The authors noted that according to the ongoing trend, there could be a doubling of the global IR after twenty years. The highest continental IRs can be found in the Nordic countries, wherein Finland exhibits an IR of 52.2/100,000 (as of the time period 2015–2018; children < 15 years of age), subsequently placing Finland in the lead of global IRs (Parviainen et al., 2020). This makes the study of T1D especially relevant amongst Nordic Caucasians and in Caucasians of a European origin. Furthermore, the etiological factors which predispose or facilitate the disease development can more easily be discovered, from a prospective standpoint, in an ethnically homogenous population. This is one reason why T1D research has been more attentive to high-incidence Caucasian populations, thus, providing predictive models better fitted for Caucasian population genetics, albeit simultaneously depriving non-Caucasians subjects from similar qualitative predictive models (Noble & Erlich, 2012).

2.2 Intercepting autoimmunity, a goal of the Diabetes Prediction and Prevention Study

The longstanding aspiration to illuminate the etiology of T1D has set the stage for numerous longitudinal observational studies, prospective studies that still today are at the forefront of diabetes research. The Finnish Diabetes Prediction and Prevention study (Ilonen et al., 1996), a prospective study that was initiated in 1994, is one of the forerunners in the field. It started as an observational prospective study enrolling newborns in the areas of Turku, Tampere, and Oulu. The DIPP study has now established a HLA genotype-based risk-classification system (Hermann, Turpeinen, et al., 2003) from results published by the study's clinical predecessors and from data available in the Finnish Pediatric Diabetes Register (FPDR) (Parkkola et al., 2013); in essence, forming a screening system that can detect individuals genetically predisposed to T1D (Ilonen et al., 2016). The DIPP community has since 1994 screened over 250,000 children for eligibility, with ~8000 children becoming screened each year (R. Veijola, personal communication, September 1, 2021). Around 700 of all children screened each year are eligible for follow-up with ~500 children becoming enrolled in the study annually. The current follow-up cohort consists of over 8000 children under the age of 15, and 766 of these children have developed seropositivity for one AAb and 868 for two or more AAbs (as of September 2021). Finally, over 500 of the enrolled children have to date progressed to clinical T1D with approximately 20 new cases appearing each year.

Parents can enroll their child in the DIPP study if the child is born at the aforementioned centers, when one of the parents is of a Caucasian origin, and when the caregivers speak either Finnish, Swedish or English (Kupila et al., 2001). The genetic screening is performed with cord blood samples extracted after delivery, however, children born with a severe systemic disease or during circumstances where a cord blood sample cannot be taken are not screened nor enrolled. A full description of the HLA-based enrollment criteria is presented in Section 4.1. Children compatible with the inclusion criteria can take part in regular follow-up visits with a physician every three months during the first year and every three to twelve months until the age of fifteen, until the development of clinical T1D or until the individual exits the study (e.g., by relocating to an area out of reach for regular follow-up visits). The follow-up includes the collection of standard healthcare data and the extraction of a venous blood

sample that is tested for AAbs against islet cells (ICAs), insulin antigens (IAA), protein tyrosine phosphatase-like antigen (IA-2A), glutamic acid decarboxylase (GADA) and zinc transporter 8 (ZnT8A). Moreover, additional blood samples are taken for the collection of PBMCs which become cryopreserved as a part of the study's data repository. Subjects who become seropositive for at least one persistent AAb, with AAb-persistence defined as two successive positive samples, have their first-phase insulin response (FPIR) to intravenous glucose tested at 6–12-month intervals. A full description of AAb-screening methods is given in Section 4.1.

The HLA-based enrollment criteria and the follow-up intervals for AAb-screenings have changed over the years, although, the most recent methodologies for included tests have been described earlier (Ilonen et al., 2016; Koskinen et al., 2018). In addition to the collection of basic healthcare data, there have been multiple subcohorts formed under the DIPP umbrella which studied maternal and newborn health after birth or factors such as newborn diet (Erkkola et al., 2005), thus creating numerous additional categories of healthcare data. Finally, siblings of enrolled children, as well as the parents of the newborn, are encouraged to become screened for T1D susceptibility. Subjects who later on developed T1D, together with their first-degree-relatives and siblings, become encouraged to enroll in the FPDR, a registry which includes around 90% of all T1D subjects in Finland (Mäkinen et al., 2008; Parviainen et al., 2020). To conclude, the DIPP study has to date been able to collect a vast amount of material, including healthcare data, PBMC samples and plasma samples, which can be used to study the diabetic pathogenesis of T1D.

The DIPP research community has published over 200 articles using data produced within the follow-up. These articles include papers describing both the genetic and environmental background of T1D susceptibility and of IA. As an example, the association of dietary factors with T1D susceptibility has been investigated by comparing T1D susceptibility amongst children who have been nursed against those that received milk formula (Kimpimäki et al., 2001; Vaarala et al., 1999). Moreover, there have been numerous studies investigating the introduction of dietary factors or known allergens, such as milk products or gluten, into the diets of young children (Hakola et al., 2019; Niinistö et al., 2020). Different pathogens' associations with T1D have also been studied, in a longitudinal manner, via the genomic analysis of childrens'

viromes and microbiomes. The goal of the metagenomic studies has been to investigate whether any specific bacteria or virus taxa are associated with T1D or IA. The proposed immunomodulatory effects of pathogens has been investigated since some pathogens are believed to modify the immunophenotype, an effect that can alter disease susceptibility or disease progression rate (Cinek et al., 2017; Honkanen et al., 2017). Furthermore, the potential effects of enterovirus and cytomegalovirus (CMV) infections on T1D susceptibility have been extensively studied, especially amongst infants (Ekman, Vuorinen, et al., 2019; Lonnrot et al., 2000). The immunophenotyping of subjects' immune cell populations is a more recent addition to the DIPP study's research spectrum; this area aims to determine and quantify the downstream effects which genetic factors, such as coding and non-coding T1D associated polymorphisms, exert onto the phenotype (Valta et al., 2020). Within cell-based research, there has been an especial effort put into studying alterations in different immune cell population frequency parameters, alterations which can be associated with IA or T1D (Ekman, Ihantola, et al., 2019; Ihantola et al., 2018; Viisanen et al., 2019). Lastly, the characterization of T cells which recognize pancreatic autoantigens and the screening for novel self-reactive T-cell epitopes are both longstanding areas of DIPP research (Ihantola et al., 2020; Öling et al., 2005).

Clinical diagnostics is an important area of functional DIPP research. This area includes the technical and methodological development of genetic tests, specifically, the development of PCR-based screening systems that can accurately genotype individuals for T1D associated gene variants (Kiviniemi et al., 2005, 2007). The development includes research into novel screening criteria, such as the potential use of metabolomic data (Lamichhane et al., 2018), non-HLA-based genetic data (Lempainen et al., 2015) and epigenetic data (Acevedo et al., 2015) to predict disease susceptibility and disease progression rate. Importantly, the research conducted by the DIPP study community is performed cooperatively, and in common interest, with other national and multinational prospective T1D studies, such as the German BABYDIAB and BABYDIET studies (Hummel & Ziegler, 2011), the USA-based DAISY study (Barker et al., 2004), the T1D research network TRIALNET (Bingley et al., 2018) and the international TEDDY study (Hagopian et al., 2011). Novel research made by the DIPP study has also served as the basis for clinical trials of interventional medical

treatments; clinical trials wherein DIPP study subjects have been enrolled. One example of a preventative trial is that of a nasal immunization with insulin to induce insulin tolerance (Näntö-Salonen et al., 2008; Ryhänen et al., 2011).

2.3 Rise of autoimmunity: the immunopathogenesis of type 1 diabetes summarized

The autoimmune pathogenesis of T1D can be divided into four stages of disease development. The founding stage, stage 0, is a pre-clinical phase during which individuals may carry a hereditary risk of developing T1D and become exposed to a diverse set of environmental factors (Insel et al., 2015). Immunological self-tolerance is breached as individuals progress to stage I, immune cell infiltration and seroconversion, when self-reactive T cells targeting β-cell autoantigens start to infiltrate β-islets and autoreactive B cells start to produce AAbs directed against self-antigens (Ilonen et al., 2019). Stage I is asymptomatic, normoglycemic, and a stage manifested by the initiation of progressive β-cell mass loss. Most importantly, stage I establishes the longest period of disease development, a phase which can last from a few months up to many years. Stage II, in opposite, is defined by the asymptomatic onset of clinical, measurable dysglycemia. During stage III, the child will eventually start to exhibit the clinical symptoms of diabetes, such as polyurea, polydipsia, polyphagia, and fatigue. The symptoms will become progressively morbid, as the disease progresses, culminating in the obligate need for exogenous insulin injections to allow for the continued survival of the child. The pathogenesis and the main pathophysiological characteristics of T1D are summarized in Figure 1.

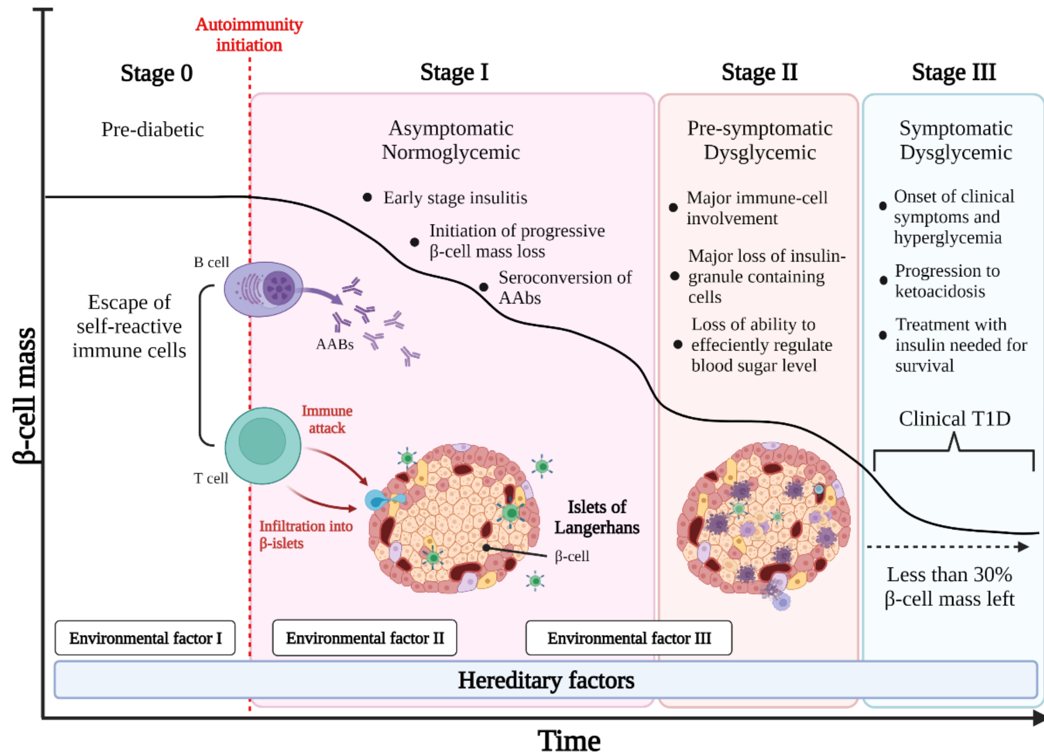

Figure 1. The pathogenesis of T1D. The disease development is staged according to the phase of immunological involvement, i.e., into the pre-autoimmune stage 0 and in three autoimmune stages according to the degree of immunological involvement and the morbidity of the symptoms. The pathological phases, stages I–III, are initiated through the induction of autoimmunity against β-islets. The autoimmune stages are further divided into asymptomatic and symptomatic T1D. The progressive loss of β-cell mass is depicted as a descending solid line. The genetic etiological background affects the individual throughout life, hence, affecting disease susceptibility. The environmental factors can temporarily or for a longer period exert an effect on disease susceptibility or disease-progression rate. Clinical T1D is manifested when there is approximately 30% of the total β-cell mass left. Created with BioRender.

Disease heritability is the first etiological factor that is to be covered when discussing T1D disease development. However, no genetic factors have to date been shown to exert a dominant causative effect on autoimmune T1D development, with the exception of rare gene variants that cause monogenic diabetes (M. B. Johnson et al., 2019). Nevertheless, genetic factors demonstrably modulate certain aspects of the disease development, such as the archetypical example of the MHC molecules that presents antigens to T-cell receptors (TCR). The HLA loci that encode MHC molecules have been consistently shown to confer the highest risk effect for various autoimmune diseases. However, it is still not understood how the HLA genes exert a causative effect in the phenotype. Nevertheless, the risk effects conferred by the MHC are often limited to a certain HLA genotype, which would indicate that the MHC has an antigenic affinity that promotes the expression of self-peptides, a bias which would

facilitate the presentation of self-antigens to self-reactive T cells (Ilonen et al., 2019). It has been suggested that certain MHC molecules are able to present self-antigens with a disparate binding affinity during the negative selection of T cells. This could enable the escape of self-reactive T cells from the thymus, consequently, breaching the immunological self-tolerance (Wallet et al., 2017). Furthermore, it is during the pre-diabetic phase that the environmental factors may exert the most significant effect onto the immunophenotype. For example, genetic factors may systematically modulate the function and the homeostasis of the innate immune system; a change that could affect the susceptibility to viral infections or the ability to initiate and resolve an immune response. The main etiological constituents of the pre-diabetic phase, the hereditary and the environmental factors, are further discussed in Section 2.4. The first stage of T1D disease development, a stage initiated via the induction of self-reactivity, is presented next.

2.3.1 Immune cell self-reactivity, when cells break the rules of self-tolerance

The first stage of type 1 diabetes development is usually completely asymptomatic. Nevertheless, progressive β-cell dysfunction can be detected when measuring FPIR, which indicate that the β-cell mass is declining (Richardson et al., 2011). The process of how self-reactivity initiates is not fully understood, however, there are multiple theories which state that the initiation of autoimmunity can occur due to stochastic or environment-mediated perturbations of central and peripheral self-tolerance mechanisms. T1D is also commonly referred to as a T-cell mediated autoimmune disease since it is mainly self-reactive cytotoxic T cells (Tc) and helper T (Th) cells that infiltrate pancreatic islets and facilitate the targeted killing of β-cells (Vandamme & Kinnunen, 2020). The immunological mechanisms which control immune cell homeostasis and the cell types which break the rules of self-tolerance are reviewed next.

The process of positive and negative selection, the main constituents of central T-cell tolerance, should first eliminate double-positive T cells that do not recognize HLA-peptide complexes through clonal deletion in the cortical thymic region (Klein et al., 2014). Self-reactive single-positive T cells are subsequently eliminated in the medullary thymic region through the clonal deletion of T cells that exhibit an increased affinity for self-antigens (Figure 2). Accordingly, low-avidity tolerogenic interactions

allow for the T-cell clone to survive. Moreover, through still uncategorized cellular mechanisms, mid-avidity interactions produce a subset of immunomodulatory cells called regulatory T cells (Treg) (The Treg cells are further described in Section 2.3.2). Negative selection occurs through a process where medullary thymic epithelial cells (mTEC) present a wide range of tissue-specific antigens (TSA). In this process, master transcription factor AIRE and the immune regulator forebrain expressed zinc finger 2 (Fezf2) initiate the expression of TSAs, leading to the surface expression of TSAs by MHC class I and class II complexes (Clark et al., 2021). The presentation of alternate TSAs, e.g. autologous chimeric proteins or low-affinity TSAs, could allow for the escape of alternate TSA-specific or self-antigen specific T cells (Pugliese, 2017). In the periphery, the self-reactive T cells can attack self-tissues and proliferate, thus, initiating a state of autoimmunity. Negative selection is also mediated by medullary dendritic cells (mDC) and B cells, both of which are able to present circulating antigens acquired from the medullary environment. These antigens can have a peripheral origin, be acquired from mTECs through trogocytosis or from apoptotic mTECs (Clark et al., 2021).

The process of central B-cell self-tolerance is first initiated in the bone marrow (Figure 2). The mechanism is mediated via clonal deletion or anergy induction of autoreactive B cells and the clone-specific editing of B cells' autoreactive or polyreactive BCRs (Brooks et al., 2020; X. Chen & Jensen, 2008). Ultimately, this process should eliminate the self-reactive B cells before the cells migrate to the periphery. The selective process of B cells is also extended to the periphery since mature B cells migrate upon antigen recognition into the germinal centers of the lymph nodes (Figure 3). The recognition of antigens presented by follicular DCs (fDC) initiates a process of clonal expansion and somatic hypermutation, a clonal expansion that is restricted to clones which demonstrate increased affinity for target antigens (Tsubata, 2017). Consequently, B cells which show unsatisfactory avidity to the target antigen become selected against; a negative selection that is mediated through the competition with high-avidity clones, subsequently causing the low-avidity clones to die by anergy (Hinman et al., 2014). Still, clonal expansion is canonically dependent on trans-cellular interactions with follicular Th (Tfh) cells that stimulate the B cells and fDCs which present the clone-specific antigen. However, perturbations in the cellular functions

which regulate the process of clonal expansion and affinity maturation could allow for the proliferation of self-reactive B cells which have a high affinity to self-antigens (Hanley et al., 2017). Nonetheless, the existence of self-reactive Tfh cells or peripheral Th cells (Tph) is perceived to be a necessity for the positive selection of self-reactive B cells in the germinal centers.

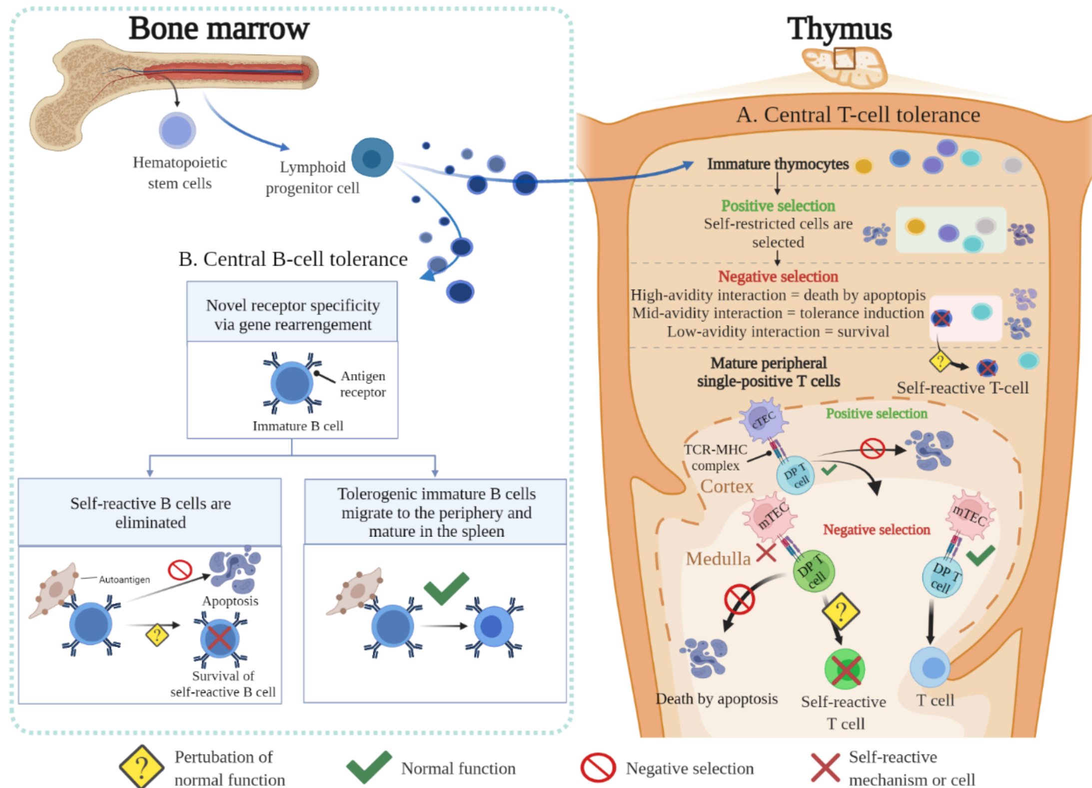

Figure 2. The main mechanisms of central tolerance. A. Central T-cell tolerance is mediated via the positive selection of self-restricted T cells in the cortical thymic region. The selected cells are thereafter tested for affinity to self-antigens in the medullary thymic region. Cells that show affinity to autoantigens become selected against. Perturbations in the process of negative selection could allow for the escape of self-reactive T cells. B. The process of central B-cell tolerance occurs in the bone marrow where B cells expressing autoreactive or polyreactive BCRs become selected against. Negative selection is mediated via direct deletion or death by anergy. Perturbations in the negative selection of autoreactive B cells could allow for the escape of self-reactive B cells to the periphery. Created with BioRender.

Peripheral tolerance is multifaceted and relies on multiple cellular mechanisms to limit the activation and expansion of self-reactive naïve T cells in the periphery (Figure 3). Traditional T-cell activation requires that the interaction between a naïve conventional T cell (Tconv) and a MHC-antigen complex presented by an APC is of a high enough avidity (Clark et al., 2021). Moreover, T-cell activation is dependent on costimulatory signaling through the CD28–CD80/86 axis and on cytokines expressed by the APC. Genetic perturbations of cellular activating pathways could change the kinetic threshold level of BCR and TCR activation, a change that could be coupled with the aberrant MHC presentation of a self-antigen. Hence, in theory, facilitating a breach in the aforementioned stimulatory mechanisms which would allow for a rare self-reactive cell clone to proliferate upon activation (Clark et al., 2021; Pugliese, 2017). Furthermore, systemic or temporal changes in cytokine-receptor function or in cytokine-expression levels can modify a cell population's ability to become activated, to expand or to resolve an immune response (Jeker et al., 2012). Lastly, changes in the function or expression of immune cell checkpoint inhibitors may cause perturbations to the threshold levels of immune cell activation or in the ability of a regulatory cell to downregulate effector cells (Shibru et al., 2021). Essentially, perturbations of the aforementioned cellular mechanisms can collectively change how the cumulative stimulatory effect between a T cell and an APC is interpreted. This can cause an otherwise harmless self-antigen that should not activate any circulatory effector T cells (Teff) to produce an immune response that facilitates the expansion of self-reactive immune cells, subsequently initiating a state of autoimmunity. Examples of genetic and environmental factors which can facilitate perturbations of central and peripheral self-tolerance mechanisms are presented in Section 2.4.

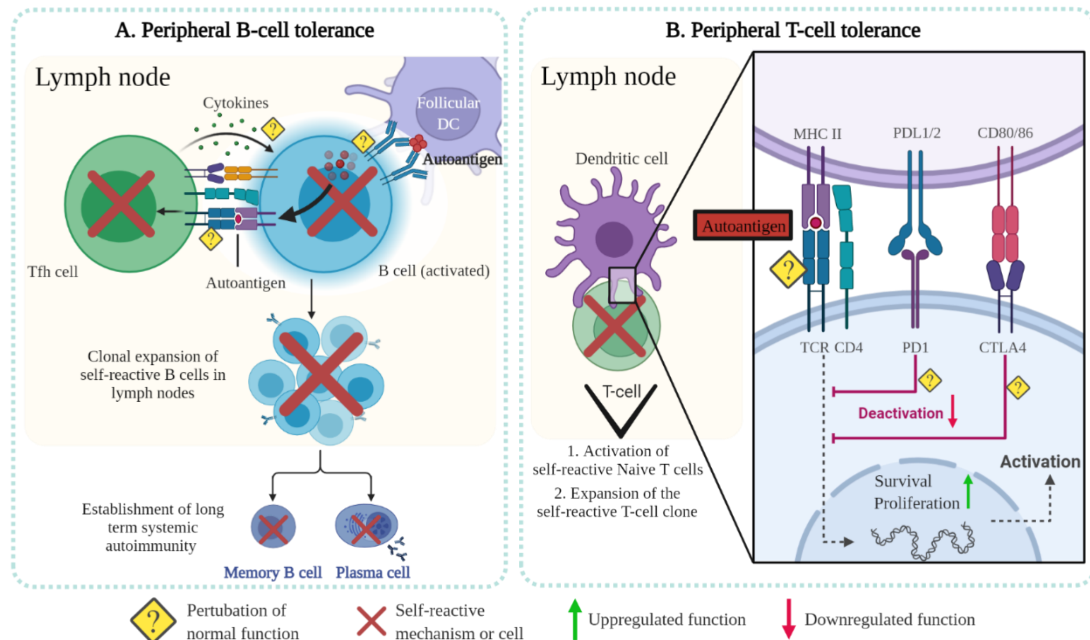

Figure 3. The main mechanisms of peripheral tolerance. A. Peripheral B tolerance is centralized to the germinal centers of the lymph nodes where activated B cells undergo somatic hypermutation and clonal expansion to produce a broad pool of experienced memory B cells and plasma cells. Peripheral tolerance should restrict the inappropriate expansion of B cells showing affinity for autoantigens presented by follicular dendritic cells. Follicular Th cells facilitate the expansion of B cells which show increased avidity for presented target antigens. The restriction of B-cell expansion is mainly facilitated by anergy induction in those cells which develop BCRs' that do not interact with the target antigen. B. Peripheral T-cell tolerance include processes which restrict the expansion of activated T cells in the periphery. Peripheral T cell tolerance is mainly restricted to the time period when naïve T cells first encounter their antigen, after which the T cell clone begins to proliferate in the T-cell areas of the lymph node. Clonal expansion is mediated by DCs and Th cells, with self-reactive cells either becoming deleted or anergic. Perturbations of cell-cell and intracellular signaling pathways could lead to the aberrant expansion of self-reactive T cell clones. Created with BioRender.

