UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE IN MEDICINA MOLECOLARE

Curriculum in basic and applied immunology and oncology

TARGETING THE GLUCOSE TRANSPORTER GLUT1 TO CONTROL T CELL ACTIVATION

DoS: Dr. Paolo Monti

Second Supervisor: Dr. Marinos Kallikourdis

Tesi di DOTTORATO di RICERCA di Carla Di Dedda

Matr.017132

Ciclo di dottorato XXXVI

SSD MED/04 Patologia generale

Anno Accademico 2022/2023

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

Lla sottoscritta/I	CARLA DI DEDDA
Matricola / registration number	017132
nata a/ born at	BARI
il/on	07/11/1993

autore della tesi di Dottorato di ricerca dal titolo / *author of the PhD Thesis titled*

TARGETING THE GLUCOSE TRANSPORTER GLUT1 TO CONTROL T CELL ACTIVATION

☒ AUTORIZZA la Consultazione della tesi / AUTHORIZES *the public release of the thesis*

□ NON AUTORIZZA la Consultazione della tesi per ..... mesi /DOES NOT AUTHORIZE *the public release of the thesis for ..... months*

a partire dalla data di conseguimento del titolo e precisamente */ from the PhD thesis date,*

specifically

Dal / *from* ...../...../..... Al / *to* ...../...../.....

Poiché /*because*:

□ l'intera ricerca o parti di essa sono potenzialmente soggette a brevettabilità/ *The whole project or part of it might be subject to patentability;*

□ ci sono parti di tesi che sono già state sottoposte a un editore o sono in attesa di pubblicazione/ *Parts of the thesis have been or are being submitted to a publisher or are in press;*

□ la tesi è finanziata da enti esterni che vantano dei diritti su di esse e sulla loro pubblicazione/ *the thesis project is financed by external bodies that have rights over it and on its publication.*

Data /Date .....24/10/2023..... Firma /Signature .....Carla Di Dedda.....

DECLARATION

This thesis has been:

- composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.
- has been written according to the editing guidelines approved by the University.

Permission to use images and other material covered by copyright has been sought and obtained.

All the results presented here were obtained by myself, except for:

1) ***Insulin secretion test of EndoC-βH1.***

Perifusion experiments (Results, paragraph 3.4.5 , figure 39), were performed by with Dr. Antonio Citro, Diabetes Research Institute, San Raffaele Scientific Institute, Milano.

2) ***Inhibition of GLUT1 in preclinical model of T1D.***

In vivo administration of WZB117 (Results, paragraph 3.4.5, figure 41), was performed by Dr. Paolo Monti in collaboration with Dr. Antonio Citro, Diabetes Research Institute, San Raffaele Scientific Institute, Milano.

All sources of information are acknowledged by means of reference.

ACKNOWLEDGEMENTS

I would like to thank all the members of the Transplantation Immunology lab, who have been a guiding light and source of inspiration for me during these years of PhD.

In particular, I would like to express my deepest gratitude to Dr. Paolo Monti, Director of Studies for this thesis. His firm dedication, expertise, enthusiasm, and profound knowledge in the field have been invaluable.

Most importantly, I am immensely grateful to Dr. Paolo Monti for the incredible opportunity he gave to me and the faith and trust he invested in my work during these years.

Special gratitude to Prof. Alberto Pugliese, who welcomed me in his lab in the United States, making me feel at home when I was far from home. My time in his lab has been a period of profound personal and professional growth. I consider it a great privilege to have had the opportunity to work at the side of a giant in the field of diabetes research.

Paolo Monti and Alberto Pugliese not only provided me with invaluable advices and ideas but also granted me the opportunity to attend international congresses. These experiences allowed me to connect with other professionals in the field and share the progress of my project.

I express my gratitude to Dr. George Burke for generously providing samples from pancreas transplanted patients and their associated characteristics for this thesis.

I would also like to express my sincere thanks to Isaac Snowhite, always available in every situation, who contributed to this work with his knowledge and who never hesitated to offer help whenever I needed it.

A mia nonna Dora

ABSTRACT

Beta cell replacement, through pancreas or islet transplantation, represents a therapeutic option for patients with severe T1D. However, its success is limited by the host immune response. Introducing beta cell replacement therapy into an individual with pre-existing autoimmunity presents an immunological challenge. This scenario involves the simultaneous coexistence of immune responses—transplant rejection and tissue-specific autoimmunity— with the potential for reactivation of autoreactive memory T cells, which poses an additional set of therapeutic challenges.

T cell activation relies on a metabolic switch from oxidative phosphorylation to glycolysis. Upregulation of GLUT1 and therefore increased cellular glucose uptake is considered key for this metabolic switch. My thesis work aims to test pharmacological GLUT1 blockade as a potential strategy to control T cell activation.