2.3.2 Pancreatic insulitis, the infiltration of immune cells into islets of Langerhans

Insulitis, the immune cell-mediated inflammation of β-cells, is caused by the infiltration of primarily $CD8^{+}$ Tc cells and secondarily $CD4^{+}$ Th cells (Figure 4). Macrophages, DCs, B cells, Treg cells and natural killer cells (NK) are to a lesser extent observed in the immune infiltrate (Leete et al., 2016; Richardson et al., 2011; Vandamme & Kinnunen, 2020). The medical field's current understanding of insulitis as a pathological event is still based on the observational study of pancreatic tissue samples, tissue samples which are most often of a cadaveric origin and rarely from biopsies. Consequently, it is largely unknown how insulitis facilitates β-cell mass loss since there is no sufficient method to study the pathology ex vivo without the use of tissue samples taken from individuals who actively experience insulitis. Nevertheless, insulitis is believed to be initiated by self-reactive Tc cells since they are most often observed in the proximity of β-islets during the early-stages of insulitis (Richardson et

al., 2011; Roep et al., 2021). As the inflammation progresses, there is a significant increase in the number of immune cells surrounding the islets and a noticeable reduction of β-islet size. During the final stage, there is a large infiltrate of immune cells visible inside the islets and there can be a total lack of insulin-granule containing cells. β-cell death is believed to be preliminary mediated via the direct cytotoxic killing by Tc cells; a β-cell death induced by granzyme and perforin, and by FASL-mediated killing (Richardson et al., 2011; Roep et al., 2021). Furthermore, intrinsic apoptosis is partially contributing to the decline in β-cell mass, either through becoming activated by elevated levels of interleukin-1β (IL-1β) and interferon-γ (IFN-γ), or by becoming activated via extrinsic factors secreted by inflammatory macrophages and Th1 cells (Vives-Pi et al., 2015) (Figure 4). The cumulative effect of local extrinsic and intrinsic cell-apoptosis activation is highly analogous to a classical type 1 immune response against an intracellular pathogen, causing the β-cells to undergo programmed apoptosis in an attempt to save neighboring cells.

The role of pAPCs is becoming more dominant during periods of chronic inflammation since there is an increase in systemic inflammatory-factor prevalence, therefore, facilitating an increase in the presentation of endo- and exogenous antigens in lymphoid tissues (Clark et al., 2017). The increase in antigen presentation might in autoimmune disease promote a more efficient presentation of novel antigens and self-antigen epitopes, antigens which can be recognized by naïve T cells. Notably, novel epitopes have not been presented as a part of the self-peptide repertoire during the negative selection of single-positive T cells (James et al., 2020). It is, hence, likely that novel epitopes cause an immune response since the equivalent TCRs have not been eliminated through the process of negative selection. Moreover, local inflammation increases intracellular IFN-γ production which facilitates an increased surface expression of MHC I and possibly the presentation of aberrant antigens (Richardson et al., 2011). The increase in MHC I expression also mimics a prototypical type 1 inflammatory response where an immunological reaction increases antigen presentation by APCs, thus, promoting antigen-recognition by T cells and subsequently the clonal expansion of the experienced T cells.

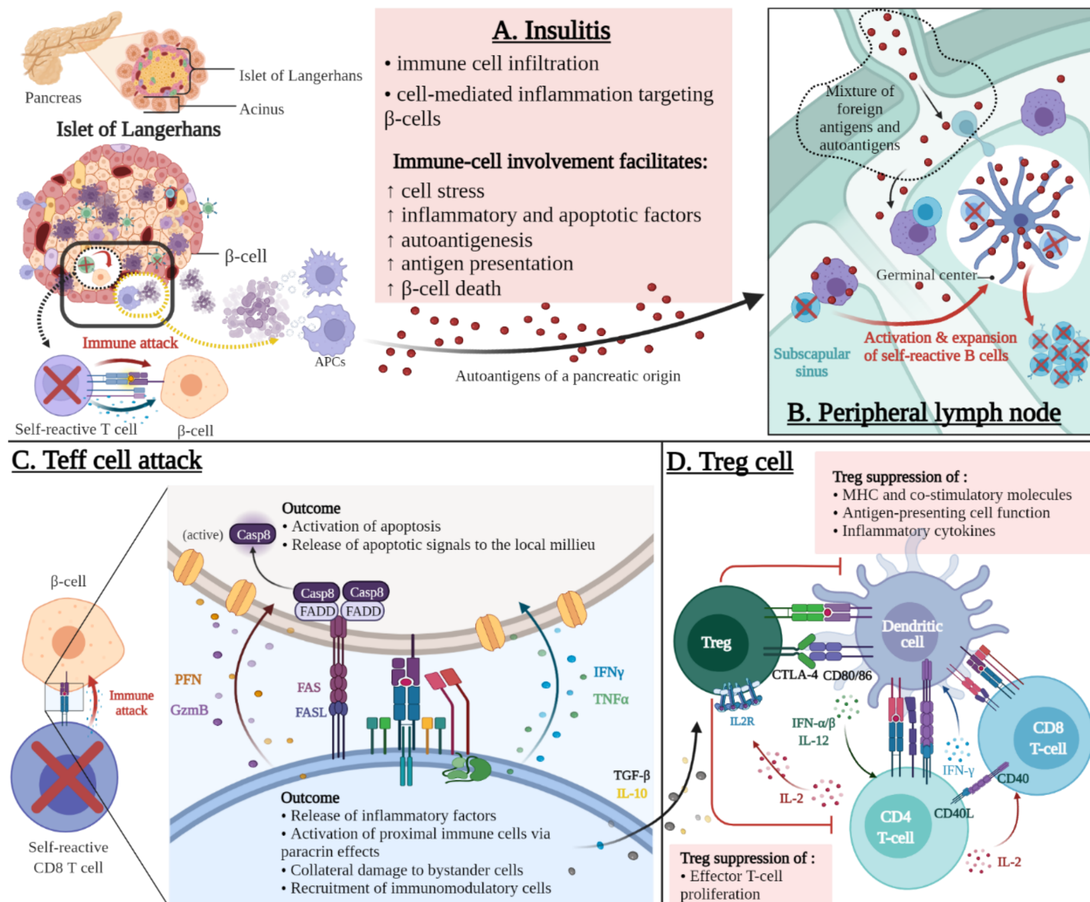

Figure 4. The facilitators of islet inflammation and β-cell death. A. Islet autoimmunity is initiated via the infiltration of immune cells into the islets of Langerhans. The immune cell involvement facilitates cell stress, insulin degranulation and β-cell death. B. Cell stress can cause the upregulated expression of antigens and the production of neo-autoantigens, both of which can result in the activation of self-reactive B cells and T cells in the periphery. C. The killing of β-cells is preliminary mediated by direct cytotoxic killing and contact-dependent killing, however, increased levels of cell stress in an inflammatory milieu may allow for intrinsic apoptosis to occur. D. The immunomodulatory involvement of Treg cells is crucial for the downregulation of effector-cell activation and for the resolution of immune cell activation in an inflammatory niche. Treg cells are recruited by increasing levels of inflammatory factors, nevertheless, some Teff cells can achieve an immunoregulatory profile in the presence of IL-10 and TFG-β. Created with BioRender.

Furthermore, coordinated T-cell–mediated attacks on β-islets will increase the β-cells' inherent stress levels, and concurrently increase the rate of β-cell mass loss by programmed cell death (Figure 4). Programmed cell death also enables the release of apoptotic bodies into the environment, extracellular vesicles which function as a good source of self-antigens (Vives-Pi et al., 2015). Both the inflammatory milieu and cell stress can increase the aberrant expression of self-antigens or the production of chimeric antigens (Piganelli et al., 2021). The pathway of neo-autoantigen formation is still poorly understood, although, neo-autoantigen production is believed to be a by-product of major endoplasmic reticulum and metabolic stress (Clark et al., 2017;

Piganelli et al., 2021). Nevertheless, neo-autoantigen production in β-cells could produce novel antigens from aberrant insulin peptides, thus forming a pool of novel pancreas-specific autoantigens. Furthermore, since blood-derived antigens have been found to be presented by mDCs during the negative selection of T cells, peripherally-sourced pancreas-specific antigens could be presented in the thymus (Atibalentja et al., 2011; Vollmann et al., 2021). However, it is still unknow whether blood-derived antigens affect tolerogenicity induction and T-cell selection.

During the development of insulitis, some individuals have an increased prevalence of B cells. B cells have been found in the proximity to β-islets during insulitis, and B cell positive insulitis correlates with a more prevalent Tc involvement, a more rapid islet degeneration, and a younger age at diagnosis (Leete et al., 2016). The B cells are not themselves believed to contribute to the pathogenesis, although, when B cells gain the ability to produce low- and high-avidity AAbs, the disease will start to gain progressively more speed (Richardson et al., 2011; Vandamme & Kinnunen, 2020). Nevertheless, B cells might aid the local inflammation by functioning as pAPCs, hence presenting self-antigens to local self-reactive T cells. B cells could in this case present self-antigens to experienced autoreactive Th cells, thus, providing each other with co-stimulation that facilitate B-cell differentiation or class switching (Leete & Morgan, 2021). Moreover, the Th cells are simultaneously provided with costimulatory signals, hence facilitating further clonal expansion (Armitage et al., 2021; X. Chen & Jensen, 2008). The recognition of soluble neoantigens by BCRs is another mechanism analogous to the presentation of neo-antigens to TCRs. A low avidity neo-autoantigen–BCR interaction occurring in an inflammatory milieu could allow for B-cell activation, hence, enabling the B cell to more efficiently present autoantigens to surrounding T cells (Hinman et al., 2014). Nevertheless, it is still not understood how self-reactive B cells escapes protective peripheral self-tolerance mechanisms, such as receptor editing and the negative selection of self-reactive cells during somatic hypermutation. However, the self-tolerance mechanisms could be perturbated by underlaying genetic and environmental factors (Ilonen et al., 2019). The B-cell compartment has been observed to be altered in diabetic individuals, although, the alterations are highly heterogonous and change as the disease progresses (Habib et al., 2019; Hanley et al., 2017). Furthermore, the serological appearance of AAbs during the first stage of T1D

development, followed by a decline in FPIR, function as the main markers of autoimmunity initiation and progression (Ilonen et al., 2019). A pooled analysis of multinational cohorts observed that 69.7% of individuals with multiple AAb and 14.5% of individuals with one AAb progressed to T1D after 10 years (Ziegler et al., 2013). In comparison, only 0.4% of children with no AAbs developed T1D after 15 years.

The autoimmune condition in T1D is also mediated by an aberrant prevalence of immune regulatory cells, mainly regulatory T cells, that during normal physiological circumstances should be able to maintain the body's immunological homeostasis. Thymic Treg cells (tTreg) are produced during the negative selection of single positive T cells in the thymus (Clark et al., 2017). It has been determined that certain $CD4^+$ single-positive T cells, showing higher affinity for TSAs presented by mTECs, start to express FOXP3 (Sprouse et al., 2018). This makes $FOXP3^+$ T cells, carrying a self-reactive TCR, to undertake a highly immunomodulatory cell profile and to express a more diverse set of inhibitory receptors and ligands. This, in turn, enables Treg cells to suppress conventional T cells and pAPCs presenting the TCR-specific epitope, either through direct cell-cell interactions or indirectly through the production of cytokines such as IL-10, IL-35 and TGF-β (Amit Sharma & Rudra, 2018) (Figure 4). Treg cells can also be formed in the periphery from conventional T cells in the presence of IL-10 and transforming growth factor β (TGF-β). These Treg cells are coined peripheral Treg cells (pTreg). pTreg cells can, therefore, be formed in an inflammatory environment where bystander cells express IL-10 and TGF-β in an attempt to reinstate homeostasis and to resolve an immune response. Treg cells also have a high-expression of the trimeric high-affinity IL-2-receptor which deprives surrounding naïve and effector T cells from IL-2, a necessary cytokine for T-cell proliferation and survival (Clark et al., 2017). Tissues with a less effective or a smaller Treg-cell population may be prone to autoimmunity, mainly since the existing Treg-cell pool is unable to reintroduce immunological homeostasis (Mohr et al., 2019). Functional studies of Treg-cell populations amongst individuals with autoimmunity have observed major phenotypic changes in Treg-subpopulation frequencies, thus, indicating that there might be a pathological alteration in Treg cell homeostasis (Ohkura & Sakaguchi, 2020).

The emergence of AAbs and the phenotypic alteration of immune cell population parameters function as the most accurate markers of autoimmunity. This marks the first stage of T1D disease development as a phase during which medical preventative and interventional treatments might have the greatest effect in either delaying or stopping the disease development (Warshauer et al., 2020). The development of interventional treatments has to this point focused on the targeting of cell-mediated pathological mechanisms; all in an attempt to deplete self-reactive immune cell subsets or to reinstate immunological homeostasis.

2.4. The predisposing genetic factors and proposed environmental triggers of T1D

The immunogenetics community has established a clear causative connection between hereditary immunogenetic factors and T1D. The ability to predict the likelihood of T1D development using genetic factors is no longer limited to one event, clinical diagnosis; genetic factors can now be used to estimate the probable time point of seroconversion, the number and type of autoantibodies (Siljander et al., 2009) and the progression rate of the disease (Pöllänen et al., 2017). Gene variants in loci both within and outside the HLA region can remodulate the immune system through variant-mediated perturbations of immune system functions. They are, hence, in theory able to produce a susceptible immunophenotype in which the disease could be initiated through various stochastic immunological events. Furthermore, if an individual has an intrinsic genetic predisposition to T1D, it is also possible that extrinsic environmental factors could more easily act as a trigger for the disease. These predisposing factors and proposed triggers of T1D will be discuss in the following sections.

2.4.1 HLA-based risk factors

The main genetic factors which predispose individuals to T1D are located within the MHC loci (6p 21.31), i.e. in genes coding for proteins that are responsible for antigen presentation in adaptive immunity (Noble & Valdes, 2011). The MHC loci, annotated I through III, establish one of the most gene-dense and polymorphic regions of the human genome, consequently producing vast polygenic sections in high linkage disequilibrium (LD) (Figure 5). The class I region contains the classical HLA genes, HLA-A, -B and -C, in addition to the non-classical and non-polymorphic genes HLA-E, -F and -G. The class II region encodes the HLA-DPA1, -DPB1, -DQA1, -DQA2, -DQB1, -DQB2, -DRA, -DRB1, -DRB2, -DRB3, -DRB4 and -DRB5 genes (Dendrou

et al., 2018). HLA-DRB2 is a non-coding pseudogene and the DRB2 through DRB5 loci are not necessarily found on all haplotypes due to copy number variations (Noble & Erlich, 2012). The class II region also encodes multiple invariant non-classical MHC complexes, such as HLA-DM and HLA-DO, which are important for antigen preparation and antigen loading onto classical MHC complexes. The third MHC region contains numerous genes important for antigen processing, complement activation and immunological signaling. Nevertheless, the study of antigen-presenting non-classical MHC complexes has been largely overlooked, mainly due to a longstanding disinterest in the invariant presentation of antigens by the gene products and due to the genes' monomorphic nature.

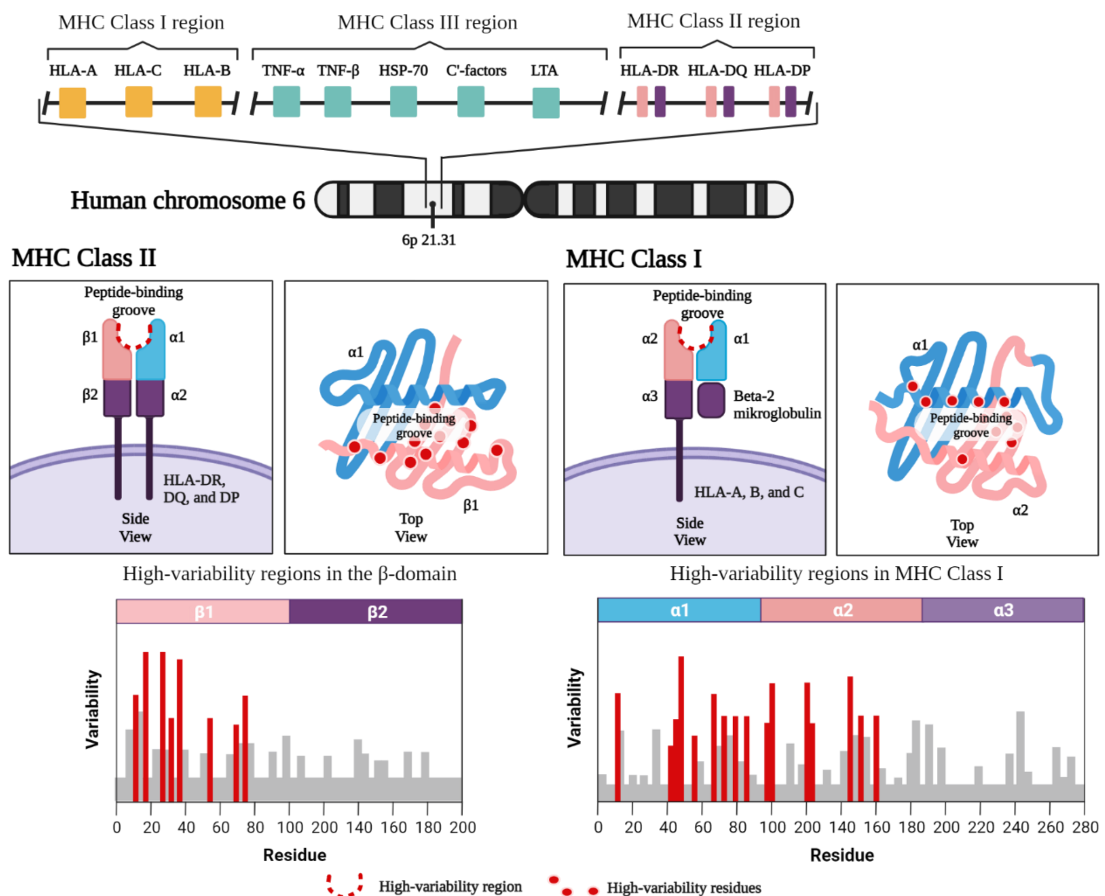

Figure 5. The chromosomal placement of the HLA loci and the structure of the classical MHC molecules. The three regions of the MHC are located on chromosome 6. The region is highly polymorphic, and the genes are densely packed. The side view of respective MHC class I and II molecules demonstrates the secondary structure of the complexes. The top view depicts the secondary structures of the MHC molecules which forms the peptide-binding groove. The variable residues are illustrated with red dots (top and side view) and the generalized distribution of high-variability residues is illustrated in the bar chart. The figure is based on data from Noble & Valdes (2011) and on a illustrative concept from Janeway's Immunobiology (Janeway et al., 2012). Created with Biorender.

The MHC I molecule contains a transmembrane helix domain with a short cytoplasmic tail and three extracellular immunoglobulin domains (α1, α2 and α3), of which the α1–2 domains form the peptide-binding groove (Wieczorek et al., 2017) (Figure 5). The peptide-binding groove is formed by eight antiparallel β-sheets that contain peptide-anchoring residues at conserved positions and a wide range of residues which exhibit high genetic variability (Noble & Erlich, 2012). The anchoring residues are less variable and form the strongest interactions with the antigen, thus, determining a MHC's bias to bind a particular antigen. The high-variability residues are sites which demonstrate the most variation when comparing different HLA alleles, hence modifying the MHC-antigen avidity. The anchoring residues of the MHC class I complex are located closely to the terminal domains of either immunoglobulin domain. Furthermore, the peptide-binding groove is close-ended, hence, fitting a peptide of 8–10 amino acids (Wieczorek et al., 2017). The MHC class II complex, in contrast, is formed by a heterodimeric interaction between the A and B domains of respective HLA-DP, DQ or DR components (Wieczorek et al., 2017). Both the A and B units are composed of a transmembrane helix domain with a short cytoplasmic tail and two extracellular immunoglobulin domains, α1–2 and β1–2, respectively. The peptide-binding groove of the MHC class II complexes is formed by the combined surface of respective α1 and β1 immunoglobulin domains, both contributing with four antiparallel β-sheets (Noble & Erlich, 2012). In comparison to the MHC class I molecule, the MHC class II complex's peptide-binding groove contains evenly spaced anchoring residues and is open ended, thus fitting a 13–25 amino-acid long peptide (Wieczorek et al., 2017). Codominant gene expression of both maternally and paternally inherited MHC molecules is an important inherent property of HLA genetics. This enables the combination of class II alpha-beta components in both cis and trans combinations, hence, creating an even more diverse panel of antigen-presenting MHC complexes.

Both familial linkage studies and genome-wide association studies (GWAS) have demonstrated that certain allelic genotypes of classical HLA genes confer a risk or are protective for a wide range of different autoimmune diseases, e.g. T1D, rheumatoid arthritis (RA), celiac disease (CeD) (Bakay et al., 2019). It is primarily variants of the HLA class II genes HLA-DRB1, -DQA1 and -DQB1 that in T1D exert the strongest

risk effect (Noble & Erlich, 2012). Moreover, alleles of DRB1, DQA1 and DQB1 loci form haplotypes which within a population are conserved by the process of LD, thus making it highly unlikely to find allelic components in other combinations than those seen in the most common genotypes. For example, the HLA-DRB1*03-DQA1*05-DQB1*02 (referred to as DR3-DQ2) and DRB1*04-DQA1*03-DQB1*0302 (DR4-DQ8) haplotypes are on a panethnic level associated with T1D susceptibility. However, some high-risk haplotypes, including the risk associated DRB1, DQA1 and DQB1 alleles, are less frequent in certain ethnic populations. Consequently, partially explaining why the IR of T1D can be lower in some ethnic populations or geographical regions which have significantly lower frequencies of high-risk HLA genotypes. (Erlich et al., 2008; Nakaoka & Inoue, 2015). Furthermore, certain allele subtypes of HLA loci, such as the risk-associated DRB1*04:01 allele versus the protective *04:03 allele, exert effects which are specific to a defined haplotype (Ilonen et al., 2016). This adds an additional level of complexity to the determination of disease susceptibility amongst genotypes carrying both protective and risk associated HLA alleles.

In accordance with published linkage studies of Finnish DIPP material, the DR3-DQ2 haplotype confers an approximately three-fold increase in disease risk (OR = 2.8; CI = 2.50–3.17), and the DR4-DQ8 haplotype confers a ten-fold increase in disease risk (OR = 10.11; CI = 8.66–11.54) (Ilonen et al., 2016). Interestingly, the combined DR3-DQ2/DR4-DQ8 genotype confers a significant increase in T1D susceptibility (OR = 14.69; CI = 10.46–20.62), and this has been contributed to the phenotype expressing both the cis and trans conformations of the aforementioned risk-associated HLA-DQ/DR components. Nevertheless, there are also haplotypes that are associated with strong protection, such as the DRB1*15-DQB1*06:02 (OR = 0.03; CI = 0.03–0.05) haplotype (Ilonen et al., 2016). HLA haplotypes generally adhere to the rule of additive effects, although, there are certain genotype combinations in where one haplotype seemingly exerts a dominant effect (protective or risk effect) over the other haplotype. These types of deviations from the traditional rules of additive effects have not been widely characterized, and it is still unknown why certain alleles become dominant in certain haplotypic combinations (Dendrou et al., 2018).

In addition to the classical HLA class II genes, class I alleles in the HLA-A and -B loci are also associated with T1D (Nejentsev et al., 2007). The strongest predisposing T1D-

associated MHC class I alleles are B*18, B*39 and A*24 (Mikk et al., 2014). The predisposing effects of the MHC class I alleles are best seen as an association with increased progression rate from seroconversion to T1D, a risk effect also correlating with a faster decline in FPIR. (Koskinen et al., 2020). The HLA class I variants' associations to later events in the disease development imply that the Class I molecule is not as important for the triggering of autoimmunity; instead, variants in Class I loci are thought to modulate disease progression characteristics after IA initiation.

To conclude, it is now commonly agreed upon that it is the DRB1, DQA1 and DQB1 genes which confer the highest risk effect to T1D when compared to other HLA genes. This association has been observed in multiple international and national studies, such as those conducted by the Type 1 Diabetes Genetics Consortium (T1DGC) (Erlich et al., 2008), the TEDDY group (Hagopian et al., 2011) and the DIPP study group (Ilonen et al., 2016). It has been possible through similar prospective studies to allot other less powerful HLA alleles, e.g. in HLA-DP and HLA-B loci, to a T1D risk (Mikk et al., 2014; Noble & Erlich, 2012). Most importantly, specific genotypic and haplotypic combinations of DRB1-DQA1-DQB1 alleles are now recognized to be the main contributors to the genetically conferred risk. The classification of genotypes into risk-associated DRB1-DQA1-DQB1 haplotypes now forms the basis for a majority of the adapted T1D genetic risk-scoring systems (Noble & Valdes, 2011).

2.4.2 Genetic risk factors outside the HLA region

The risk effects contributed by non-HLA factors are overshadowed by the overpowering effects of HLA-based factors. However, SNPs proximal to risk-associated HLA loci can exert a significant effect on HLA gene expression as an expression quantitative trait locus (eQTL) (Aydemir et al., 2019; Nygård et al., 2021); an epigenetic effect that can be exerted in cis or trans (Nyaga et al., 2018). In other words, it is possible that there are additional undetected HLA-proximal non-coding polymorphisms which significantly modify the risk conferred by HLA loci. However, the process of finding new modifiers has proven to be difficult since HLA-proximal modifiers are in strong LD with already disease-associated HLA loci. Furthermore, recent studies have also determined that the methylations status of the HLA-situated epigenome significantly modifies the risk conferred by HLA loci (Kindt et al., 2018).

However, there is also a plethora of non-HLA polymorphisms, such as the PTPN22 rs2476601 (G>A) SNP, a SNP that has been associated with an increase in risk of developing T1D (Sharp et al., 2015) and with an increased T1D progression rate (Pöllänen et al., 2017). Furthermore, variable number tandem repeats (VNTR) in the insulin (INS) gene can either increase or decrease the susceptibility to T1D (Durinovic-Belló et al., 2010). It has been determined that the risk effect conferred by INS VNTR type I is due to a decreased AIRE-activated insulin expression in the thymus (Cai et al., 2011). The lower expression of insulin-type TSAs could allow for the escape of self-reactive T cells during the negative selection of T cells; auto-reactive cells which could have an high specificity for insulin-epitopes (Bettini & Bettini, 2017; Durinovic-Belló et al., 2010). The most recent T1D GWAS have found approximately 90 gene variants that are associated with T1D and which encompass almost 70 non-HLA loci (Nyaga et al., 2018; Sur, 2020). However, the T1D associated SNPs' functional effects have not been thoroughly studied, except in cases of rare polymorphisms in already well-studied loci, such as TYK2 (Pellenz et al., 2021), or in the more common or powerful gene variants such as SNPs in the locus of PTPN2 (Wiede et al., 2017), PTPN22 (Valta et al., 2020), CTLA-4 (Y. Chen et al., 2018) and IL2RA (Rainbow et al., 2017). Notably, these are all immunologically important genes which in the global population harbor multiple polymorphisms associated with various forms of autoimmune disease.

The study of exonic coding polymorphisms is of uppermost interest in T1D research since it is a more straightforward approach to formulate a preliminary hypothesis of SNP causality if the polymorphism's translation results in a structural change to the protein. Nevertheless, intronic coding polymorphisms can cause downstream effects by modifying protein-splicing patterns or micro-RNA binding (de Almeida et al., 2018; Sharp et al., 2021). Moreover, both coding and non-coding polymorphisms can exert epistatic effects via alterations to the expression patterns of close and distant genes. Researchers have been committed to integrating HLA and non-HLA risk factors to produce more accurate systems of risk scoring. Studies have observed that certain combinations of genes may significantly increase the disease risk through synergistic and epistatic mechanisms, although these types of combinatory effects were difficult

to corroborate and the synergistic effects were often best observed amongst high-risk HLA genotypes (Maziarz et al., 2015; Perry et al., 2018; Winkler et al., 2012).

Still, a highly relevant aspect of future research into the risk effects conferred by non-coding SNPs relates to their highly pleiotropic nature, i.e., that they have an ability to exert hard-to-detect distal effects on other genes by remodulating their expression. Non-coding polymorphisms can be clustered in super-enhancer regions proximal to large gene clusters (Afzali et al., 2017; Onengut-Gumuscu et al., 2015). These types of polymorphisms may influence expression by affecting the methylation and acetylation rates of those enhancer regions (Gao et al., 2019). Moreover, such polymorphisms may exert more visible changes to the cell phenotypes since they can influence cell subset-specific expression patterns and cell fate (Soskic et al., 2019). Non-coding polymorphisms may also affect the binding of transcriptional factors or the formation of transcriptional complexes through effects on complex formation (Schwartz et al., 2017; Soderquest et al., 2017). Lastly, non-coding polymorphisms may also affect epigenetic functions, such as heterochromatin formation, by changing acetylation and methylation patterns, or by modifying a loci's ability to recruit chromatin modifiers. Crucially, alterations to cell subset-specific chromatin-modification patterns can inflict changes to higher-order chromatin structures (Acevedo et al., 2015). However, there are still only a few papers that cover the epigenetic downstream effects of non-coding polymorphisms, and the field's current understanding of cells subset-specific epigenetics is still primarily based on archaic in-silico analysis methods of GWAS data, methods which only impute the probability of an epigenetic effect occurring. For this thesis, ten different SNPs are presented in Section 2.5 in order to describe the functional polymorphisms' suggested alterations to the immunophenotype.

2.4.3 Environmental factors

The contribution of environmental factors to the formation of T1D and to the heterogeneity of the disease has been implied earlier in this literature review. As mentioned, physiological factors such as birthweight, obesity, growth pattern and nourishment status have been thoroughly studied, although the prospective study of physiological characteristics has often produced counter-indicative results with effects that are either weak or change direction depending on the study population (Krischer

et al., 2015, 2019). The study of dietary factors, such as the introduction of dietary fibers or milk products, has suggested that they contribute with a causal effect, albeit the results have been difficult to replicate (Hakola et al., 2019; Niinistö et al., 2020). A more recent hypothesis on this matter, which has been supported by independent authors, suggests that a dietary factor's timepoint of introduction determines whether the factor confer a risk or not (Rewers & Ludvigsson, 2016). The immune system has to adapt to a novel non-pathogenic antigen or to a change in diet every time when a new dietary factor is introduced. Thus, indicating that a dietary factor's risk effect stem from a highly temporal perturbation in the tolerogenic immune response. This could become especially relevant at a time point when children are weaned off from breast milk or when solid food are introduced to the diet.

The most thoroughly studied unit of environmental factors is that of viral infections, which can exert broad downstream effects on both immune cell and β-cell functions. It was noted early on that pancreatic sections of deceased T1D subjects had higher viral loads of certain virus taxa (Krogvold et al., 2015; Richardson et al., 2014). Additionally, observational studies have associated enterovirus and adenovirus infections with the onset of β-cell autoimmunity (Faulkner et al., 2020). The connection between enteroviral infections and T1D is well documented, although, the suggested causal nature of either acute or latent infections is obscure (Honkanen et al., 2017; Lonnrot et al., 2000; Oikarinen et al., 2011). The interaction between viral infections and T1D susceptibility or protection could be related to the direct infection of pancreatic tissue, an infection which could increase β-cell stress and facilitate the engagement of immune cells (Richardson et al., 2014). Furthermore, viral infections have been shown to modulate the immune system via indirect effects. Certain viruses have the ability to suppress and remodulate the function of the innate and adaptive immune system (Ghazarian et al., 2013). For example, perinatal CMV infections have been reported to confer protection from T1D (Ekman, Vuorinen, et al., 2019); moreover, some viruses in the herpes family produce lifelong latent infections that exert strong immunomodulatory effects (Picarda & Benedict, 2018).

Approximately 25% of all Finnish children have been infected with CMV postnatally or during early childhood (Aarnisalo et al., 2008; Hiltunen et al., 1995). Moreover, it has been shown that latent CMV infections exert a significant effect on the

immunophenotype, especially on T cell, natural killer (NK) cell and macrophage frequencies (Picarda & Benedict, 2018). To conclude, the study of environmental factors and their causal effects is ongoing, and the topic remains opinionated. Nevertheless, the field's most recent observations in this area have already been extensively reviewed and are considered out of scope for this literature review (Faulkner et al., 2020; Ilonen et al., 2019; Rewers & Ludvigsson, 2016).

2.5 Functional polymorphisms and their proposed effects in the immunophenotype

There has been a renewed endeavor to study the downstream functional effects of polymorphisms on immune cell population parameters. The increase in interest has come with recent advancements in bioinformatics, advancements including novel computational methods better able to distinguish causal polymorphisms with functional downstream effects from non-causal polymorphisms that are in LD with the causal disease-associated loci. New in silico methods, such as GWAS fine-mapping, combine computational imputation and loci prioritization to enable one to statistically discern locus that only tag for a disease-associated locus (nicknamed 'stowaway loci') from true causal gene variants (Farh et al., 2015; Onengut-Gumuscu et al., 2015). Multiple published algorithms have shown promising efficiency and accuracy in the detection of possible downstream phenotypic effects; such as effects on transcription-factor binding, gene silencing or the likelihood of a site undergoing an epigenetic modification (Huang et al., 2017; Robertson et al., 2021; Westra et al., 2018). Nevertheless, causal gene variants which have shown strong associations to IA or T1D are usually of chief interest. Importantly, analysis of genetic data have clearly shown that many T1D associated polymorphisms are clustered in pathways that regulate immune cell function and fate (Onengut-Gumuscu et al., 2015). In order to emphasize the functional polymorphisms' known and proposed effects on the immunophenotype, the following sections will depict ten different SNPs located in ten separate genes. The summary is authored to review the published literature of the ten polymorphisms' downstream effects, and concurrently declare the rationale behind the selected SNPs' inclusion into the thesis project. The reviewed SNPs' MAFs, T1D odds ratios or hazard ratios are presented in Table 1. Furthermore, the SNPs' proposed effects on cellular functions and cell-cell interactions are illustrated in Figure 6.

Table 1. Selected T1D associated SNPs and their proposed effects on the immunophenotype.

Gene	Code	Major > Minor	Risk Allele	MAF	OR (HZ*)	Disease association	Affected cell types
PTPN22[1]	rs2476601	G>A	A	0.094	1.81	T1D, CD, RA, MG, VI, JIA	T cells, B cells, Myeloid cells
CTLA-4[1]	rs231775	A>G	G	0.359	1.20	T1D, RA, CD, UC, JIA	Treg cells
IFIH1[1]	rs1990760	T>C	T	0.605	1.14	T1D, UC, Ps, CD, MS, MG	T cells, B cells
SH2B3[1]	rs3184504	C>T	T	0.464	1.27	T1D, CeD, CD, Ps, UC, RA, JIA	Monocytes, DCs, T cells
FUT2[1]	rs601338	G>A	A	0.441	1.12	T1D, CD, MS, Ps, MG	MAITs, iNKT, Myeloid cells
CD226[1]	rs763361	C>T	T	0.473	1.10	T1D, UC, CD, CeD, MS, RA	MAITs, iNKT
BACH2[2]	rs72928038	G>A	A	0.170	1.20	T1D, RA	T cells, B cells
UBASH3A[2]	rs11203203	G>A	A	0.330	1.16	T1D, VI, RA	CD4+ T cells, Treg cells
IL2RA[2]	rs61839660	C>T	C	0.100	0.62	T1D, RA, JIA	T cells, Treg cells
PTPN2[3]	rs45450798	C>G	C	0.156	1.20*	T1D, JIA, LADA	T cells, B cells

The odds ratio (OR) describes the SNP's association to T1D. The hazard ratio (HZ) annotated (*) describes the SNP's association to islet autoimmunity. The column "affected cell types" lists the cell types which cellular function or frequency could potentially be affected by the SNP. Sources for numerical data: 1. Shapiro et al. (2021), 2. Onengut-Gumuscu et al. (2015) and 3. Lempainen et al. (2015). The information on the SNPs' disease associations stems from previously cited sources and from data available in the online SNPedia database. CD = Crohn's disease, RA = rheumatoid arthritis, MG = myasthenia gravis, VI = vitiligo, JIA = juvenile idiopathic arthritis, UC = ulcerative colitis, Ps = psoriasis, MS = multiple sclerosis, CeD = celiac disease, LADA = latent autoimmune diabetes in adults.

2.5.1 PTPN22

PTPN22 encodes protein tyrosine phosphatase non-receptor type 22 and is commonly referred to as lymphoid tyrosine phosphatase (LYP). LYP acts as a negative regulator of TCR, BCR, and innate immune signaling by dephosphorylating a wide range of receptor kinases, such as Lck, Fyn and ZAP70 (Rieck et al., 2007). Additionally, LYP functions as a negative regulator of LFA-1 and type 1 IFN signaling (Armitage et al., 2021). The online SNPedia database lists 21 different PTPN22 SNPs, of which rs2476601 (G>A) is associated with multiple autoimmune diseases, such as T1D, RA, systemic lupus erythematosus (SLE) and Latent Autoimmune Diabetes in Adults (LADA) (Cousminer et al., 2018; Ferreira et al., 2019; Márquez et al., 2018; Westra et al., 2018). It has been proposed that a change in the inhibitory function of LYP could shift the threshold of immune receptor activation or inhibition in cell subsets expressing the aforementioned kinases; a change which either produce a gain-of-

function or a loss-of-function phenotype (Armitage et al., 2021). The downstream phenotypic effects of rs2476601 on lymphocyte and myeloid cell functionality have been extensively reviewed in a recent article by Armitage and colleagues (2021). The rs2476601 SNP is associated with T1D, with a younger T1D onset, and with a lower degree of β-cell functionality at diagnosis (Kordonouri et al., 2010; Petrone et al., 2008; Vehik et al., 2019). The SNP exerts a strong risk effect for T1D and it has been observed to cause changes in Treg-cell frequencies (Ferreira et al., 2019; Valta et al., 2020). Moreover, the rs2476601 polymorphism confers an increase in risk for seroconversion by potentially perturbating BCR-receptor signaling and increasing cell resistance to apoptosis, subsequently decreasing B-cell self-tolerance by enabling the escape of self-reactive B cells (Armitage et al., 2021; Habib et al., 2012). Furthermore, the rs2476601 SNP has consistently been associated with an increase in risk of seroconversion, seroconversion of IAA and GADA AAbs, initial IAA seroconversion, and an increased progression rate to seropositivity (Brorsson & Pociot, 2015; Krischer et al., 2015, 2017, 2019; Lempainen et al., 2015; Steck et al., 2009).

2.5.2 CTLA-4

CTLA-4 encodes an inhibitory checkpoint molecule that is constitutively expressed on Treg cells, and upregulated on activated conventional T cells (Y. Chen et al., 2018). The receptor is contained intracellularly when the cell is in a resting state and becomes expressed on the cell surface upon cell activation, whereafter it starts to shuttle between the cytosolic and membrane-bound phase. Activated $CD4^+$ cells have also been shown to excrete a soluble CTLA-4 isoform, however, the physiological function of the soluble form is still under investigation (Esposito et al., 2014). CTLA-4 competes with its activating and costimulatory counterpart, CD28, for the binding to B-7 molecules (CD80/CD86) on APCs. A recent study demonstrated that Treg cells were able to deplete CD80/CD86 molecules on APCs via CTLA-4–dependent trogocytosis (Tekguc et al., 2021). Moreover, the authors were able to demonstrate that CTLA-4 disrupts cis-CD80/programmed death ligand-1 (PD-L1) heterodimers through the formation of CTLA-4/CD80 interactions. This increases the prevalence of free PD-L1 on DCs, ligands now enabled to interact with and inhibit programmed death-1 (PD-1)–expressing effector T cells. A previous functional study observed that the Treg cells of SLE patients expressed CTLA-4 levels corresponding to that of

healthy subjects, however, the CTLA-4 molecules were less clustered in the lipid microdomains of the Treg cells (Jury et al., 2010). This is one direct connection between the role of CTLA-4 and the dysfunction of Treg cells in a systemic autoimmune disease. T1D GWAS have reported six different SNPs in CTLA-4 wherein the rs231775 (A>G), the most studied variant, has a suggested modifying effect on CTLA-4-expression levels (Robertson et al., 2021; Shapiro et al., 2021). It is proposed that the intragenic A/G substitution in exon 1 of CTLA-4, causing a T17A amino acid exchange, impairs CTLA-4 surface expression via changes to protein glycosylation patterns (Haseda et al., 2011; Mäurer et al., 2002). Exon 1 codes for the CTLA-4 protein's leader peptide, which have led researchers to believe that the rs231775 SNP mainly affects protein transport, turnover, or surface expression (Zhao et al., 2018). A lower expression of CTLA-4 could decrease the negative regulatory activity of Treg cells, and, therefore, increase the risk of self-reactive T cells escaping peripheral immunomodulatory mechanisms. A population study of Han-Chinese observed a reduced intracellular CTLA-4 expression in $CD4^{+}CD45RA^{-}FOXP3^{high}$ Treg cells of healthy individuals carrying the GG and GA genotype (Y. Chen et al., 2018). Furthermore, they also observed a higher frequency of IA-2A positive subjects amongst GG-genotypes as compared to AA-genotypes. The rs231775 allele has been associated with an initial seroconversion of GADA AAbs and with a later age of T1D onset in a TEDDY cohort, but the association was not found in a later analysis of similar material (Krischer et al., 2015, 2017). Moreover, the SNP did not function as a reliable predictor for IA in transethnic DAISY cohorts (Frohnert et al., 2017; Steck et al., 2014) The causal downstream effects of the polymorphism are poorly understood, nevertheless, the potential functional effects on Treg cell suppressor activity supports the assertion that the polymorphism needs to be studied further.

2.5.3 CD226

CD226, also known as DNAX, is a costimulatory molecule in the immunoglobulin superfamily known to modulate the activation of effector and memory T cells, and NK cells (Shapiro et al., 2021). The CD226 complex is competing with its immunoregulatory counterpoint, TIGIT, to bind CD155 and CD112 expressed on APCs during antigen presentation (W. Li et al., 2021; Shapiro et al., 2021). The function of CD226 is equivalent to CD28 since it activates Th-cell engagement, and

its competition with TIGIT creates an interaction that is highly analogous to the CTLA-4/CD28 immunoregulatory checkpoint pathway. A recently published study reported an upregulated expression of CD226 amongst $TIGIT^{+}CD226^{+}CD4^{+}$ Th cells in patients with dermatomyositis (W. Li et al., 2021). The CD226 SNP rs763361 (C>T) has been associated with T1D, RA, SLE and multiple sclerosis (MS) (Bai et al., 2020). The rs763361 variation produces a G307S missense mutation introducing a novel serine phosphorylation site at the cytoplasmic tail (Shirakawa et al., 2005; Wallet et al., 2017). In vitro studies have found an increase in MAPK/ERK downstream signaling amongst rs763361 genotype selected primary $CD4^{+}$ T cells (Gaud et al., 2018). This was best observed as an increase in IFN-γ and IL-17A production upon stimulation with anti-CD3/CD28 antibodies. The CD226 polymorphism rs763361 has been associated with IA, GADA-positivity and a faster disease progression rate from birth to T1D (Lempainen et al., 2015; Mattana et al., 2014; Törn et al., 2015).

2.5.4 SH2B3

SH2B3 encodes the lymphocyte adaptor protein (LNK), a phosphatase which is expressed in hematopoietic and endothelial cells (Wallet et al., 2017). LNK negatively regulates several JAK/STAT signaling pathways, pathways which are central during hematopoiesis, cytokine signaling, TCR-signal transduction, and cell migration (Shapiro et al., 2021). A recent study of $LNK^{-/-}$ mouse models has shown that LNK is an important negative regulator of lymphoid homeostasis in adipose tissue and that LNK reduces the risk of developing diabetes by regulating the expansion and activation of IL-15-dependent adipose G1 innate lymphoid cells (ILC) (Mori et al., 2018). LNK is strongly expressed in monocytes and DCs, and the SNP rs3184504 (C>T) has been associated with T1D, IA and with an altered expression of IFN-γ and STAT1 amongst T1D cases (Ashok Sharma et al., 2018; Westra et al., 2018). The rs3184504 polymorphism produces a R262W missense mutation in exon 3, an exon coding for a pleckstrin homology domain important for protein docking (Shapiro et al., 2021). It has been predicted that the rs3184504 SNP could disrupt a splicing enhancer motif important for splicing regulator binding, thus, producing a protein with perturbed functionality (Shapiro et al., 2021). Extensive analysis of EBV-transformed cells and peripheral blood transcriptome profiles have determined that the rs3184504 SNP functions as a trans-eQTL that affects the expression of multiple genes over a 1

Mb distance (Nyaga et al., 2018; Ram & Morahan, 2017). Moreover, rs3184504 has been shown to exert a cis-eQTL effect where it increases peripheral blood LNK levels in RA patients (Xingbo Mo et al., 2020). The SNP's functional effect is thought to be caused by a structural change to LNK's own phosphorylation site; a change that could affect LNK-activity levels and possibly the phosphatase's ability to dephosphorylate other protein complexes. The rs3184504 SNP has been associated with changes in IL-1β and IL-6 production due to an activation of the NOD2-pathway (Maslah et al., 2017). The SNP has also been associated with seroconversion amongst individuals predisposed to T1D in multiple prospective studies (Krischer et al., 2017; Törn et al., 2015; Vehik et al., 2019). The rs3184504 polymorphism's downstream effects on different hematological parameters have already been extensively reviewed (Maslah et al., 2017).

2.5.5 FUT2

FUT2 encodes fucosyltransferase 2 an enzyme required to secrete ABO-blood group antigens. The H-antigens are expressed on intestinal mucosa, and they are actively secreted in bodily fluids such as saliva, tears, milk, semen, and urine. Individuals able to secrete these antigens are called secretors (Shapiro et al., 2021). Disruption of FUT2 results in the absence of ABO secretion, which can be observed amongst non-secretor individuals homozygous for the T1D associated FUT2 variant rs601338 (G>A), a variant that produces a W154Stop nonsense mutation. ABO-antigen expression by the mucosal epithelium heavily impacts the binding of environmentally acquired pathogens, and, therefore, the formation of the commensal microbiota. However, studies have yet not been able to find a significant causative or correlative relationship between viral or bacterial infections and a definitive risk of developing T1D in non-secretors (Giampaoli et al., 2020). Nevertheless, similar relationships have been studied in diseases such as measles and mumps with significant findings surrounding the SNP's effect on infection susceptibility (Azad et al., 2018). The non-secretor AA genotype has been determined to be more susceptible to T1D (Smyth et al., 2011) and exhibit a faster decline in FPIR (Koskinen et al., 2020). Conversely, homozygosity for the FUT2 major-G SNP has in high-risk individuals been associated with a secretor, rapid progressor disease phenotype who after seroconversion have high AAb-titers and are positive for multiple AAbs (Pöllänen et al., 2017). Furthermore, one functional

study of mice demonstrated that the induction and maintenance of FUT2 expression in epithelia requires the stimulation of mucosal class 3 innate-lymphoid cells (called ILC3 cells) expressing RORγt, the presence of IL-22 and a pool of lymphotoxin provided by the microbiota (Goto et al., 2014). Hence, indicating that also secretors have a phenotype that secrete variable levels of H-antigens. It is possible that the prevalence of mucosal-associated immune cells, such as MAIT cells gamma-delta T cells or ILCs, could be different when comparing secretors with non-secretors since environmental factors could affect the individuals differently. Thus, depending on secretor status, individuals may be more likely to interact with different predisposing or protective environmental factors.