Using a collection of pancreas transplanted patients I characterized T cell subsets that are involved in autoimmunity recurrence and whether the expression of GLUT1 is associated with autoimmune reactivation. Moreover, using the small molecule GLUT1 inhibitor WZB117 I studied phenotypic, differentiation, and metabolic changes of T cells activated under GLUT1 blockade *in vitro*. As GLUT1 is broadly expressed in several tissues I started investigating the potential off-target effects that may limit GLUT1 blockade as an immune-modulatory strategy.

Collectively the data of my thesis support the hypothesis that GLUT1 blockade can control T cell activation and that GLUT1 is highly expressed in autoreactive T cells from patients experiencing autoimmunity recurrence post-transplant. Moreover, no significant adverse effects have been identified, prompting us to test this novel strategy in pre-clinical models to determine its effectiveness.

TABLE OF CONTENT

ACRONYMS AND ABBREVIATIONS

2-DG	2-deoxy-D-glucose
AAB	Autoantibodies
AGEs	Advanced Glycation End-Products
AID	Autoimmune Drug
APC	Antigen Presenting Cell
ATG	Anti- Thymocyte Globulin
CD	Cluster of Differentiation
CFSE	Carboxy-Fluorescein Succinimidyl Ester
CLIP	Class II Associated Invariant Chain Peptide
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DHA	Dehydroascorbic Acid
DIV	Days In Vitro
DKA	Diabetic Ketoacidosis
DMF	Dimethyl Fumarate
DN	Diabetic Nephopathy
DR	Diabetic Retinopathy
DRiP	Defective Ribosomal Product
EAE	Experimental Autoimmune Encephalomyelitis
ECAR	Extracellular Acidification Rate
EDTA	Ethilenediaminetetraacetic Acid
ESC	Embryonic stem cells
ESRD	End-Stage Renal Disease
FACS	Fluorescence Activated Cell Sorter
FBS	Fetal Bovine Serum
GAD65	Glutamic Acid Decarboxylase 65
GLU1-DS	GLUT1 Deficiency Syndrome
GLUT	Glucose Transporter
GRS	Genetic Risk Scores
GvHD	Graft-Versus-Host Disease
GWAS	Genome-Wide Association Studies
HbA1c	Glycated Hemoglobin
HCC	Hepatocellular Carcinoma
HIF	Hypoxia-Inducible Factor
HIPs	Hybrid Insulin Peptides
HLA	Human Leukocyte Antigen
IA-2	Insulinoma Associated Antigen 2
IFN	Interferon
IL#	Interleukin
INS	Insulin
iPS	Induced Pluripotent Stem

JAK	Janus Kinase
KCl	Potassium Chloride
Km	Michaelis-Menten Constant
LN	Lymph Node
mAb	monoclonal Antibody
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MMF	Mycophenolate Mofetil
MMTT	Mixed Meal Tolerance Test
NGS	Next Generation Sequencing
NGT	Normal Glucose Tolerance
NK	Natural Killer
NOD	Non-Obese Diabetic
nPOD	Network For Pancreatic Organ Donors With Diabetes
OCR	Oxygen Consumption Rate
PAK	Pancreas After Kidney
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PEC	Pancreatic Endoderm Cells
PFKFB3	6-Phosphofructo-2-Kinase
PI	Propidium Iodide
Pi3K	Phosphoinositide 3-Kinase
PPI	Pre-Proinsulin
PTA	Pancreas Transplant Alone
PTPN22	Protein Tyrosine Phosphatase, Non-Receptor Type 22
RIA	Radioimmunoassays
RRMS	Relapsing-Remitting Multiple Sclerosis
SNP	Single Nucleotide Polymorphisms
SPK	Simultaneous Pancreas-Kidney
STAT5	Signal Transducer And Activator Of Transcription 5
T1D	Type 1 Diabetes
T1DR	Type 1 Diabetes Recurrence
TCF-1	T Cell Factor 1
TCR	T Cell Receptors
TNF	Tumor Necrosis Factor
VHL	Von Hippel-Lindau
ZnT8	Zinc Transporter 8

LIST OF TABLES

LIST OF FIGURES

Chapter 1
INTRODUCTION

1.1 Type 1 Diabetes (T1D)

1.1.1 T1D is a chronic autoimmune disease

Type 1 diabetes (T1D) is a chronic autoimmune disorder caused by the destruction of insulin-producing beta cells by autoreactive T cells. This process results in severely impaired insulin secretion and lifelong dependence on exogenous insulin administration [1]. Both $CD4^+$ and $CD8^+$ T cells are involved in the beta cells destruction. The presence of $CD4^+$ and $CD8^+$ T reactive to multiple beta cell antigens was noted in islets obtained from T1D patients [2, 3].

T1D represents 5-10% of the global diabetes cases and its incidence is increasing at a rate of 2 to 4% per year [4]. More recently, growing evidence suggested that a notable increase in T1D incidence was associated with the COVID-19 pandemic [5-7].

While T1D can manifest at any age, it usually occurs during childhood or early adulthood. Unfortunately, there is no cure for T1D, and daily insulin administration is required to control glucose metabolism. Adopting a healthy lifestyle and adhering to the treatment regimen can help in maintaining normal blood glucose levels, but patients with T1D can still experience episodes of hyperglycemia as well as hypoglycemia.

The exact causes of T1D are not fully understood, but both genetic susceptibility and environmental factors may contribute to its development. Certain histocompatibility antigens in the human leukocyte antigen (HLA) region, or the major histocompatibility complex (MHC), have been associated with an increased risk of developing diabetes, indicating a genetic link.

Although T1D is primarily considered a disease mediated by T cells, other immune cell types, including macrophages, dendritic cells, natural killer (NK) cells, and B lymphocytes, play integral roles in the development of the disease, especially during the early disease stages (**Fig. 1**). Macrophages function as antigen-presenting cells, facilitating the priming of naïve T cells in the draining lymph nodes (LN), and as effector cells, producing tumor necrosis factors (TNFs), when islet inflammation is established [8]. NK cells infiltrate beta cell islets and extravasate into the pancreas, having the capacity for beta cell cytolysis [9]. Dendritic cells participate in T1D development by migrating from pancreatic islet to pancreatic draining LN after antigen uptake, and

physically activating T cells. B lymphocytes are also associated with the initiation and progress of the disease [10, 11].