2.5.6 IFIH1

IFIH1, the interferon induced with helicase C domain 1, is an innate immune receptor that senses intracellular RNA and facilitates the initiation of an antiviral response by inducing IFN type I expression (Domsgen et al., 2016; Shapiro et al., 2021). IFIH1 is important for bodily IFN regulation since rare GOF mutations have been reported to cause interferonopathies, while LOF mutations provide protection from T1D (M. B. Johnson et al., 2019). The IFIH1 SNP rs1990760 (T>C) causes a missense mutation in the proteins C-terminal domain, and the polymorphism has been associated with protection from T1D on a GWAS level (Barrett et al., 2009). It has been determined that the rs1990760 minor allele does not affect the receptor's ability to bind to double-stranded RNA. However, the risk associated major T-allele has been linked to an elevated basal and ligand-induced type I IFN response. The protective effect, conferred by the minor C-allele, could come from a more efficient detection and clearance of entero- and coxsackie-viral infections; both of which are common viral infections associated with T1D (Gorman et al., 2017; Nejentsev et al., 2009). In vitro studies of pancreatic islets have demonstrated that the protective rs1990760 genotype provides a more efficient response to viral infections via a prominent type III IFN response (Domsgen et al., 2016). The increase in anti-viral response was also found in cells infected with T1D associated virus strains, suggesting that the IFIH1 SNP confers protection by improving viral response in pancreatic tissue. Interestingly, multiple studies have shown that TC-heterozygotes, as compared to TT-homozygotes, are more susceptible to T1D development (Dean et al., 2020; Schulte et al., 2016). This

dominant effect amongst heterozygotes could be observed in IFN and IFIH1 levels; an effect possibly facilitated by molecular heterosis. One study of T1D cases found that the rs1990760 risk-associated phenotype exhibited higher numbers of NK cells (Dean et al., 2020). Moreover, an eQTL analysis found a reduced CD25 expression on total and $CD56^{bright}$ NK cells amongst rs1990760 risk genotypes. Interestingly, the major rs1990760-T has also been associated with AAb-positivity and an increased IA-to-T1D progression rate (Brorsson et al., 2015; Brorsson & Pociot, 2015; Lempainen et al., 2015; Steck et al., 2014).

2.5.7 BACH2

BACH2, BTB domain and CNC homolog 2, is a highly conserved member of the basic leucine zipper domain (bZIP) superfamily of transcription factors, and it is an important regulator of both T- and B-lymphocyte differentiation and maturation (Afzali et al., 2017). BACH2 coordinates the transcription, activation, and repression of multiple lymphocyte-regulatory mechanisms, and it can induce cell apoptosis in response to oxidative stress. A knock-out of BACH2 will in mouse models reduce Treg cell numbers while simultaneously increasing effector T cell numbers, hence, causing fatal autoimmunity (Afzali et al., 2017). BACH2 appears to be critical for Treg-cell development in the thymus and it restrains effector T-cell differentiation in the periphery (Igarashi et al., 2017). BACH2 also restrains plasma-cell differentiation and promotes B-cell proliferation and memory B-cell formation (Igarashi et al., 2017). Patients with BACH2-deficiency develop common variable immune deficiency (CVID) and colitis (Afzali et al., 2017). Treg cells from these patients have reduced FOXP3 expression and their effector T cells have an increased expression of T-bet (Th1-cell master transcription factor) as well as the gut-homing receptors CCR9 and B7 integrin. The BACH2 SNP rs3757247 (G>A) appears to be associated with the primary seroconversion of GADA AAbs and with IA (Krischer et al., 2017; Lempainen et al., 2015). One widely studied BACH2 non-coding SNP, rs11755527, has been implicated to exert extensive pleiotropic effects, and the SNP is associated with T1D. Moreover, the rs11755527 SNP is in LD (r^2 = 0.94) with rs3757247, suggesting that these SNPs could exert synergistic effects (Krischer et al., 2017; L. Yang et al., 2019). Nevertheless, it should be further investigated if both SNPs are causative or if one only tag for the other’s risk effect. Recent reviews have discussed

the role of BACH2 in lymphoid fate determination and different BACH2 polymorphisms' potential roles in autoimmunity (Igarashi et al., 2017; L. Yang et al., 2019).

2.5.8 UBASH3A

UBASH3A encodes the ubiquitin-associated SH3A protein which is dominantly expressed in T cells and serves as a negative regulator of TCR signaling (Shapiro et al., 2021). UBASH3A interferes with the Cbl-mediated down-regulation and degradation of receptor tyrosine kinases (Shapiro et al., 2021), and UBASH3A can inhibit the activation of the IKK complex through a ubiquitin-dependent mechanism (Ge et al., 2017), subsequently inhibiting NFκB-signaling. The rs11203203 (G>A) SNP is a UBASH3A polymorphism that is located in intron 4, an intron including a possible $CD4^+$ T-cell enhancer region or super-enhancer region (Onengut-Gumuscu et al., 2015). The rs11203203 SNP is associated with IA, T1D and a faster disease-progression rate from IA to T1D (K. Johnson et al., 2012; Steck et al., 2014; Ziegler et al., 2013). Functional studies have demonstrated that the rs11203203 SNP increases UBASH3A levels in TCR-activated primary $CD4^+$ T cells, resulting in a decreased NF-kB signaling and subsequently to a reduced IL-2 production (Ge et al., 2017). It is, therefore, also possible that the SNP could affect the stability of Treg-cell populations via modifications to the IL2/IL2 receptor α (IL2RA) regulatory axis. Moreover, the rs11203203 is in complete LD (r^2 =1) with rs80054410 (T>C), another SNP modifying the same super-enhancer. A recent article showed that the risk associated rs11203203-A with rs80054410-C forms a common T1D associated risk haplotype (haplotype frequency = 0.292) (Ge & Concannon, 2018). It has also been shown that the UBASH3A SNP rs883868, which is in LD with rs11203203 (D' = 0.85), disrupts the binding of retinoic acid receptor α (RARA), an important transcriptional factor in Treg cells (Gao et al., 2019).

2.5.9 IL2RA

IL2RA encodes the interleukin 2 receptor chain alpha that together with the beta-subunit forms the IL2-receptor. The IL2RA locus belongs to one of the strongest non-HLA susceptibility-factor loci, an association observed in multiple autoimmune diseases (Rainbow et al., 2017). SNPs of both IL2 cytokine and IL2-receptor genes are thought to facilitate homeostatic changes to the IL2/IL2RA regulatory axis (J. Yang et

al., 2015), changes which could affect the activation of T cells, especially in naïve Treg cells (Garg et al., 2012; Tang et al., 2015). One rare IL2RA SNP of particular interest, rs61839660 (C>T), is associated with T1D, inflammatory bowel disease (IBD) and Crohn's disease (Huang et al., 2017; Rainbow et al., 2017; Soderquest et al., 2017). The SNP has been shown to reduce receptor subunit expression as a response-eQTL that becomes activated in response to diverse transcriptional factors, although, additional functional studies are needed to investigate which T-cell populations are affected by the SNP (Rainbow et al., 2017; Simeonov et al., 2017). Furthermore, the rs61839660 SNP was determined to be located in a T-cell enhancer region, where the minor-T allele disrupts the binding of the transcriptional factor MEF2A/C (Schwartz et al., 2017). Additionally, the rs61839660 gene locus has been predicted to bind T-bet, a transcription-factor interaction that could be modified by the SNP (Soderquest et al., 2017)

2.5.10 PTPN2

PTPN2 encodes the protein tyrosine phosphatase non-receptor type 2, a phosphatase that has a analogous function to the PTPN22 product since it is an important negative regulator of the JAK/STAT pathway (Wiede et al., 2017). Interestingly, early in vitro studies of β-cells demonstrated that decreased PTPN2 availability exacerbated STAT1-activated IL-1β signaling, concurrently turning an intrinsic IFNγ-signal into an apoptotic signal (Moore et al., 2009). Studies have shown that deficiency of PTPN2 may allow for Th-independent acquisition of Tc cytotoxic activity (Wiede et al., 2014). This is reflected in the gene's connection to T1D development since the PTPN2 SNP rs45450798 (G>C) is associated with islet autoimmunity and with a faster disease progression after seroconversion (Lempainen et al., 2015). The SNP, located in the PTPN2 3'-untranslated region, has been in-silico predicted to produce a novel microRNA binding site for miR-453 (de Almeida et al., 2018). However, it was also predicted that the novel binding site would not affect the translation of the product. Nonetheless, the authors pointed out that other non-coding SNPs repressing PTPN2 are associate with several immune related diseases, including T1D (de Almeida et al., 2018). The risk effect conferred by the rs45450798 SNP could be contributed to a decreased sensitivity for apoptotic stimuli in B cells, although, one functional study could not find an SNP-mediated alteration in the B-cell frequencies of individuals with

T1D (Thompson et al., 2014). Furthermore, functional studies of rs45450798 carriers with T1D have not found any significant difference in Treg cell subpopulation frequencies (Valta et al., 2020). However, one study found that the rs45450798 SNP was significantly associated with an more rapid decline in FPIR amongst children with multiple AAbs (Koskinen et al., 2020).

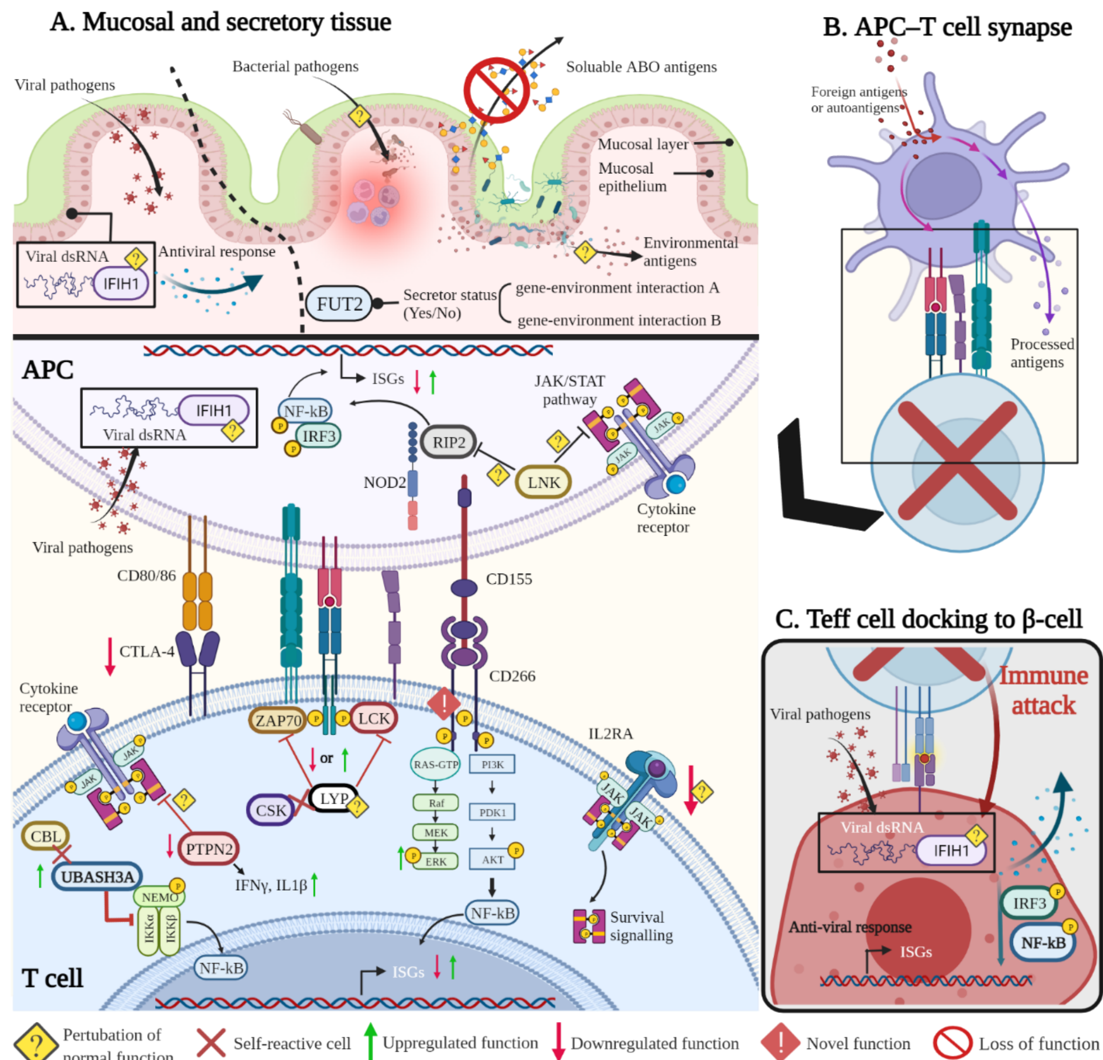

Figure 6. The reviewed polymorphisms' placements in functional pathways and their suggested effects on cellular function. A. Perturbations in the function of IFIH1 and FUT2 modify the organism's interactions with the environment and the pathogens in it. The modification of antiviral response time via the activation of interferon stimulating genes (ISG) can affect the organism holistically, therein modifying the virome or modify the antiviral response of β-cells (C.). B. The majority of the reviewed SNPs can be placed in the functional pathways of immune cells, especially in the pathways of T cells and APCs. Nonetheless, it should be noted, the reviewed SNPs' functional effects are not limited to the illustrated cell subsets since the functional effects can be observed in other cell types expressing the same locus. The figure is conceptually adapted from Shapiro et al. (2021). Created with BioRender.

3. The background and the objectives of the thesis study

3.1 Background

The DIPP study group was recently able to observe an effect where the PTPN22 polymorphism rs2476601 (G>A) significantly modified Treg cell frequencies in both non-diabetic and diabetic children (Valta et al., 2020). The authors found a significantly higher $CD4^+CD25^+CD127^{low}FOXP3^+$ Treg cell frequency (GG vs. AA; $p = 0.014$) and $CD4^+CD25^+CD45RO^-CD127^{low}FOXP3^+$ naive Treg-cell frequency (GG vs. AA; $p = 0.041$) amongst rs2476601-A risk-allele carriers when comparing ten non-diabetic GG, AG, and AA genotypes in a validation cohort. This Treg cell modifying effect was also observed in a similar clinical study of SLE, however, only amongst healthy adult individuals of a non-SLE–susceptible genotype (Ferreira et al., 2019). Ferreira and colleagues observed that the rs2476601-A allele conferred a significantly higher frequency of total $CD4^+CD127^{low}CD25^{hi}$ Treg cells ($p = 3.3\times10^{-4}$) and $CD4^+CD127^{low}CD25^{hi}$ memory Treg cells ($p = 1.0\times10^{-4}$). It should be noted that the DIPP study was able to detect the SNP's cell frequency modifying effects in a much smaller cohort (N = 30 vs. N = 486), which can potentially be explained by the DIPP cohort examining younger children at risk for diabetes. In contrast, the SLE study detected a corresponding effect when examining > 450 healthy adults; adults who have a more heterogeneous and experienced immunophenotype. Notably, the SNP's effect could not be observed when examining SLE cases due to a persistent in-group variation that most likely derived from the heterogeneity of the disease. High in-study variation usually stems from the study populations' phenotypic and genotypic heterogeneity, and this concurrently increases the need for a larger samples size so as to achieve more statistical power and more robust results. In conclusion, the quantification of weak genetic risk effects in adults usually requires large sample sizes to compensate for in-study variation, thus, lowering the practicability of immunophenotyping adults when attempting to quantify small variations in immune cell parameters or in immune cell functionality.

CTLA-4, the key inhibitory immune-checkpoint protein expressed by conventional T cells and Treg cells, competes in cis with its costimulatory counterpart CD28 for the binding to CD80/86 expressed on antigen-presenting cells (APCs). It is evident that the sequestration of CD80/86 on APCs suppresses the APC–T-cell interactions by

lowering the propensity and avidity of CD28–CD80/86 interactions (Y. Chen et al., 2018). The competitive-inhibitory interaction between the aforementioned components is well studied, and the kinetic-mechanistic pathway of CTLA-4 suppression was recently elucidated (Tekguc et al., 2021). The rs231775 (A>G) SNP has been shown to confer a significant increase in risk of developing T1D, and the risk effect is determined to stem from an alteration in the protein's expression level (Y. Chen et al., 2018; Mäurer et al., 2002; Robertson et al., 2021; Shapiro et al., 2021; Wang et al., 2017). The SNP has been observed to modify $CD4^+$ and $CD4^+CD25^+$ cell frequencies in children at risk for T1D (Jonson et al., 2006). Conversely, one functional study could not find a SNP-mediated effect in the intracellular expression levels of CTLA-4 amongst individuals with fully developed T1D (Haseda et al., 2011). Nonetheless, a lowered expression of surface-bound CTLA-4 would constitute a major alteration to the T cells ability to downregulate CD28–CD80/86 interactions (Y. Chen et al., 2018), a activatory axis important for normal conventional T-cell–APC interactions. The polymorphism's functional downstream effects on an immunophenotype level are still unknown. Furthermore, the SNP's effect on a broader spectrum of T-cell subsets has not been investigated, even when the polymorphism increases susceptibility to T1D and other autoimmune diseases.

3.2 Study hypothesis

We postulate that it would be more efficient to detect and quantify the immunological effects exerted by non-HLA polymorphisms in a cohort of younger children (< 5 years of age); mainly since environmental effects have not had the time to modify the immunophenotypes of young children in a similar manner to adults. Genetic factors could, therefore, be perceived to exert a greater risk effect on the immunophenotype of younger children since environmental factors, such as vaccinations and infection history, have not caused a persistent alteration in the immunophenotype. Consequently, the quantification of hard-to-detect phenotypic effects could possibly be more efficient when studying younger children, even within a cohort of a smaller sample size, since one is simultaneously eliminating the heterogeneity caused by various acquired environmental effects. This hypothesis sets up a proposition to study genotype-to-immunophenotype effects in younger children (< 5 years of age) by utilizing the databank material produced and managed by the DIPP study group.

It should be attainable to quantify the CTLA-4 rs231775 SNP's downstream effects on multiple immune cell subsets by utilizing the DIPP study's data repository. Proper case-control matching, and the elimination of other powerful genetic and environmental effects could enable one to better quantify previously discussed downstream effects, although, not only on protein expression levels, but also on a wider scale of cells subset frequencies; frequencies which can be affected by the inherent perturbation of CTLA-4's inhibitory role in the stimulatory CD28–CD80/86 axis.

3.3 Study objectives

This thesis study contains two main objectives. The first objective is to generate a novel DIPP subject cohort that allows for the future execution of genotype-to-immunophenotype studies which aim to quantify non-HLA polymorphisms' effects on various PBMC population parameters. Our preliminary aim is to generate a dataset through the genotyping of ten different non-HLA polymorphisms amongst 700 healthy, AAb-negative, at-risk children who are enrolled in the DIPP study. All subjects are genotyped, utilizing the TaqMan qPCR platform, for the SNPs rs2476601 (PTPN22), rs231775 (CTLA-4), rs1990760 (IFIH1), rs3184504 (SH2B3), rs601338 (FUT2), rs763361 (CD226), rs72928038 (BACH2), rs11203203 (UBASH3A), rs61839660 (IL2RA) and rs45450798 (PTPN2). Additionally, all subjects will be screened for CMV-IgG antibodies with seropositivity functioning as a marker for an active or latent CMV infection.

The second objective of the thesis study is to conduct an immunophenotyping pilot of twelve matched case-control pairs homozygous for the wild type or variant form of CTLA-4 (rs231775). The immunophenotyping pilot is conducted to demonstrate the feasibility and practicability of the study design. The functional downstream effects of the rs231775 SNP on immune cell population parameters will be quantified using flow cytometry amongst the following cell types: monocytes, DCs, NK cells, innate natural killer T lymphocytes (iNKT), B cells, T cells and Treg cells. Furthermore, the polymorphism's effect on intracellular CTLA-4 levels will be quantified amongst $CD4^{+}CD25^{+}CD127^{low}FOXP3^{+}$ Treg cells utilizing flow-cytometric panels developed and optimized for the thesis project. Finally, surface CTLA-4 expression will be

quantified on isolated $CD3^+$ T cells after 1, 4, and 24 hours of PMA/Ionomycin stimulation.

4. Study design and methods

4.1 Cohort generation

The study cohort was generated from children enrolled in the DIPP study, but who have not developed AAbs or clinical T1D during the follow-up period (Parkkola et al., 2013). All subjects included were born between 2005 and 2017 and were HLA-genotyped in accordance with the most recent DIPP inclusion criteria (Ilonen et al., 2016).

The DIPP study's HLA class II-based eligibility criteria, in short, are developed to define major haplotypes associated with susceptibility to or protection against T1D. HLA typing was initiated with genotyping for frequent DQB1 alleles, with four-digit resolution, followed by a panel of probes defining three DQA1 alleles (DQA1*02:01, DQA1*03, DQA1*05) that were differentiated into major haplotypes containing DQB1*02, DQB1*03:01, DQB1*03:02 and DQB1*03:03 alleles. When screening DRB1 alleles, DRB1*04 alleles associated with variable T1D risk were further defined into DRB1*04 subtypes amongst DQB1*03:02 positive subjects. A subset of DQB1 genotypes had their second exon hypervariable region sequenced by an off-site service provider since certain alternative genotypes cannot be distinguished with probe-based genotyping. To summarize, eligible HLA genotypes include those homozygous or heterozygous for the DQA1*05-DQB1*02 (DR3-DQ2) and DRB1*04:01/02/04/05-DQA1*03-DQB1*03:02 (DR4-DQ8) haplotypes. Individuals carrying the DR3-DQ2/DQA1*03-DQB1*03:03 (DR9-DQ9) combination or the high-risk DR4-DQ8 haplotype in combination with the DQB1*05:01 (DR1-DQ5), DQA1*02:01-DQB1*02 (DR7-DQ2), DQB1*04 (DR8-DQ4), DR9-DQ9, or DQB1*06:04 (DR13-DQ6.4) haplotype are also eligible (Mikk et al., 2020). The DQB1 and DQA1 assays are in-house homogeneous PCR assays (Kiviniemi et al., 2007). The HLA-DRB1 assay is an in-house assay that is based on the commercial dissociation-enhanced lanthanide fluorescence (DELFIA) platform (Kiviniemi et al., 2007; Lehmusvuori et al., 2014; Nejentsev et al., 1999).

All subjects included in the thesis cohort have at least two or more cryopreserved PBMC samples which were drawn between the ages of six months and five years. The PBMCs were isolated from heparinized whole blood by gradient centrifugation in accordance with an in-house protocol, whereafter the cells were cryopreserved in -80 °C or in liquid nitrogen (Öling et al., 2005). Serotyping for GADA, IA-2A, IAA, and ZnT8A was performed with AAb-specific radio-binding assays using blood serum (Juusola et al., 2016; Siljander et al., 2009). Screening for ICA positivity was performed with an indirect immunofluorescence staining method using human pancreas sections (Bottazzo et al., 1974). The standardized limits for seropositivity have been described earlier (Pöllänen et al., 2019).

The selection of SNPs for genotyping was based on four criteria:

1. The gene product should have a determined or suggested functional relationship to the activities of immune cells, especially T and B cells.
2. The SNP should affect the functionality of the protein or the expression of the gene.
3. The MAF should be sufficient for the detection of homozygotes in a cohort of fewer than 1000 individuals, i.e., so that each SNP would yield at least 15 to 20 minor SNP homozygotes.
4. The SNP should have an association to multiple autoimmune diseases.

The selected SNPs and their suggested functions and effects on the immunophenotype are presented in Section 2.5.

This study was authorized by the ethical committee of the North Ostrobothnia's Hospital District in accordance with the Declaration of Helsinki. All samples and data were collected with informed consent from the subjects' legal guardians and are stored according to the protocols of the DIPP study.

4.2 Study population characteristics

The generated cohort consisted of 694 DIPP study subjects of which 99% (690/694) were recruited at the Turku Universal Hospital Research Centre and the remaining 1% (4/694) at the Tampere University Hospital Research Centre. The whole-cohort male-to-female ratio was 50.3% and 49.7%, respectively. Furthermore, in accordance with the most recent DIPP HLA-risk categories, 28.2% of the subjects are placed in a high-

risk group, 53.9% in a moderately increased risk group and 16.0% in a slightly increased risk group (Ilonen et al., 2016). One out of the 694 subjects belonged to a neutral risk group, and twelve subjects carried an unresolved haplotype.

The subcohort intended for wide-scale immunophenotyping and quantification of CTLA-4 expression was formed from twelve pairs matched for rs231775 (A>G) SNP genotype (risk allele homozygote = GG; wild type allele homozygote = AA), sex, research center, birthdate (± 5 months) and sample date (± 6 months). All subjects were of a PTPN22 GG-homozygous genotype, and the subjects were on average heterozygous for two of the other eight analyzed SNPs (number of heterozygous SNP loci: min = 0; max = 6). None of the subjects were homozygous for any other studied SNP, apart from the subcohort's rs231775-GG risk-allele homozygotes. The immunophenotyped PBMC samples of the subcohort pairs were taken at the age of 6 months, 9 months, 1 year, 2 years, 3 years, or 4 years; hence, producing two matched pairs for respective time point. Moreover, as of the whole subcohort, six pairs were of respective sex, however, the sex was not matched between respective pairs when considering a specific time point.

4.3 Genotyping

Sample preparations were initiated with the aliquotation of diluted and undiluted stock DNA samples. DNA was alternatively extracted from freezer-stored cord blood samples using a standard in-house salting-out method (Olerup & Zetterquist, 1992), from stored bloodspot cards using an in-house extraction method (Nejentsev et al., 1999) or from stored PBMC samples using the commercial NucleoSpin Tissue DNA extraction kit (Macherey-Nagel, ref. 740952.50). Visibly impure samples were purified with a NucleoSpin gDNA Clean-up kit (Macherey-Nagel, ref. 740230.50). DNA concentrations were measured with a Qubit 4 Fluorometer (Thermo Fisher, cat. Q33226).

SNP genotyping was performed by TaqMan qPCR using on-the-shelf and custom-made TaqMan probes (Thermo Fisher, cat. 4351379) for rs2476601 (Assay ID: C__16021387_20), rs231775 (Assay ID: C___2278044_10), rs1990760 (Assay ID: C___2780299_30), rs3184504 (Assay ID: C___2981072_10), rs601338 (Custom assay), rs763361 (Assay ID: C___1464836_20), rs72928038 (Assay ID:

C__97128303_10), rs11203203 (Assay ID: C__31891467_20), rs61839660 (Assay ID: C__90618163_10) and rs45450798 (Assay ID: C__86382390_10). The used methods, as modified from the manufacturers protocol, have been described earlier (Nygård et al., 2021). The genotyping results were analyzed with QuantStudio Designs & Analysis Desktop Software (v1.5.1) with analytical call settings set at "analyze Real-time Rn-Median (Rna to Rnb)". A summary of all calls was made with Thermo Fishers Connect cloud software for genotyping with previously specified call settings.

4.4 Serological testing for latent cytomegalovirus infection

The subjects had their in-cohort oldest plasma sample analyzed, i.e., the sample extracted in tandem with the last available PBMC sample taken at or before the age of five years. The serological CMV-ELISA assay was performed by an off-site service provider (Laboratory of Clinical Microbiology, Turku University Hospital) that utilized the commercial VIDAS CMV IgG assay kit (bioMérieux, cat. 30204-01). The specifications of the assay kit and the threshold values for seropositivity have been described earlier (Murat et al., 2013)

4.5 PBMC aliquotation, CD3$^+$ T-cell fractioning and PMA/Ionomycin stimulation

The preparations for flow cytometry and CD3$^+$ magnet-activated bead sorting (MACS) was initiated with the thawing and aliquotation of cryopreserved PBMC samples. PBMC thawing was conducted according to a standard in-house procedure, and the cells were treated with DNAse I. The matched pairs had their samples concomitantly prepared and analyzed to limit the influence of batch variation. The used cell staining methods are described in the next section and the panels are described in Appendix I, tables 1-5. The cells, 0.5–2.0×10^6 cells for each panel, were analyzed in the following order of prioritization using the following panels: Treg-cell panel, stimulated CD3$^+$ T cell CTLA-4–expression analysis panel, CD4$^+$/CD8$^+$ chemokine panel, B-cell panel, and myeloid and innate lymphoid cell panel. Samples which included a suboptimal total number of PBMCs were excluded from panel analysis in the order of increasing prioritization. The cells aliquoted for the Treg cell, CD4$^+$/CD8$^+$ chemokine, B-cell, and myeloid and innate lymphoid cell panels, were analyzed with flow cytometry on the same day as they were thawed. Meanwhile, the cells aliquoted for CD3$^+$ MACS fractioning were incubated (37 °C, 5% CO_2) in suspension with 5% AB serum supplemented RPMI-1640 (supplemented RPMI-AB; Appendix II, Table 1) overnight.

The magnetic separation of $CD3^+$ T cells was performed with CD3 Microbeads (Miltenyi Biotec, cat. KM-12636) and with the use of MS columns (Miltenyi Biotec, cat. 130-042-201) fitted for Miltenyi's MS MACS separator. Fractioning was performed according to the manufacturers protocol. The isolated $CD3^+$ T cells were subsequently aliquoted in 5×10^4 cell subsets to duplicate flat-bottomed 96-well 250 uL plates with aliquots intended for 1, 4 and 24 hours of incubation. Furthermore, additional cells were aliquoted for 24 hours of incubation (and for the remaining time points when possible) and stained as fluorescence-minus one controls (FMO). One sample set functioned as an unstimulated control with cells suspended in 200 uL supplemented RPMI-AB medium. The samples aimed for stimulation were suspended in 200 uL supplemented RPMI-AB including 50 ng/mL phorbol myristic acid (PMA) (Sigma-Aldrich, P8139-1MG) and 1 ug/mL Ionomycin (Sigma-Aldrich, I0634-1MG). The plates were incubated (37 °C, 5% CO_2) for the aforementioned time periods, whereafter the samples were stained and studied with flow cytometry.

4.6 Flow cytometric studies

Flow cytometry was performed according to the guidelines published by the European Journal of Immunology (Cossarizza et al., 2019). The staining and cytometry procedure followed a published in-house strategy optimized for on-site equipment (Valta et al., 2020; Viisanen et al., 2019). Moreover, internal control PBMC samples from healthy adult individuals were used to assess technical variation. Cell viability analysis of all panels was performed with Zombie Aqua dye (BioLegend, cat. 4231) according to the manufacturer's protocol. Surface immunostaining was performed with fluorochrome-labeled antibodies selected to define each cell subtype of interest. Antibodies for intracellular markers in the Treg-cell panel were added after fixation and permeabilization with the eBioscience FOXP3/Transcription kit (Invitrogen, cat. 00-5523-00). The myeloid and innate lymphoid panel included pretreatment with the TruStain FcX Fc-receptor blocker (BioLegend, cat. 422301). Both procedures were performed in accordance with respective manufacturer's instructions. The stimulated cells intended for CTLA-4–expression analysis were stained for 30 minutes (in incubator; 37 °C, 5% CO_2) in ad 50 uL of phosphate buffer saline (PBS) supplemented with 5% bovine serum albumin (BSA), subsequently washed with FACS-buffer (PBS, 0.5% BSA and 0.1% sodium azide) and run on a NovoCyte Quanteon flow cytometer

(Agilent, cat. 2010097). The remaining panels were incubated with their markers for 20 minutes at room temperature and subsequently treated according to the aforementioned procedure. The data was analyzed with Becton Dickinson's FlowJo Software (Version. 10.8.0) in accordance with predetermined gating strategies (appendixes III–VII).

4.7 Statistical analysis

The statistical analysis was performed with GraphPad Prism 9 (Version. 9.2.0). The level of significance for lognormal distributed data was calculated using ratio paired t-test. Wilcoxon's matched pair signed-rank test was applied when the dataset deviated from lognormal distribution or included null values. A two-tailed p-value ≤ 0.05 was considered significant.

5. Results

5.1 Genotyping and CMV-IgG serotyping results

TaqMan genotyping yielded a complete set of SNP data for 678 subjects, hence, producing a successful call rate of 99.8% (for all tests combined). Out of 694 subjects, 38.5% (267/694) were homozygous for one SNP. Furthermore, when excluding PTPN22 (rs2476601-A) SNP carriers, 38.6% (207/536) of the subjects were homozygous for one SNP. The genotyping results are presented in Table 2. CMV-IgG serotyping found that 24.0% (166/693) of all subjects were seropositive. The cutoff value for seropositivity was set at ≥ 5 IU/mL.

Table 2. The allelic distribution of genotyped SNPs

Genotype	**SNP homozygotes**	**Heterozygotes**	**WT homozygotes**	**No. of subjects | MAF**
Gene (SNP)	N-genotype (%)	N-genotype (%)	N-genotype (%)	N | MAF (%)
PTPN22 (rs2476601)	18-AA (2.6)	139-AG (20.1)	536-GG (77.3)	693 (12.6)
CTLA-4 (rs231775)	179-GG (25.8)	327-AG (47.2)	188-AA (27.1)	694 (49.4)
IFIH1 (rs1990760)	97-CC (14.0)	329-CT (47.5)	266-TT (38.4)	692 (37.8)
SH2B3 (rs3184504)	139-TT (20.1)	326-CT (47.2)	218-CC (31.5)	683 (44.2)
FUT2 (rs618338)	82-AA (11.8)	354-GA (51.1)	258-GG (37.2)	694 (37.3)
CD226 (rs763361)	137-TT (19.8)	356-CT (51.4)	201-CC (29.0)	694 (45.4)
BACH2 (rs72928038)	19-AA (2.7)	126-AG (18.2)	549-GG (79.2)	694 (11.8)
UBASH3A (rs11203203)	51-AA (7.4)	289-GA (41.7)	354-GG (51.1)	694 (28.2)
IL2RA (rs61839660)	—	CT-54 (7.8)	640-CC (92.4)	694 (3.9)
PTPN2 (rs45450798)	17-GG (2.5)	168-CG (24.2)	509-CC (73.4)	694 (14.6)

The allelic distribution of the SNPs [N-genotype (%)] is presented as the number of genotypes (N), the allele combination, and the genotype frequency (%). The total number of called genotypes for respective SNP is presented in the rightmost column together with the MAF.

5.2 CTLA-4 expression patterns in PMA/Ionomycin stimulated CD3$^+$ T cells

The quantification of CTLA-4 surface expression was performed with PMA/Ionomycin stimulated CD3$^+$ T cells. The matched pairs' samples were treated and analyzed in tandem, consequently, producing data for 8 pairs at the 1-hour time point and for 9 pairs at the 4 and 24-hour time points, respectively. The activation marker CD69 was analyzed to verify proper technical performance and to measure the level of T-cell activation (gated populations; Appendix III, Figure 1). Unstimulated control samples expressed no CTLA-4 after each time period of incubation (figures 7 and 8). CD69 was expressed by unstimulated cells during all time points, thus, producing a background level of marker expression with peak expression occurring at the 4-hour time point (Figure 7; geometric mean: 4 hours = 36.56%). The PMA/Ionomycin stimulated samples demonstrated an exponential increase in CD69 expression (Figure 7; geometric mean: 1 hour = 90.79%, 4 hours = 97.55%, 24 hours = 99.59%) with CTLA-4 surface-expression levels peaking at the 4-hour time point (geometric mean = 10.99%).

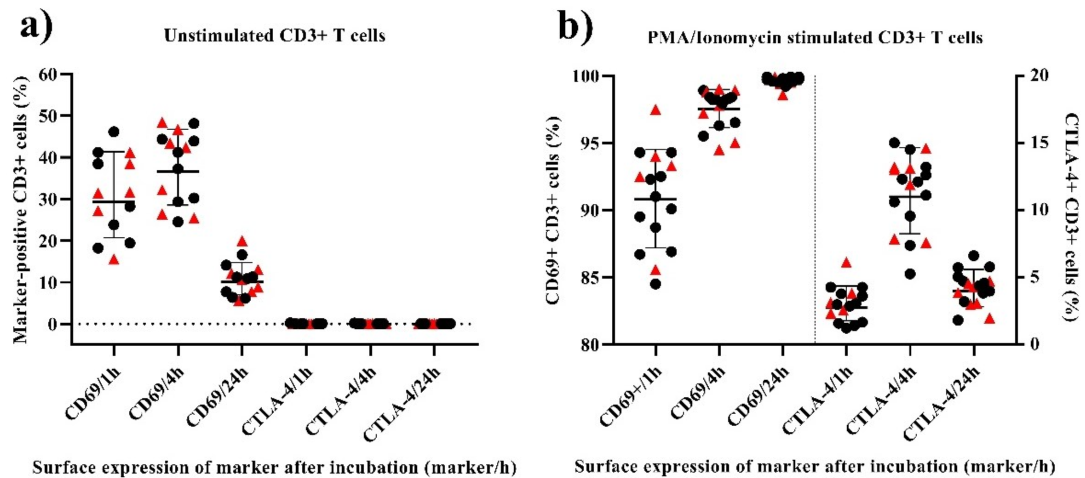

Figure 7. Expression patterns of CD69 and CTLA-4. The distribution of surface-marker positive cell frequencies for respective time points is visualized with red triangles for GG-genotypes and black circles for AA-genotypes. The 1-hour mark includes 13 samples (6-GG; 7-AA); the 4 and 24-hour marks include 15 samples (7-GG; 8-AA). The data is presented with the combined geometric mean and geometric SD for both GG and AA genotypes. a) Frequency of unstimulated marker-positive T cells as a fraction of all CD3$^+$ T cells. b) Frequency of PMA/Ionomycin stimulated CD69$^+$ T cells and CTLA-4$^+$CD3$^+$ T cells as a fraction of all CD3$^+$ T cells.

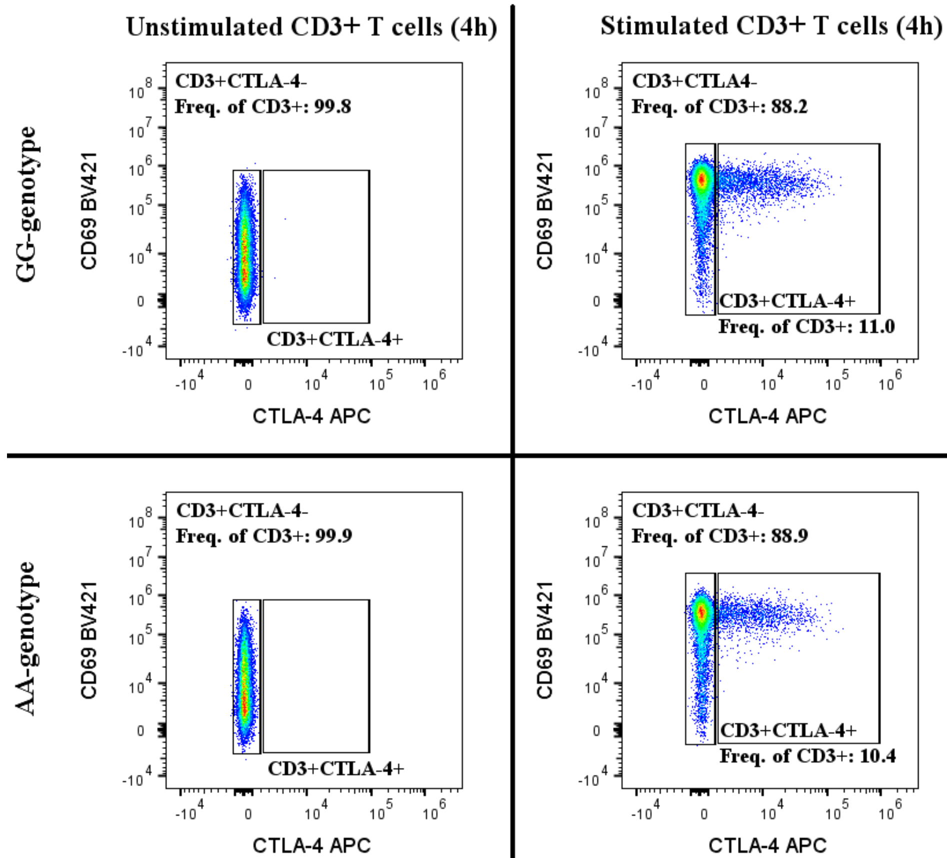

Figure 8. Comparison of a matched pairs' CTLA-4^{+}CD3^{+} cell fractions. The rows show a representative pair of samples with either CTLA-4 GG or AA genotype, and the columns present the sample type. The presented samples were incubated with or without PMA/Ionomycin for 4 hours. The frequency of CD3^{+}CTLA-4^{-} cells and CD3^{+}CTLA-4^{+} cells is given as the frequency of the total CD3^{+} cell fraction.

When comparing the matched pairs' data, there were no significant differences in the frequency of CTLA-4^{+} cells after any period of stimulation (example data; Figure 8). Furthermore, there were no apparent effects on CTLA-4^{+} cell frequencies when examining CD4^{+} Th and CD8^{+} Tc cells separately, or when analyzing naïve and memory T cells separately (Figure 9). Furthermore, a separate analysis of the CD69^{+} activated cell fraction (CD69^{-} cells excluded) did not produce any differing results from that found in the in-text presented total CD69$^{+/-}$ fraction.

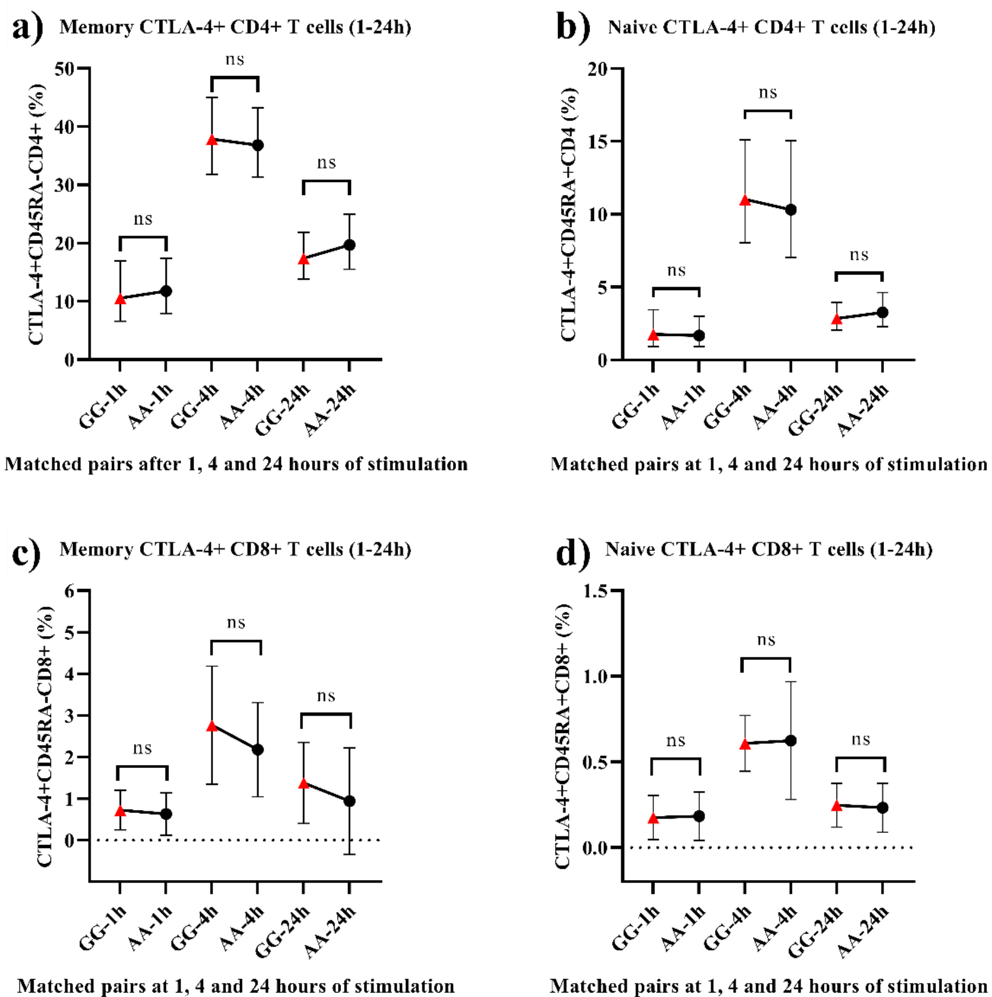

Figure 9. The frequency of the matched pairs' CTLA-4^{+} cell subsets at 1–24 hours of stimulation. The geometric means for the CTLA-4^{+} cell fractions are visualized with red triangles for GG-genotypes and black circles for AA-genotypes. The whiskers present the geometric SD. The fractions are presented as % of CTLA-4^{+} cells per parent population. The 1-hour mark includes 8 matched pairs while the 4 and 24-hour marks include 9 matched pairs. a) and b) present the fractions of CTLA-4^{+}CD4^{+} cells, memory and naïve cells, respectively. c) and d) present the fractions of CTLA-4^{+}CD8^{+} cells, memory and naïve cells, respectively. Tests for significance were performed with a ratio paired t-test in population types a) and b). Wilcoxon's matched paired signed-rank test was used for population types c) and d).

5.3 The rs231775-G polymorphism modifies CTLA-4 expression in Treg cells

The immunophenotyping of the 12 matched pairs yielded 11 complete pairs which were included in the final analysis of the Treg-cell compartments (gated populations; Appendix IV, figures 1–3). One pair was excluded from further analysis since one individual expressed a rare CD45 gene variant which disrupts the transcriptional splicing of CD45RA, consequently, rendering one unable to visualize CD45RA and to distinguish naïve cells from memory cells. In short, the Treg-cell panel stained the intracellular compartment for CTLA-4, Ki-67, FOXP3 and Helios; thus, enabling the measurement of intracellular CTLA-4 storage levels. Other phenotypical markers for Treg activation and differentiation were analyzed in parallel. There were no significant differences in the frequency of $CD3^+CD4^+CD25^+CD127^{Low}FOXP3^+$ Treg cells when analyzing the matched pairs' data (Figure 10). However, there was a significant difference in the frequency of CTLA-4$^+$ Treg cells when studying total CTLA-4$^+$ Treg cells ($p = 0.0091$; $t(10) = 3.223$; geometric mean: GG = 29.15%, AA = 35.75%), and CD45RA$^+$ naïve Treg cells ($p = 0.0136$; $t(10) = 2.998$; geometric mean: GG = 16.39%, AA = 23.32%) (Figure 11). Moreover, when examining the CTLA-4$^+$ CD45RA$^-$ memory Treg cells, the difference in population frequency reached a borderline significance ($p = 0.0631$; $t(10) = 2.090$; geometric mean: GG = 72.29%, AA = 77.68%) (Figure 11). A representative pairs' genotypic difference in Treg cell CTLA-4 expression is shown in Figure 12.

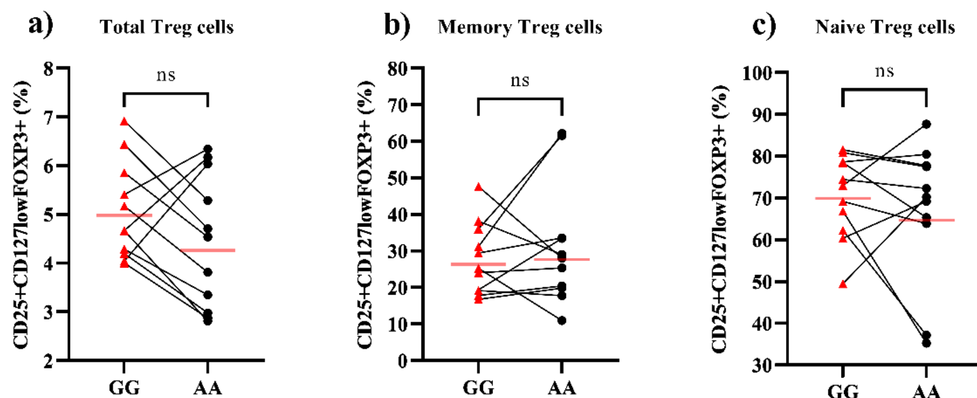

Figure 10. The frequency of $CD4^+CD25^+CD127^{Low}FOXP3^+$ Treg cells. The level of significance was calculated with ratio paired t-tests and the red line presents the geometric mean of the genotype-specific distribution. a) The frequency of Treg cells as a fraction of all CD4$^+$ T cells. b) The frequency of CD45RA$^-$ memory Treg cells as a fraction of all Treg cells. c) The frequency of CD45RA$^+$ naïve Treg cells as a fraction of all Treg cells.

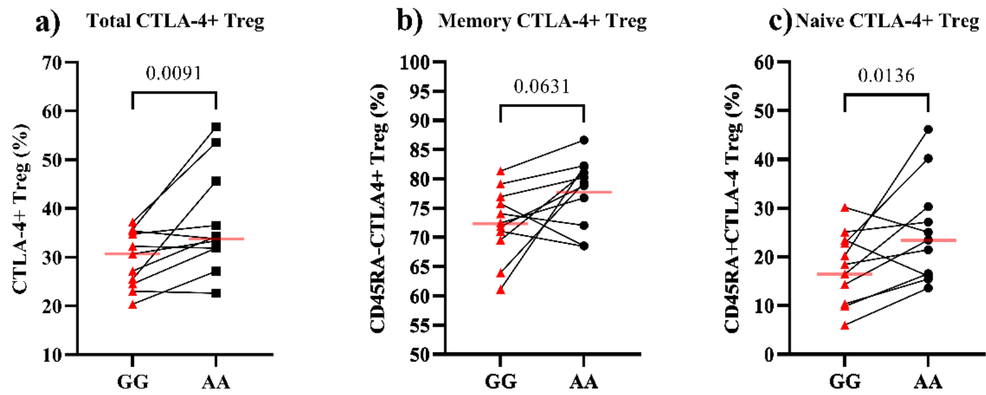

Figure 11. The frequency of CTLA-4$^+$ CD4$^+$CD25$^+$CD127^{Low}FOXP3$^+$ Treg cells. The level of significance was calculated with ratio paired t-tests and the red line presents the geometric mean of the genotype-specific distribution. a) The frequency of CTLA-4$^+$ Treg cells as a fraction of all Treg cells. b) The frequency of CTLA-4$^+$CD45RA$^-$ memory Treg cells as a fraction of all CD45RA$^-$ Treg cells. c) The frequency of CTLA-4$^+$CD45RA$^+$ naïve Treg cells as a fraction of all CD45RA$^+$ Treg cells.

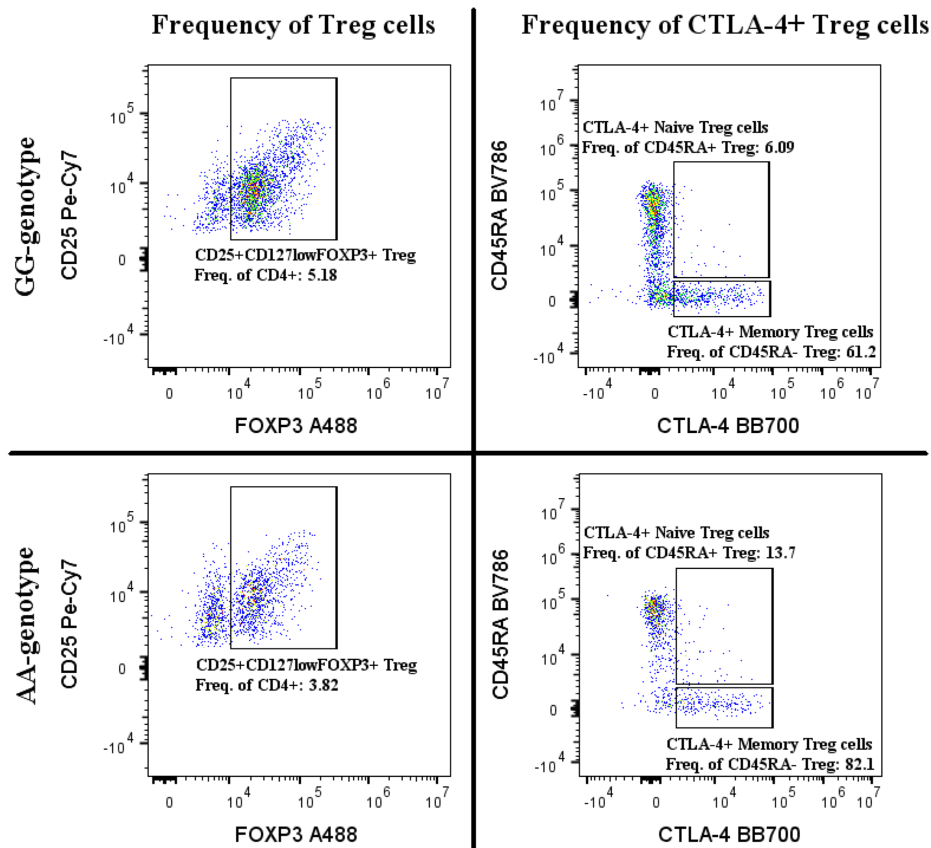

Figure 12. Comparison of a matched pairs' Treg cell and CTLA-4$^+$ Treg-cell frequencies. The rows specify the genotype, and the left column shows the frequency of Treg cells in one representative pair with either CTLA-4 GG or AA genotype. The right column shows the frequency of CTLA-4$^+$ naïve and memory Treg cells. The frequency of CD4$^+$CD25$^+$CD127^{Low}FOXP3$^+$ Treg cells is given as the fraction of total CD4$^+$ cells. The frequency of CTLA-4$^+$ naïve or memory cells is given as the CTLA-4$^+$ fraction of respective CD45RA$^+$ or CD45RA$^-$ parent populations.

The Treg-cell panel also enables a separate analysis of $CD4^+$ non-Treg cells; a $CD4^+$ T-cell fraction which excludes $CD4^+CD25^+CD127^{low}$ cells (non-Treg gate; Appendix IV, Figure 2). When examining CTLA-4^+ memory $CD4^+$ non-Treg cells separately, there was a significant difference in intracellular CTLA-4 expression ($p = 0.0386$; $t(10) = 2.380$; geometric mean: GG = 10.27%, AA = 13.12%) between the genotypes (Figure 13). A weak trend ($p = 0.0702$; $t(10) = 2.027$; geometric mean: GG = 0.333%, AA = 0.469%) could also be observed in the CTLA-4^+ naïve $CD4^+$ non–Treg-cell fraction (Figure 13). Whole $CD8^+$ and $CD4^+$ T-cell populations were also examined separately (gating strategy; Appendix IV, Figure 1). There were no significant differences in the CTLA-4^+ fractions within the $CD8^+$ T-cell compartment. However, a significant difference ($p = 0.0314$; $t(11) = 2.501$; geometric mean: GG = 13.06%, AA = 15.90%) could be observed in the CTLA-4^+ memory $CD4^+$ T-cell compartment. Lastly, no trends were detected when examining the expression of other phenotypic markers within the Treg-cell compartment (gating for phenotypic markers; Appendix IV, figures 2-3).

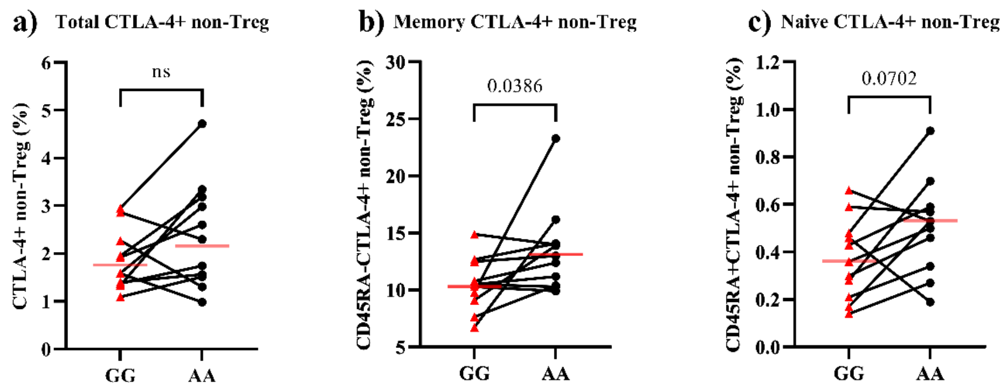

Figure 13. The frequency of CTLA-4^+ $CD4^+$ non-Treg cells. The level of significance was calculated with ratio paired t-test and the red line presents the geometric mean of the genotype-specific distribution. a) The frequency of CTLA-4^+ $CD4^+$ non-Treg cells as a fraction of all $CD4^+$ non-Treg cells. b) The frequency of CTLA-$4^+CD45RA^-$ memory $CD4^+$ non-Treg cells as a fraction of all $CD45RA^-$ $CD4^+$ non-Treg cells. c) The frequency of CTLA-$4^+CD45RA^+$ naïve $CD4^+$ non-Treg cells as a fraction of all $CD45RA^+$ $CD4^+$ non-Treg cells.

Further immunophenotyping was performed on the samples from the 12 matched pairs with the myeloid and innate lymphoid staining panel, the B-cell panel and the $CD4^{+}$/$CD8^{+}$ chemokine panel. This was done in accordance with the pre-determined order of panel prioritization, hence, excluding some individuals' panel-specific analysis due to a shortage of cells. Four pairs had their samples studied with the myeloid and innate lymphoid panel, six pairs with the B-cell panel and six pairs with the $CD4^{+}$/$CD8^{+}$ chemokine panel. Nevertheless, the phenotyping did not reveal any indicative trends or differences between the matched pairs. The analyzed cell subtypes of respective panel have been highlighted in the panels' descriptions (appendixes V–VII).

6. Discussion

The main objective of the thesis study was to produce a novel DIPP data repository which in the future can be used to conduct the genotype-targeted immunophenotype profiling of DIPP subjects and to study the polymorphisms' effects on immune cell functions. Naturally, the genotyped SNPs were selected based on their associations with autoimmune disease, especially to T1D, and due to their presumed effects on immune cell function. The number of preliminary subjects (N = 694) and the high average number of stored PBMCs (Number of subjects with > 2 stored PBMCs = 85%; > 4 stored PBMCs = 50 %) allows for the time-efficient inclusion of additional genotyped SNPs in the future. Furthermore, since the PTPN22 rs2476601 SNP causes both determined and still poorly characterized modifications in the immunophenotype, individuals carrying the disease associated SNP will most likely be excluded when forming new cohorts. In our material, 77.3% (536/694) were homozygous for the wildtype rs2476601-G allele, thus, leaving us with 207 subjects (207/536) of a rs2476601-GG genotype who are homozygous for one additional SNP. Nonetheless, as observed within our genotyping output, the inclusion of low frequency SNPs puts to question the relative cost-effectiveness of genotyping rare polymorphisms since the frequency of homozygous carriers can be inadequate for widescale immunophenotyping of multiple functional parameters. This drawback is observed in the case of the IL2RA polymorphism (rs61839660) that according to Shapiro et al. (2021) had and MAF of 10%. However, our cohort yielded no minor allele homozygotes and a calculated MAF of 3.9%. We later noted that the 1000Genomes

study of European material had previously reported a lower MAF of 6.9% (N = 1006) for this SNP. Moreover, an Estonian population study reported an MAF of 4.8% (N = 4480) (Tasa et al., 2019), a MAF that is relatively close to that observed in our material. Hence, to yield a satisfactory number of SNP-homozygous subsets, the future genotyping of additional SNPs within the established cohort should focus on polymorphisms with MAFs over 20%. In some cases, the study of the SNP's effect on the immunophenotype can also be performed with heterozygous individuals if the investigated allele demonstrates a dominant effect.

Serotyping for CMV-IgG seropositivity found that 24% of all subjects were infected with CMV, a value similar to the most previous estimates (~25%) of equivalent material (Aarnisalo et al., 2008; Hiltunen et al., 1995). CMV-infection history was not considered when forming the matched pairs of the pilot study, an inadvertent result of the thesis project's time constraints. Still, CMV-infection history shall be considered when using the produced dataset in the future, either when forming new subcohorts or when stratifying results post hoc, since CMV infections significantly shifts the proportion of effector $CD8^+$ T cells towards an increased frequency of terminally differentiated effector T cells (TEMRA). Given that CMV infections mainly exert visible effects on the $CD8^+$ T-cell compartment, it can be assumed that CMV seropositivity does not significantly affect the cell-frequency patterns of $CD4^+$ T cells, including Treg cells.

In the pilot study, which constitutes the second objective of the thesis, the stimulation of isolated $CD3^+$ T cells and the subsequent phenotyping of CTLA-4–surface expression did not produce any data to support the assertion that CTLA-4 expression would be lower amongst rs231775 minor-G allele homozygotes. This conflicts with the only previous study that quantified surface expression of CTLA-4 amongst rs231775 genotypes (Mäurer et al., 2002). However, is should be noted, Mäurer et al. used allogenic mature DCs to stimulate isolated T cells which after 48 hours of stimulation showed a significant difference in the mean fluorescence index of CTLA-4^+ cells when comparing GG and AA genotypes in a small cohort (GG = 4; AA = 4). Nevertheless, we were able to observe CTLA-4–expression patterns which corresponded to that observed in an earlier study that used PMA/Ionomycin for T-cell activation (Jago et al., 2004). To summarize, we and Jago et al., respectively,

demonstrated that CTLA-4 is mainly expressed in the effector subsets of $CD4^+$ T cells and $CD4^+$ Treg cells. Memory T cells, as compared to naïve T cells, have demonstrably higher levels of CTLA-4, both intracellularly and extracellularly, pre-stimulation and post-stimulation (Figure 9). $CD8^+$ T cells do express CTLA-4 upon stimulation, but only at a fraction of that seen in $CD4^+$ T cells.

The activation of T cells with PMA/Ionomycin, a pharmacological activator of calcium-dependent signaling, circumvents the classical pathway of TCR-dependent activation. PMA/Ionomycin treatment activates the degranulation of vesicles which contain CTLA-4, hence, facilitating the proteins' transport to the surface (Linsley et al., 1996). Recent evidence has also shown that the transcriptional expression of CTLA-4 is regulated via a PKA/CREB-dependent pathway, via transcription factor IFN-γ and by cAMP; three mechanisms which upstream intersect with the pathways of canonical calcium-dependent signaling (J. Li et al., 2013; Xuan Mo et al., 2018). However, it is still uncertain whether pharmacological PMA/Ionomycin treatment can induce physiologically corresponding levels of CTLA-4 expression since the induction of stimulation is strictly pharmacological and highly potent. It is possible, when comparing GG and AA genotypes, that the expected physiological difference in CTLA-4 expression is lost due to our choice of stimulation method. If that is the case, the expression levels that correspond to true physiological CTLA-4 upregulation would perhaps be discernable after TCR-activation. It would, therefore, be reasonable to verify the results using a separate method of stimulation, e.g., with CD3/CD28 T-cell activation or with allogenic mature DCs, as in the prior experiments of Mäurer et al. (2002). Prior to initiation of our main experiments, T-cell stimulation with beads that facilitate TCR-based T-cell activation was tested. However, we were unable to activate the T cells reliably with the conditions used and, as a result, had to abandon this approach since no CTLA-4 surface expression was observed after activation.

The Treg-cell panel demonstrated that there was a significant difference in the frequency of CTLA-4^+ total ($p = 0.0091$) and naïve ($p = 0.0136$) Treg cells when staining for CTLA-4 intracellularly (Figure 11). Moreover, the difference between the pairs reached a borderline significance ($p = 0.0631$) in memory Treg cells. In the memory Treg-cell compartment, it is likely that the naturally high frequency of CTLA-4 positivity lowered the genotypic difference when examining intracellular storage

levels. That is to say, the expression and storage of CTLA-4 could already be at a maximum level in non-stimulated memory Treg cells. The study of $CD4^+$ non-Treg cells found a significant difference ($p = 0.0386$) in the CTLA-4^+ fraction of memory $CD4^+$ non-Treg cells (Figure 13). However, compared to the CTLA-4^+ memory Treg-cell compartment, the frequency of CTLA-4^+ memory $CD4^+$ non-Treg cells showed less variance, and two outliers present in the non-Treg fraction contributed significantly to an increase in mean difference.

There was also a significant difference in the frequency of CTLA-4^+ cells when investigating the total memory $CD4^+$ T-cell population ($p = 0.0314$), thus, suggesting that the study of intercellular CTLA-4 storage levels on a whole $CD4^+$ T-cell population level should yield significant results. However, as shown in our stimulation experiment no difference could be observed in the surface expression levels of CTLA-4, perhaps due to the low number of analyzed cells ($15–25\times10^3$ cell at the time point of measurement) and due to the fact that the panel cannot discern Treg cells from conventional $CD4^+$ T cells. The majority of CTLA-4 is expressed on Treg cells, however, Treg cells only constitute approximately 5% of all $CD4^+$ T cells. If the physiological difference between the genotypes is restricted to Treg cells, e.g. as a difference in CTLA-4 storage levels or surface expression levels, then we would most likely be unable to detect the difference when studying the whole population of $CD4^+$ T cells.

The frequency of different Treg-cell phenotypes was also investigated; however, no significant differences could be observed in our pilot material of eleven matched pairs. Still, when examining the frequency of total Treg cells, a trend could be observed amongst eight of the eleven pairs (Figure 10), wherein the GG-genotype had an approximately 1–2 percentile unit higher frequency of Treg cells. However, the statistical pairwise comparison was insignificant since three pairs demonstrated inverse trends. Moreover, the abovementioned trend was lost when stratifying the population into naïve and memory T-cell subsets. A prominent artefact was observed when accounting for the subjects' age as a covariate in linear regression (data not shown). The rs231775-GG genotypes who were ≤ 2 years of age (8/11) exhibited a higher frequency of Treg cells when compared to those ≥ 3 years of age (3/11) (Figure 10). Notably, an inverse trend could be observed amongst the rs231775-AA genotypes,

thus, revealing an artefact in the Treg-cell frequency. The trend where the frequency of Treg cells decreases with age amongst rs231775-GG genotypes could be a false signal stemming from the small sample size. Nevertheless, without further study, we cannot corroborate whether this observation is a true physiological phenomenon or an inadvertent effect stemming from population heterogeneity.

The analysis of the myeloid and innate lymphoid panel, B-cell panel and $CD4^+/CD8^+$ chemokine panel did not reveal any evident differences when comparing the genotypes. However, the results have to be interpreted with caution since only 4–6 pairs were studied. These panels need to be studied further with more material to exclude the possibility of a rs231775-inflicted downstream modification on other cell subtypes. It remains a possibility that if the rs231775 SNP also modifies the functionality of the Treg-cell population, there could be downstream alterations to the prevalence of other effector T-cell subsets. For example, an alteration in the frequency of inflammatory Th cells which are downregulated by immunomodulatory Treg cells. However, the SNP-mediated downstream alterations to a broad range of immune cells could be more prominent during a certain phase in the disease development, for example, during the early development of IA or when IA starts to progress towards an increased severity. Alterations in the Treg cell compartment have been associated with these phases; hence, when a SNP's causal effect is determined in non-diabetic individuals, it would be justified to study the SNP-mediated effects in AAb-positive individuals and in T1D cases.

The interindividual variance found in the major immune cell populations' frequencies displayed an inherent disadvantage in this small pilot study's design. The inclusion of multiple age groups (defined as age at sample retrieval) produced a scenario in which each age group showed a variance that was dependent on the subjects' past immunological involvement. Hence, the deviation from the whole-cohort's gross mean was more profound in the younger subjects, perhaps since the experience of infections can cause a significant change in the cell populations which expand as a direct consequence of immunological engagement, for example, in the ratio of naïve-to-memory cells. Consequently, when analyzing the cell frequencies of the pairs aged 6 months to 2 years, we were unable to see any of the expected linear trends, such as a progressive increase in the proportion of memory $CD4^+$ cells. Moreover, our

calculations of cell frequencies are biased towards that seen in younger children since 8/11 pairs where ≤ 2 years old. Moreover, the small sample size made further analysis using age as a covariate unreasonable. Notably, the reported geometric means of the cell populations should be interpreted with caution since they do not necessarily represent the actual biological mean of respective rs231775 genotype. As previously discussed, it would be more proper to calculate a genotypic mean difference for a specific age group since the frequency of multiple cell subsets covariate with age.

A larger sample size would allow for a more accurate study of trends and effects in different age groups and for the detection and proper evaluation of outliers. However, since the pilot study aimed to quantify the expression of CTLA-4 , a wider range of age groups was formed to verify the existence of a rs231775-SNP effect; an effect which is expected to be independent of the age. Further studies on cell frequencies, especially amongst effector cell subsets, should focus on one age group in order to limit the influence of age-dependent in-group variation. To conclude, the future study of the rs231775 SNP's effects on Treg-cell parameters should include the quantification of intracellular storage levels and surface expression levels. This could be applied to both resting and activated T cells, including Treg cells. Lastly, the SNP's possible impact on Treg-cell functionality should preferably be studied with in vitro suppression and proliferation assays.

As one of the canonical competitive inhibitors of T-cell activation, alterations to the function and availability of CTLA-4 could entail major modifications to the CD28–CD80/86 activatory axis. Even when the mechanism of CTLA-4 inhibition has been determined, there are still functional aspects which require further investigation, such as the biological function of soluble CTLA-4. The rs231775 SNP has been observed to alter the prevalence of soluble CTLA-4 in serum, although, the physiological impact of this alteration is unknown (Esposito et al., 2014; Pruul et al., 2015; Rydén et al., 2012). Changes to the CD28–CD80/86 activatory axis also affect the prospects of immunomodulatory therapy, e.g. cancer immunotherapy, since perturbations of the pathway evidently modify the T cells' immunological kinetics and, therefore, the clinical outcome (Zhao et al., 2018). For example, CTLA-4 polymorphisms have been associated with long-term survival when treating melanoma (Xuan Mo et al., 2018; Queirolo et al., 2017). Furthermore, through still undetermined effects, the rs231775

SNP has been shown to modify susceptibility for graft rejection after stem cell transplantation; an effect that most likely stem from the SNP's alterations to Treg-cell function (Hammrich et al., 2019). Lastly, from a pharmacogenetic perspective, the rs231775 polymorphism has also been shown to modify the prognosis of treating RA with Abatacept, a fusion protein carrying the extracellular domain of CTLA-4 which functions as a selective costimulation modulator (Pete et al., 2020).

It is now commonly agreed upon that T1D associated SNPs exert various modifying effects on the immunophenotype; effects which not necessarily facilitate disease by themselves but are able to modify disease susceptibility and contribute to disease heterogeneity. Lastly, from a prospective standpoint, the broad-spectrum immuno-phenotyping of genotyped individuals is expected to become a more commonly adopted research approach in functional immunogenetics. This, in turn, will hopefully create a better understanding of how various SNPs increase disease susceptibility and contribute to disease heterogeneity.

7. Conclusions

- The genotyping for ten T1D associated polymorphisms produced a cohort of 678 completely genotyped subjects. Consequently, this enables the efficient execution of future functional immunophenotyping studies within the DIPP study community.
- When comparing CTLA-4 rs231775 (A>G) GG and AA genotypes, the risk-associated G-allele did not appear to modify CTLA-4 surface expression on $CD4^+$ Th cells and $CD8^+$ Tc cells after PMA/Ionomycin stimulation.
- When staining for CTLA-4 intracellularly in non-stimulated cells, the rs231775-G allele was associated with a lower intracellular CTLA-4 expression in $CD3^+CD4^+CD25^+$ $CD127^{Low}FOXP3^+$ Treg cells.
- Our results maintain the assertion that the rs231775 SNP modifies the biological availability of CTLA-4 in Treg cells. This, in turn, warrants further functional studies of this SNP to investigate whether it affects the biological surface availability of CTLA-4 in activated Treg cells and the ability of these to suppress the immune system.

8. Acknowledgements

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9. Swedish summary

Autoimmunitets-associerade polymorfismer och deras effekt på immunofenotypen: Genprofilering och immunofenotypprofilering inom den finländska Diabetes Prediction and Prevention Study.

Avhandlingsprojektets målsättning är att producera en kohort på 700 barn värvade till den finländska prospektiva studien The Diabetes Prediction and Prevention Study (eng. förkortning, DIPP). Den skapade datasamlingen är ämnad inom DIPP-forskningsverksamheten att användas vid kartläggningen av diverse polymorfismers påverkan på immunofenotypen, och därmed möjliggöra kvantifieringen av de sjukdomsassocierade polymorfismernas funktionella effekter i immunceller. Den preliminära datasamlingen bildas genom att genprofilera icke-diabetiska, autoantikroppsnegativa, T1D-predisponerade barn för tio olika enbaspolymorfismer. Samtliga individer genprofileras med hjälp av kvantitativ TaqMan-PCR för polymorfismerna rs2476601 (PTPN22), rs231775 (CTLA-4), rs1990760 (IFIH1), rs3184504 (SH2B3), rs601338 (FUT2), rs763361 (CD226), rs72928038 (BACH2), rs11203203 (UBASH3A), rs61839660 (IL2RA) och rs45450798 (PTPN2). Respektive polymorfism har en antydd verkan på proteinets funktion eller en modifierande effekt på proteinets uttryck (Tabell 1).

Avhandlingsprojektet inkluderar även en mindre immunofenotypprofilering av tolv fenotypparade individer för att kvantifiera CTLA-4 rs231775-polymorfismens (+A49G) inverkan på CTLA-4 proteinets uttryck bland konventionella T-celler och regulatoriska T-celler (Treg). CTLA-4 är en central inhibitor till aktiveringen av konventionella T-celler då CTLA-4 konkurrerar i cis mot den T-cell-expresserade aktivatorn CD28 för att binda till CD80/86 uttryckt på antigenpresenterande celler. CTLA-4 utövar sin verkan genom att minska förekomsten av CD28–CD80/CD86-interaktioner vilket resulterar i en nedreglerad T-cellaktivering. Rs231775 polymorfismen har observerats medföra en ökad risk för T1D-utveckling och denna riskeffekt har förknippats med en lägre CTLA-4-uttryck bland konventionella $CD4^{+}$-celler samt Treg-celler (Y. Chen et al., 2018; Jonson et al., 2006; Mäurer et al., 2002). Polymorfismen har även tidvis förknippats med en modifiering av ovannämnda cellpopulationers frekvens, antigen vid ett friskt tillstånd eller hos individer med fulminant T1D.

I avhandlingens experimentella del studeras CTLA-4-polymorfismens inverkan på intracellulär CTLA-4-lagring bland $CD4^{+}CD25^{+}CD127^{low}FOXP3^{+}$ Treg-celler. Därefter studeras polymorfismens effekt på proteinets ytuttryck bland konventionella $CD3^{+}$ T-celler efter 1, 4 och 24 timmars stimulering med PMA/jonomycin. Då rs231775-polymorfismen har uppvisats modifiera ytuttrycket av CTLA-4 hos Treg-celler, vilket kan medföra en nedsatt immunregulatorisk funktionalitet, kommer även andra cellpopulationer studeras för att upptäcka möjliga förändringar på fenotypnivån. Till detta används tre separata flödescytometriska paneler för att studera konventionella T-celler, B-celler, myeloida celler och icke-variabla T-celler.

Avhandlingens kohort bildades från 694 DIPP-klienter födda mellan 2005 och 2017, individer som är genetiskt predisponerade för T1D enligt DIPP-inkluderingskriterierna. Kohorten ämnad för kvantifiering av CTLA-4-proteinets uttryck och allmän immunofenotyp-profilering formades av tolv fenotypparade individer vilka var parade enligt kön, födelseort, födelsedatum (± 5 månader) och provtagningsdatum (± 6 månader). Respektive individ av varje par var antigen homozygot för den riskassocierade rs231775-G-allelen eller vildtypsallelen rs231775-A. De valda individerna har gett blodprover vid endera åldern av 6 månader, 9 månader, 1 år, 2 år, 3 år eller 4 år, vilket gav upphov till två fenotypparade par för respektive åldersklass.

TaqMan-profileringen resulterade i en komplett polymorfismprofil hos 678 individer, vilket ger en total profileringsrat på 99,8 %. Utav 694 individer var 38,5 % (267/694) homozygot för en polymorfism (Tabell 2). Kvantifieringen av den extracellulära CTLA-4-uttrycket i nio par producerade inga resultat som skulle antyda på en modifiering av frekvensen CTLA-4-positiva konventionella $CD3^{+}$-T-celler (Figur 8). Frånvaron av polymorfismens förmodade effekt observerades även då man studerade $CD4^{+}$-hjälpar-T-celler och $CD8^{+}$-cytotoxiska T-celler separat. Detta inkluderar respektive populations naiva och minnes T-celler (Figur 9).

När elva fenotyppar studerades med en Treg-cellpanel upptäcktes ingen modifiering av frekvensen $CD3^{+}CD4^{+}CD25^{+}CD127^{Low}FOXP3^{+}$-Treg-celler (Figur 10). Man fann däremot en signifikant skillnad då man studerade den totala uppsättningen av CTLA-4-positiva Treg-celler ($p = 0,0091$; $t(10) = 3,223$; geometriskt medeltal: GG = 29,15

%, AA = 35,75 %), naiva Treg-celler ($p = 0,0136$; $t(10) = 2,998$; geometriskt medeltal: GG = 16,39 %, AA = 23,32 %), och som ett gränsfall, minnes Treg-celler ($p = 0,0631$; $t(10) = 2,090$; geometriskt medeltal: GG = 72,29 %, AA = 77,68 %) (Figur 11). När $CD25^{+}CD127^{low}$-celler exkluderas från $CD4^{+}$ populationen bildas en icke-Treg-population. Denna population uppvisade även en signifikant skillnad mellan genotyperna då man studerande den CTLA-4-positiva minnes-populationen ($p = 0,0386$; $t(10) = 2,380$; geometriskt medeltal: GG = 10,27 %, AA = 13,12 %), och med en svag skillnad i den naiva populationen ($p = 0,0702$; $t(10) = 2,027$; geometriskt medeltal: GG = 0,333 %, AA = 0,469 %) (Figur 13).

Avhandlingsprojektets första delmålsättning var att producera en datasamling som kan användas i utforskningen av polymorfismers funktionella effekter på immunofenotypen. Projektet producerade en kohort på 694 individer där alla har ett relativt högt antal lagrade prover (85 % av alla individer har fler än två lagrade prover). Detta möjliggör en effektiverad fortsatt forskning inom immunofenotypprofileringen av DIPP-deltagare. Stimuleringen av konventionella $CD3^{+}$-T-celler med PMA/jonomycin, med påföljande analys av CTLA-4-proteinets ytuttryck, gav inte några resultat som skulle understöda att G-allelen medför ett lägre ytuttryck av CTLA-4 vid stimulering. Detta står i konflikt med den enda tidigare motsvarande studien av Mäurer et al. (2002) som studerade CTLA-4-proteinets ytuttryck i $CD3^{+}$-T-celler efter 48 timmar av cellstimulering med allogena vuxna dendritiska celler (genotyper; GG = 4, AA = 4). Vi lyckades däremot replikera resultat från en tidigare studie som undersökte det relativa uttrycket av CTLA-4 bland olika konventionella T-celler efter PMA/jonomycin-stimulering (Jago et al., 2004). Det står nu i vårt intresse att replikera våra resultat med hjälp av en alternativ stimuleringsmetod, såsom stimulering med CD3/CD28-antikroppar eller med allogena vuxna dendritiska celler.

Det att vi inte fann någon signifikant inverkan av rs231775-polymorfismen på fraktionen av minnes-Treg-celler beror antagligen på populationens höga grad av lagrat CTLA-4. Det vill säga att näst intill alla minnes-Treg-celler uttrycker CTLA-4, kanske även till en biologisk maxnivå. Kvantifieringen av CTLA-4-ytuttrycket efter stimulering resulterade inte i några signifikanta resultat. Detta kan bero på att vi studerade för få celler ($15–25 \times 10^{3}$ celler vid mätning) eller på grund av det faktum att endast 5 % av alla $CD4^{+}$-celler är Treg-celler. Om den biologiska effekten av

rs231775-polymorfismen är begränsad till Treg-celler, och när deras frekvens är ~5 % av den totala $CD4^+$-populationen, är det sannolikt att man inte kan detektera polymorfismens effekt i ytuttrycket av CTLA-4 om man endast studerar $CD4^+$-fraktionen. Därtill kunde den använda panelen inte frånskilja Treg-celler från konventionella $CD4^+$-celler.

Vid framtida studier av rs231775-polymorfismens inverkan på Treg-cellers populationsparametrar borde den intracellulära och extracellulära uttrycket av CTLA-4 mätas. Detta kan utföras med stimulerade och icke-stimulerade konventionella T-celler, inklusive Treg-celler. Polymorfismens eventuella effekter på Treg-cellers funktionsduglighet kan testas med analyser som mäter cellernas frekvens, proliferationsförmåga och förmåga att dämpa konventionella T-cellers aktivering. Det är en allmän konsensus inom den immunologiska genetiken att polymorfismer har egenskapen att modifiera en immunofenotyp genom deras inbördes effekter på immuncellers funktionalitet. Metoden att immunofenotypprofilera flera immuncellspopulationer förväntas bidra till en stor ansamling originaldata, data som den kliniska medicinen kan utnyttja för att skapa en bättre överblick över autoimmunitetens immunologiska engagemang.

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11. Appendixes

Appendix I

Table 1

Myeloid and innate lymphoid panel

Fluorochrome	Marker	Clone	Company	Cat#	1x (uL)
Brilliant stain buffer plus			BD	566385	10
BV421	TCR γ-δ	B1	BioLegend	331218	1
BV510	Zombie Aqua		BioLegend	77143	
BV570	CD19	HIB19	BioLegend	302236	1
BV605	CD56	HCD56	BioLegend	318334	1
BV650	CD123	6H6	BioLegend	306020	1
BV711	CD14	MφP9	BD	563372	1
BV785	TCR Va7.2	3C10	BioLegend	351722	1
BB515	CD11c	B-ly6	BD	564490	1
BB700	CD27	M-T271	BD	566449	1
PE	TCR Va24-J18	6B11	BioLegend	342904	1
PE-Dazzle 594	CD16	3G8	BioLegend	302054	1
PE-Cy5	HLA-DR	G46-6	BD	555813	0.2
PE-Cy7	CD8	RPA-T8	BD	557746	1
APC	CD161	HP-3G10	BioLegend	339912	1
R718	CD3	UCTH1	BD	566953	1
APC-F750	CD45	HI30	BioLegend	304062	1

Table 2

B cell panel

Fluorochrome	Marker	Clone	Company	Cat#	1x (uL)
Brilliant stain buffer plus			BD	566385	10
BB515	CXCR5	RF8B2	BD	564624	1
BB700	CD38	HIT2	BD	566445	1
BV421	CXCR3	IC6/CXCR3	BD	562558	1
BV510	Zombie aqua		BioLegend	77143	
	CD14	MφP9	BD	563079	1
	CD3	UCHT1	BioLegend	300488	1
	CD16	3G8	BioLegend	302048	1
	CD56	HCD56	BioLegend	318340	1
BV570	IgM	MHM-88	Biolegend	314517	1
BV605	IgG	G18-145	BD	563246	1
BV650	CD24	ML5	BD	563720	1
BV711	CD21	B-ly4	BD	563163	1
BV785	IgD	IA6-2	BioLegend	348242	1
APC	IL-21R	2G1-K12	BioLegend	347808	1
R718	CD20	2H7	BD	566988	1
APC-F750	CD19	HIB19	BioLegend	302258	1
PE	IgA	IS11-8E10	Miltenyi	130-099-108	1
PE-Dazzle 594	CD27	M-T271	BioLegend	356422	1
PE-Cy5	CD10	HI10a	BioLegend	312206	1
PE-Cy7	CD5	UCTH2	BioLegend	300622	1

The FM4 control excludes the following markers: CXCR3 BV421, IL-21R APC, CD5 PE-Cy7 and CD38 BB700.

Table 3

CD4$^+$CD8$^+$ chemokine panel

Fluorochrome	Marker	Clone	Company	Cat#	1x (uL)
Brilliant stain buffer plus			BD	566385	10
BV605	ICOS	C398.4A	BioLegend	313537	1
BV510	Zombie Aqua		BioLegend	77143	
	CD14	MφP9	BD	563079	1
	CD19	HIB19	BioLegend	302242	1
	CD16	3G8	BioLegend	302048	1
	CD56	HCD56	BioLegend	318340	1
BV570	CD8	RPA-T8	BioLegend	301038	1
BV421	CXCR3	1C6/CXCR3	BioLegend	562558	1
BV650	CCR7	G034H7	BioLegend	353234	1
BV786	CD45RA	HI100	BD	563870	1
BB515	CXCR5	RF8B2	BD	564624	1
PE	PD-1	EH12.2H7	BioLegend	329906	1
PE/Dazzle 594	CrTh2	BM16	BioLegend	3501256	1
PE-Cy5	CD161	DX12	BD	551138	4
PE-Cy7	CD27	M-T271	BioLegend	356412	1
APC	CCR10	REA326	Miltenyi	130-120-406	1
R718	CD3	UCTH1	BD	566953	0.5
APC-F750	CD4	RPA-T4	BioLegend	300560	1
BB700	CCR4	1G1	BD	566475	1
BV711	CCR6	G034E3	BioLegend	353435	1

The FM6 control excludes the following markers

- FM3a: CCR10 APC, CXCR3 BV421 and CCR4 BB770
- FM3b: CCR6 BV711, PD-1 PE and CD161 PE-Cy5.

Table 4

Treg panel

Surface staining

Fluorochrome	Marker	Clone	Company	Cat#	1x (uL)
Brilliant stain buffer plus			BD	566385	10
BV510	Zombie aqua		Biolegend	77143	
	CD14	MφP9	BD	563079	1
	CD16	3G8	BioLegend	302048	1
	CD19	HIB19	BioLegend	302242	1
	CD56	HCD56	BioLegend	318340	1
BV570	CD8	RPA-T8	BioLegend	301038	1
BV605	CD127	HIL-7R-M21	BD	562662	1
BV786	CD45RA	HI100	BD	563870	1
BV711	CD39	A1	BioLegend	328227	1
BV650	CCR7	G034H7	BioLegend	353234	1
R718	CD3	UCTH1	BD	566953	1
APC-F750	CD4	RPA-T4	BioLegend	300560	1
PE	CD31	AC128	Miltenyi	130-118-965	1
PE-Dazzle 594	TIGIT	A15153G	BioLegend	372715	1
PE-Cy5	HLA-DR	G-46-6	BD	555813	4
PE-Cy7	CD25	M-A251	BioLegend	356107	1

Intracellular staining

Fluorochrome	Marker	Clone	Company	Cat#	1x (uL)
Brilliant stain buffer plus			BD	566385	10
A647	HELIOS	22F6	BioLegend	137218	2
A488	FOXP3	259D	BioLegend	320212	2
BB700	CTLA-4	BNI3	BD	566902	2
BV421	Ki67	Ki-67	BioLegend	350506	2

The FM4 control excludes the following markers

- Surface staining FM: HLA-DR PE-Cy5, TIGIT PE-Dazzle 594 and CD39 BV711
- Intracellular staining FM: CTLA-4 BB700.

Table 5

CTLA-4 expression panel

Fluorochrome	Marker	Clone	Company	Cat#	1x (µL)
BV510	Zombie Aqua		BioLegend	77143	
APC-F750	CD3	SK7	BioLegend	344840	1
PE-Cy7	CD4	RPA-T4	BioLegend	300512	1
PE	CD8	RPA-T8	BioLegend	30108	1
BV421	CD69	FN50	BioLegend	310929	1
FITC	CD45RA	HI100	BioLegend	304106	1
APC	CTLA-4	BNI3	BioLegend	36912	1

The FMO control excludes CTLA-4 APC.

Appendix II

Table 1

5% AB serum supplemented RPMI medium			
Reagent	Manufacturer	Cat#	Conc.
RPMI-1640	Lonza	BE12-167F	Dilutant
AB (Human male AB plasma)	Sigma-Aldrich	H4522	5%
Glutamine	Lonza	BW1760E	1%
Penicillin-streptomycin 100X	Lonza	DE-17-602E	1%
β-merkaptoetanol 5.1 M	Sigma-Aldrich	SM6250	1%
NEAA supplement	Lonza	BE13114E	1%
Sodium pyruvate 100 mM	Lonza	BE13-115E	1%
HEPES 1M in 0.85% NaCl	Lonza	BW17605E	1%

Appendix III

Figure 1

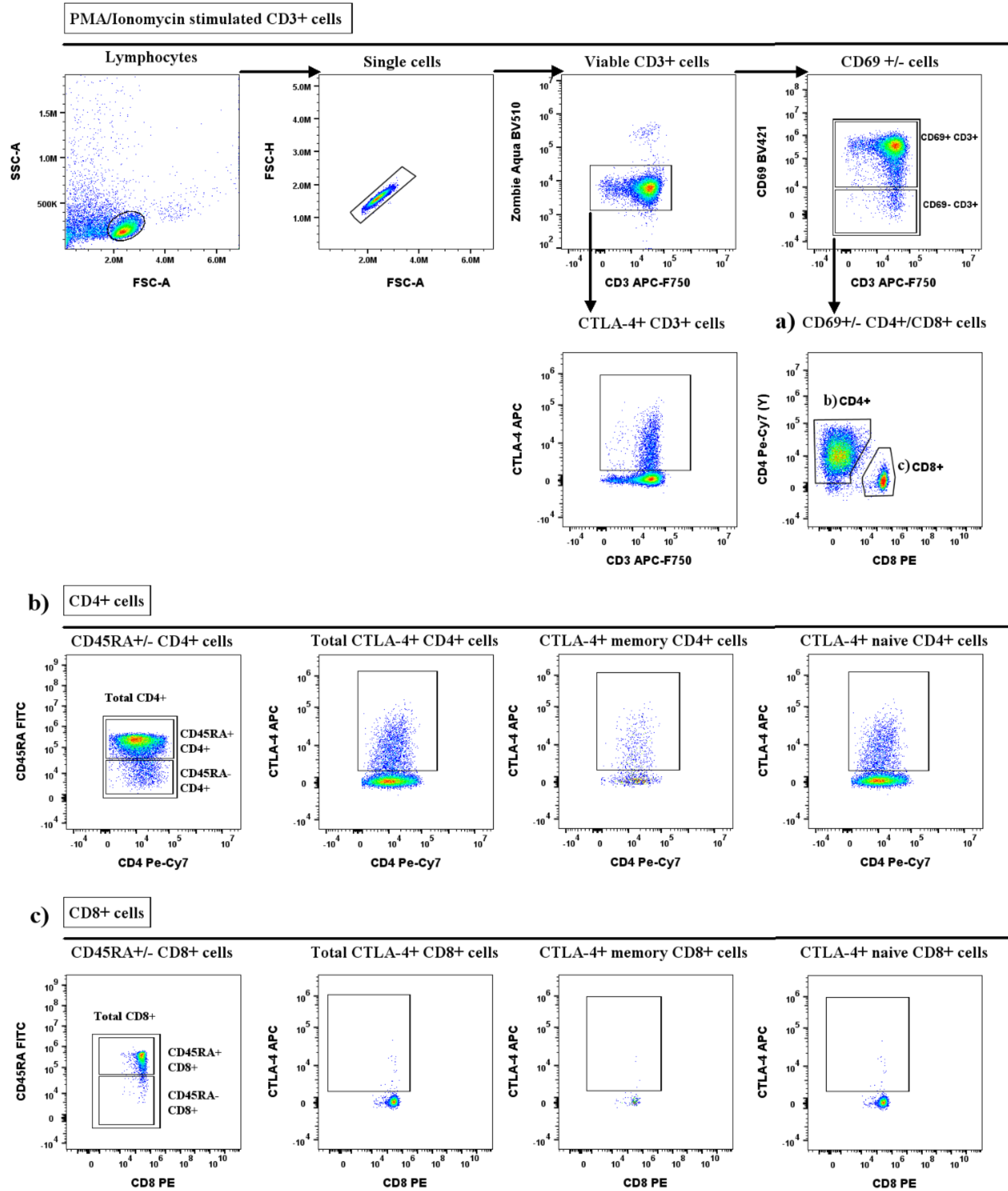

Figure 1. CTLA-4 expression panel. The displayed panel depicts a child's sample that was analyzed after 4 hours of PMA/Ionomycin stimulation. Sections a), b) and c) were analyzed separately in the CD69$^{+/-}$ and CD69^{+} fractions. FMO controls excluding the CTLA-4 APC antibody were used when gating individual samples or sample pairs.

Appendix IV

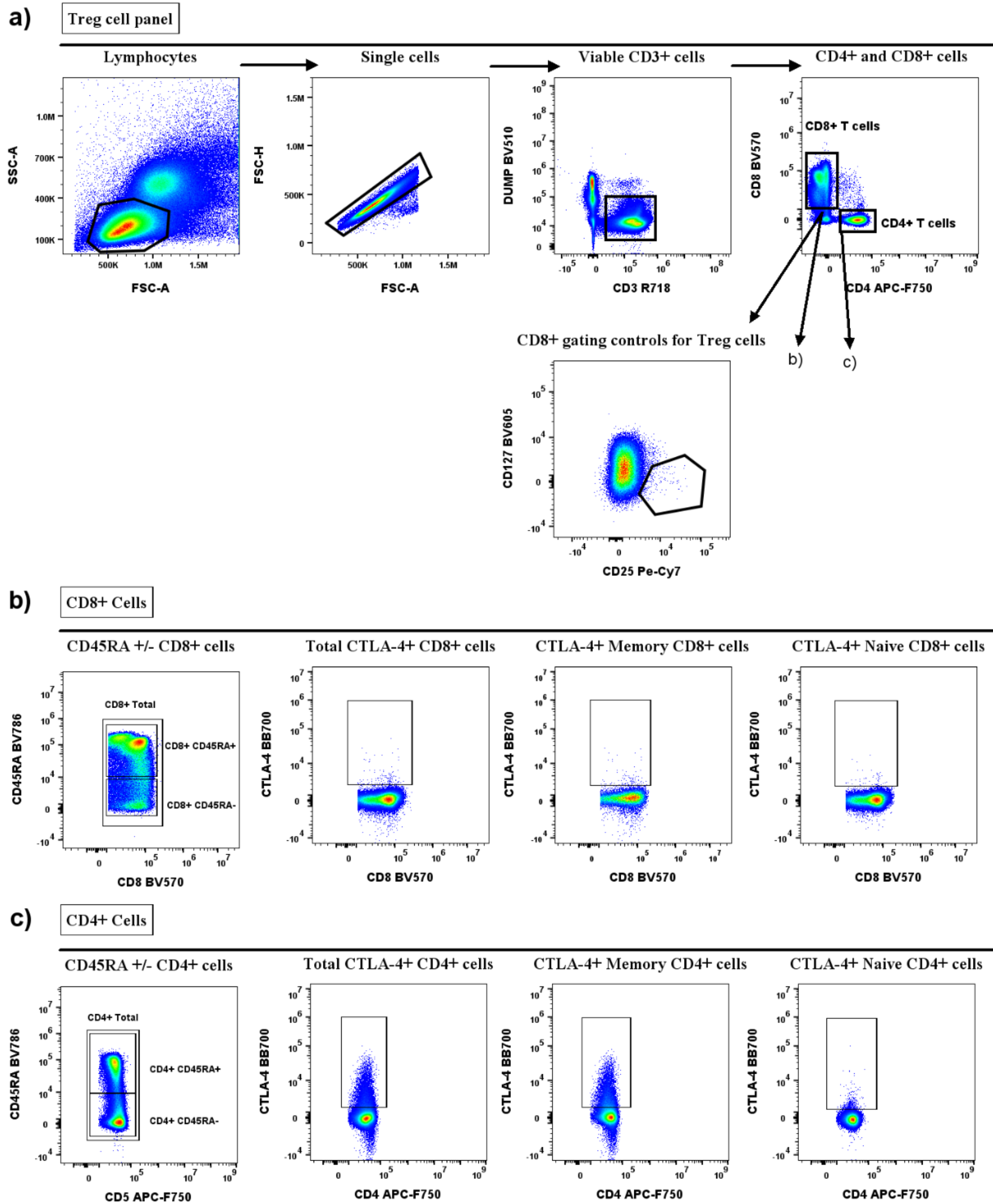

Figure 1. The Treg-cell panel, step 1. The displayed panel depicts an adult control sample. Section a) shows the preliminary steps of lymphocyte gating and the $CD8^+$ gating control for $CD25^+CD127^{low}$ cells. Sections b) and c) gate the total $CD4^+$ and $CD8^+$ T-cell populations for CTLA-4. FMOs where used when gating CTLA-4 BB700.

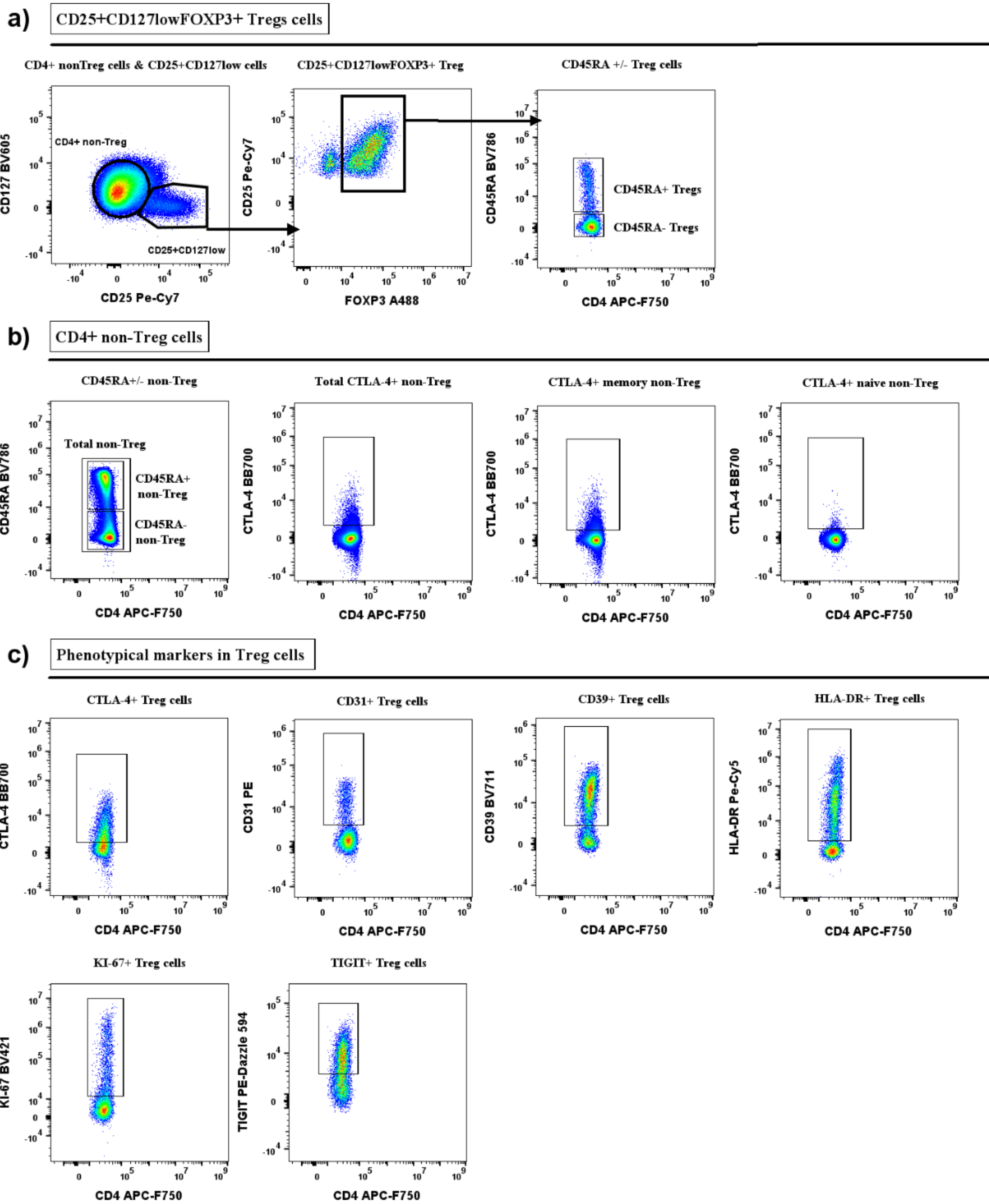

Figure 2. The Treg-cell panel, step 2. The displayed panel depicts an adult control sample. Section a) presents the preliminary steps of lymphocyte gating for $CD25^{+}CD127^{low}FOXP3^{+}$ Treg cells from $CD4^{+}$ cells. The $CD4^{+}$ population excluding the $CD25^{+}CD127^{low}$ cells were termed $CD4^{+}$ non-Treg cells. Section c) gates the total $CD25^{+}CD127^{low}FOXP3^{+}$ Treg-cell population into fractions according to the expression of phenotypical markers. FMOs where used when gating HLA-DR PE-Cy5, TIGIT PE-Dazzle, CD39 BV711 and CTLA-4 BB700.

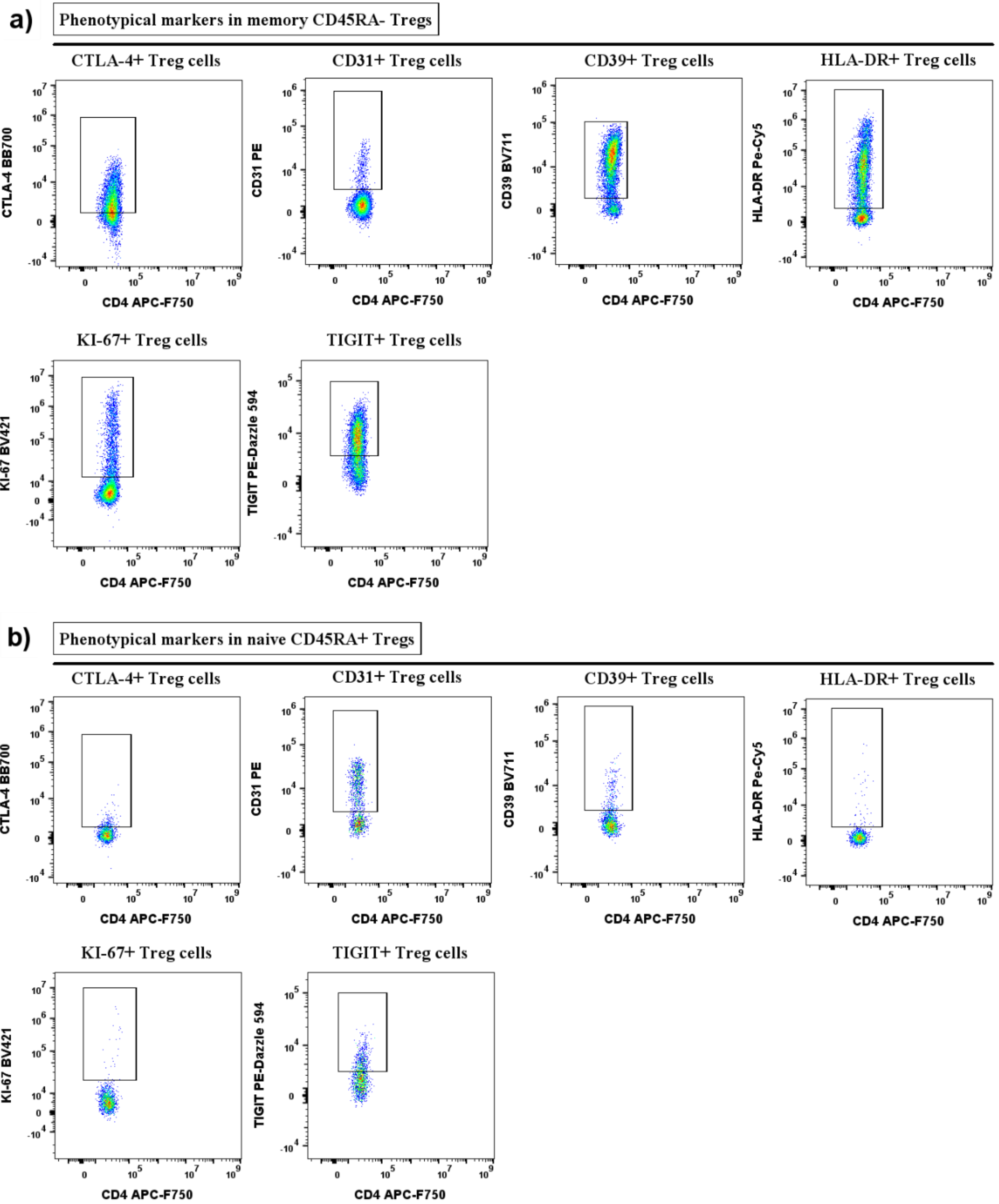

Figure 3. The Treg-cell panel, step 3. The displayed panel depicts an adult control sample. Section a) presents the gating for phenotypical markers in CD45RA⁻ memory Treg cells. Section b) presents the gating for phenotypical markers in CD45RA⁺ naive Treg cells. FMOs where used when gating HLA-DR PE-Cy5, TIGIT PE-Dazzle, CD39 BV711 and CTLA-4 BB700.

Appendix V

Figure 1

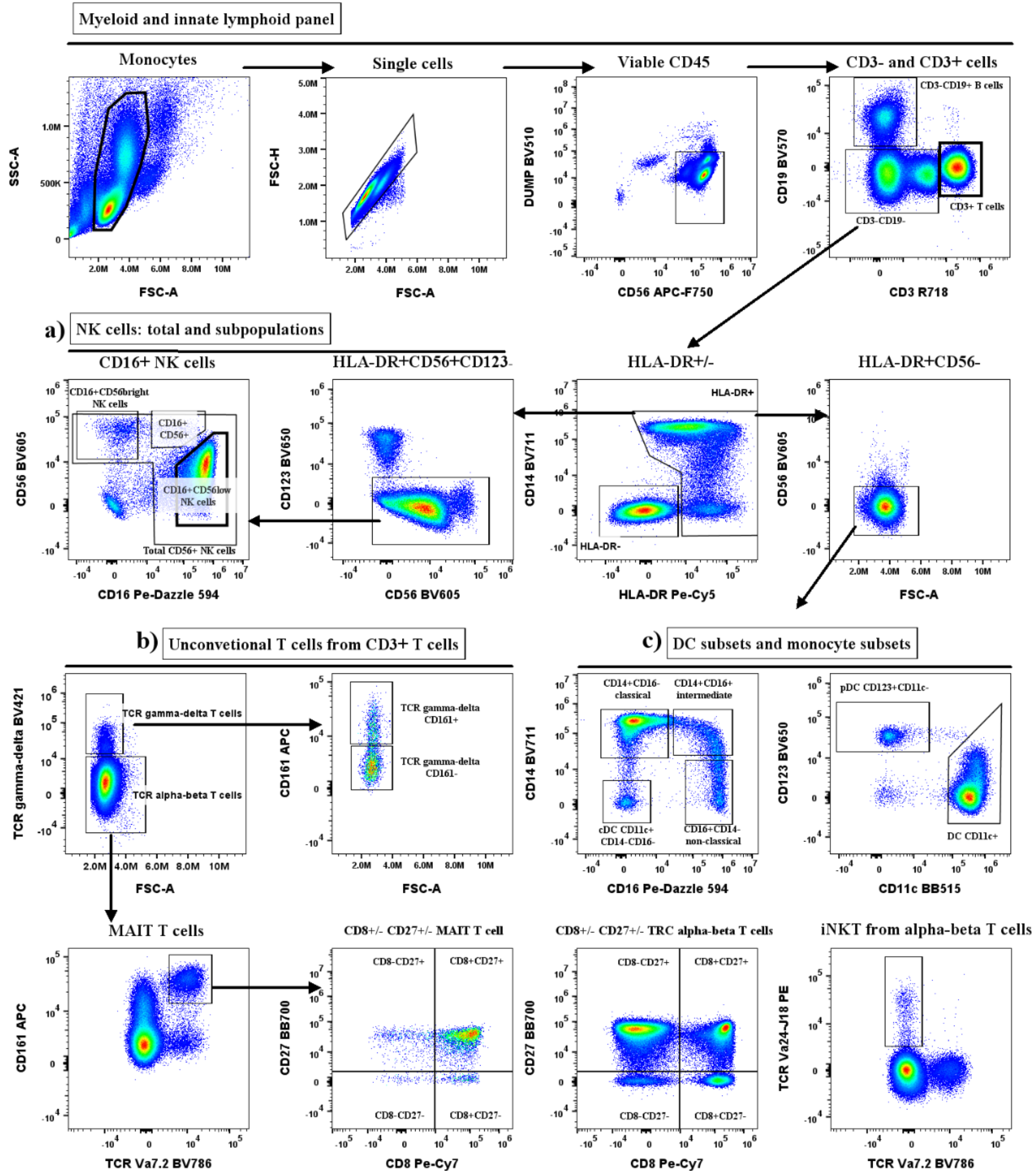

Figure 1. Myeloid and innate lymphoid panel. The displayed panel depicts an adult control sample. Section a) presents the gated NK cells. In section b), unconventional gamma-delta T cells and alpha-beta MAIT cells where gated from CD3^{+} T cells. The bottom-left figures, innate natural-killer T lymphocytes (iNKT) and CD8$^{+/-}$CD27$^{+/-}$ cells, where gated from alpha-beta T cells. Section c) displays monocytes and DCs as gated from the CD3^{-}CD19^{-}HLA-DR^{+}CD56^{-} population. The monocytes are divided into CD14^{+}CD16^{+} intermediate monocytes, CD14^{+}CD16^{-} classical monocytes and CD14^{-}CD16^{+} non-classical monocytes. Conventional DCs (cDC) are defined as CD11c+ DC cells and CD11c^{+}CD14^{-}CD16^{-} cells. Plasmacytoid DCs (pDC) are defined as CD124^{+}CD11^{-} cells.

Appendix VI

Figure 1

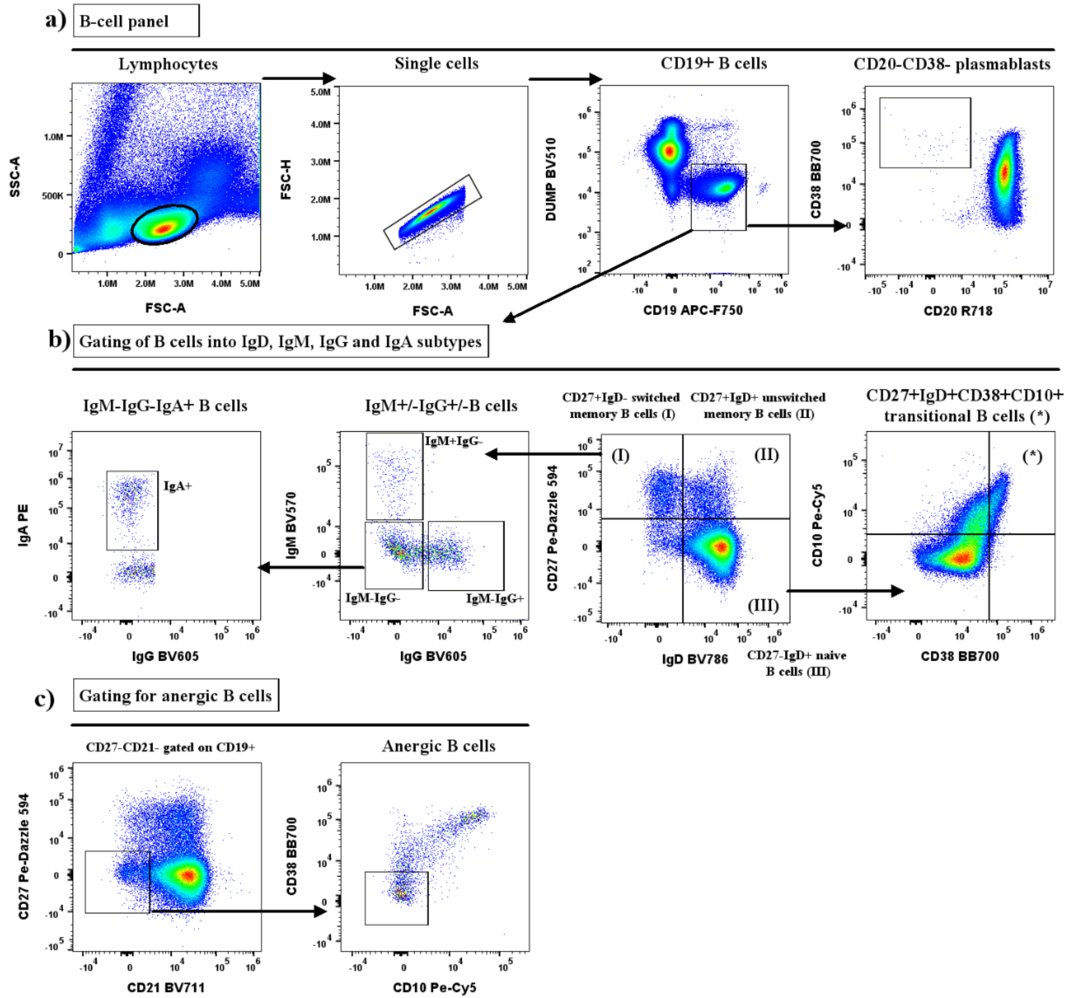

Figure 1. B-cell panel. The displayed panel depicts an adult control sample. Section a) presents the preliminary gating of CD19^{+} cells and plasmablasts. In section b), CD19^{+} B cells are gated according to their state of development and BCR class. Section c) displays the anergic B cells as gated from CD27^{-}CD21^{-}CD19^{+} B cells. FMOs where used when gating CXCR3 BV421, IL-21R APC, CD5 PE-Cy7 and CD38 BB700.

Appendix VII

Figure 1

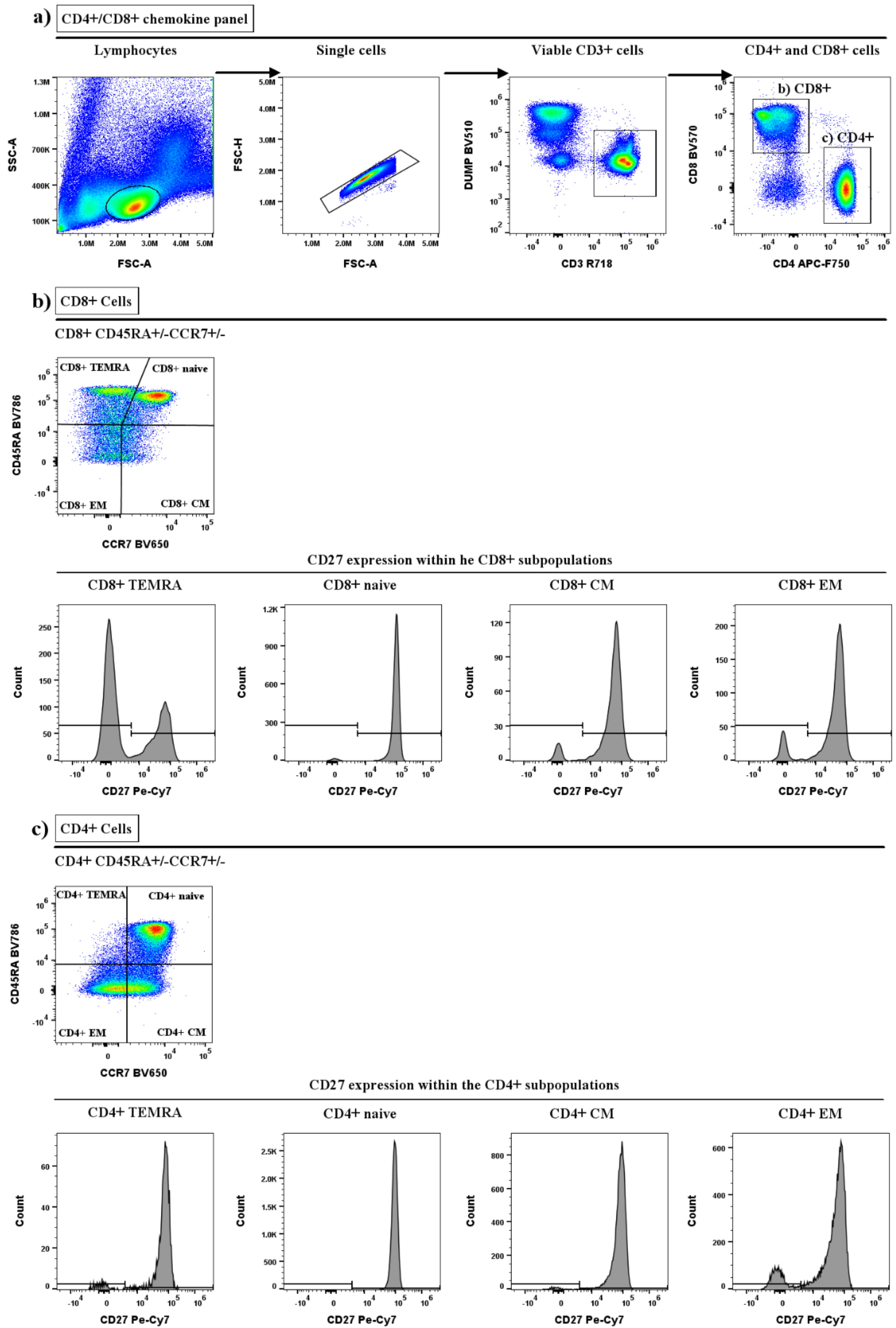

Figure 1. CD4⁺/CD8⁺ chemokine panel, step 1. The displayed panel depicts an adult control sample. Sections b) and c) gate $CD4^+$ and $CD8^+$ cells into naïve, central memory (CM), effector memory (EM), and terminally differentiated effector memory T cells (TEMRA), for $CD4^+$ and $CD8^+$ cells respectively.

Respective T cell populations in sections b) and c) are further gated for CD27 (displayed as a histogram). FMOs where used when gating CCR10 APC, CXCR3 BV421, CCR4 BB770, CCR6 BV711, PD-1 PE and CD161 PE-Cy5.

Figure 2

a) Gating for memory CD4+ cell subsets

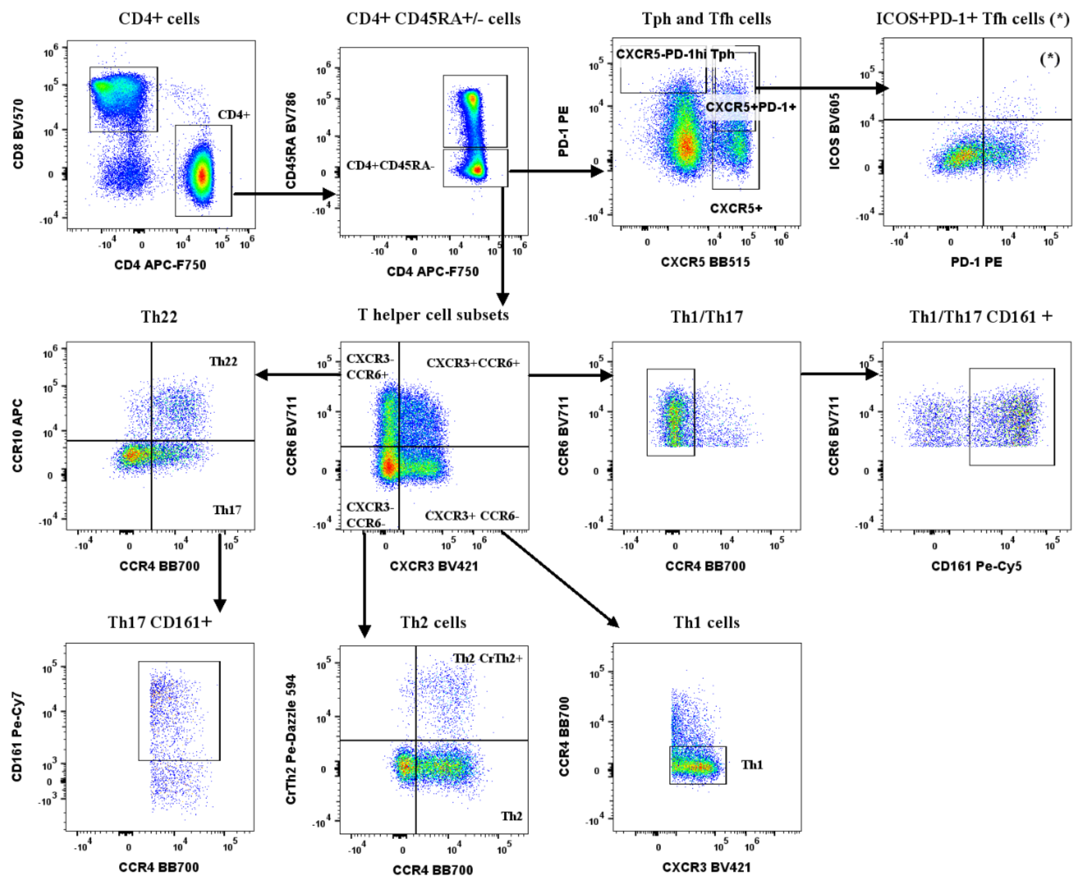

Figure 2. CD4$^+$/CD8$^+$ chemokine panel, step 2. The displayed panel depicts an adult control sample. The memory CD4$^+$ cells are first gated for peripheral Th cells (Tph) and follicular Th cells (Tfh). The Memory CD4$^+$ cells are further gated according to CCR3 and CCR6 expression, producing Th1, Th2, Th17, Th1/Th17 and Th22 cell subsets. Th1/Th17 and Th17 cells are further gated for CD161. FMOs where used when gating CCR10 APC, CXCR3 BV421, CCR4 BB770, CCR6 BV711, PD-1 PE and CD161 PE-Cy5.