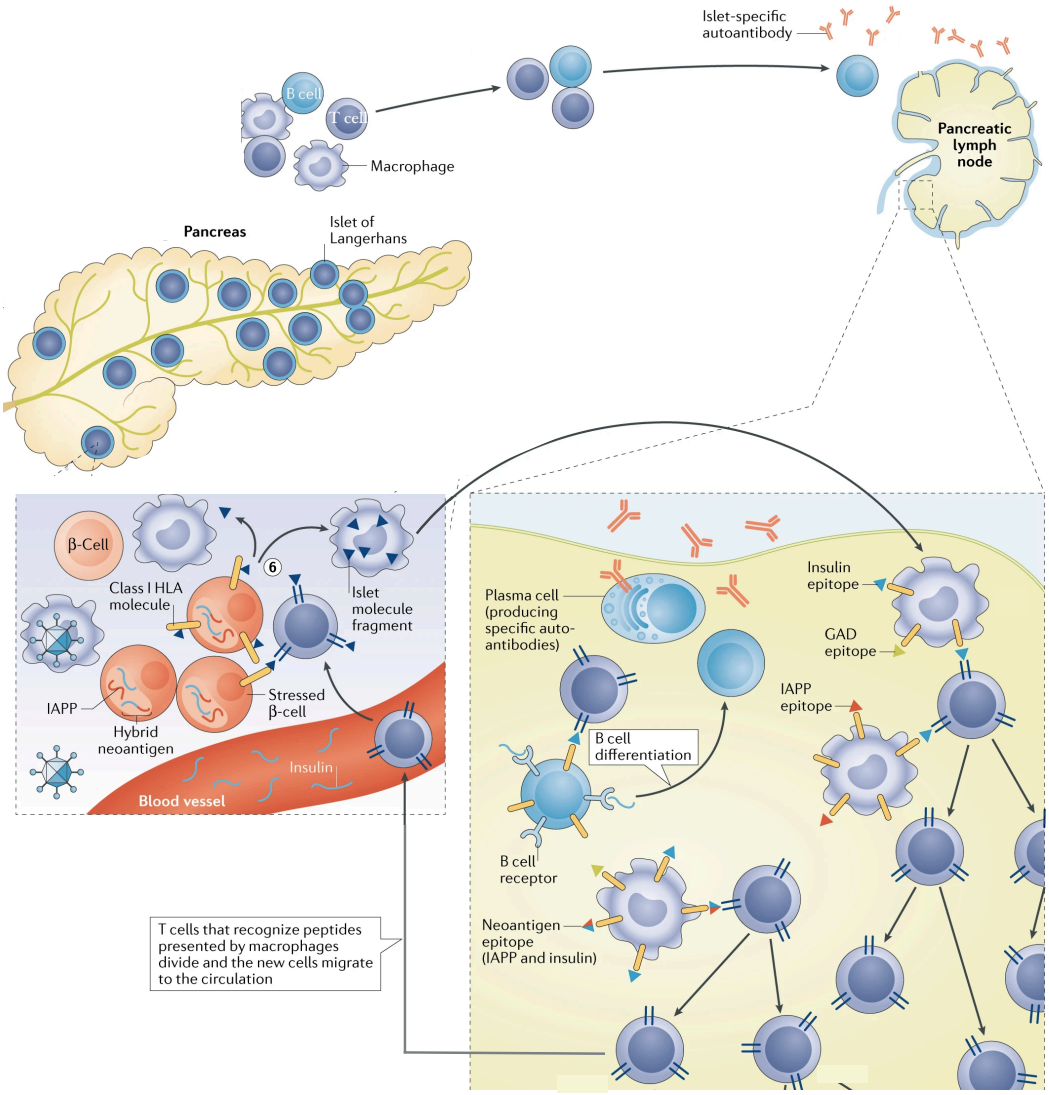


Figure 1. Immune mechanisms involved in the immunopathogenesis of T1D. *Figure modified from Ilonen, Jorma et al. "The heterogeneous pathogenesis of type 1 diabetes mellitus. "Nature Reviews Endocrinology 15 (2019): 635 - 650. [12]*

The presence of circulating autoantibodies (AABs) targeting islet cells in patients with T1D, then termed Islet Cell Antibodies (ICA), was first discovered in 1974 using indirect immunofluorescence on frozen human pancreas sections [13]. Later studies identified several antigenic targets of these ICA, which include (pro)-insulin, GAD65, islet tyrosine phosphatase 2 (IA-2) [14], Zinc transporter 8 (ZnT8) [15] and others [16]. The presence

of these AABs has been documented in both sporadic and familial cases of diabetes, independently from the age of onset. ICA can be detected months to years before clinical diagnosis and can be used for predicting the development of T1D.

Recently, it has been discovered that a variety of mechanisms lead to the formation of neoantigens that represent variants of native proteins [17] [18]. For example, post-transcriptional and post-translational modifications have been described for several islet autoantigens, such as citrullination, deamidation and oxidation; these are often triggered by cellular stress and inflammation. These are examples of modifications which can alter the protein structure generating neoepitopes, which are targeted by T cells in patients with T1D [19]. Another mechanism involves the covalent linkage between an insulin fragment with other secretory granule peptides originating hybrid insulin peptides (HIPs). The presence of autoreactive responses to HIPs has been documented in both non-obese diabetic (NOD) mice and T1D patients, emerging as potent ligands for T cells isolated from islet infiltrates [20, 21]. Because of a genetic susceptibility (mutation in the CTLA-4 gene) NOD mice spontaneously develop autoimmune diabetes that have important similarities to human T1D. Interestingly, HIPs have been detected in subjects at risk of developing T1D, showing predominantly a pro-inflammatory profile in those who eventually developed the disease [22]. Also, a defective ribosomal product of the proinsulin gene (INS-DRiP) has been identified as a neoepitope [23]. DRiP originates from the translation of an alternative open reading frame of the insulin mRNA under conditions of cellular stress and it has been identified in patients with T1D as a target of cytolytic $CD8^+$ T cells [23].

1.1.2 Stages in the natural history of T1D

The natural history of T1D is a continuum progression through distinct preclinical stages which culminate in the clinical onset of the disease [24]. The stages have been standardized on the basis on genetic risk, immunological markers, and metabolic changes [25] and are summarized in **Table 1**.

There are four identified stages in this progression:

- *Pre-stage 1* refers to the genetic and environmental factors that increase the risk of developing T1D, without any detectable signs of autoimmunity or dysglycemia [26, 27]. However, a recent study revealed that in children with high genetic risk of T1D the postprandial blood glucose levels were significantly increased already 2 months before seroconversion [28].
- *Stage 1* T1D is characterized by the presence of two or more AABs in the blood, known as seroconversion, but without any abnormalities of glucose metabolism and diabetes symptoms [29].

  Subjects with two or more islet AABs face a 70% risk over a 10-year period and an 84% risk over a 15-year period of developing clinical T1D from the time of seroconversion [30].
- *Stage 2* T1D is characterized by the presence of multiple AABs and dysglycemia, which suggests some functional impairment of beta cell function, again in the absence of clinical symptoms.
- *Stage 3* is reached when hyperglycemia is detected and it leads to the appearance of the clinical symptoms, such as weight loss, thirst, excessive urination and diabetic ketoacidosis (DKA). It is estimated that, by the time of diagnosis, a significant loss of cells has occurred, but this varies among patients based on age and other factors (estimated range 60-90%) [25]. Beta cell dysfunction is considered to play a role in the severe impairment of insulin secretion observed at diagnosis, especially when beta cell loss is not complete; however, physical beta cell mass cannot be measured at present.

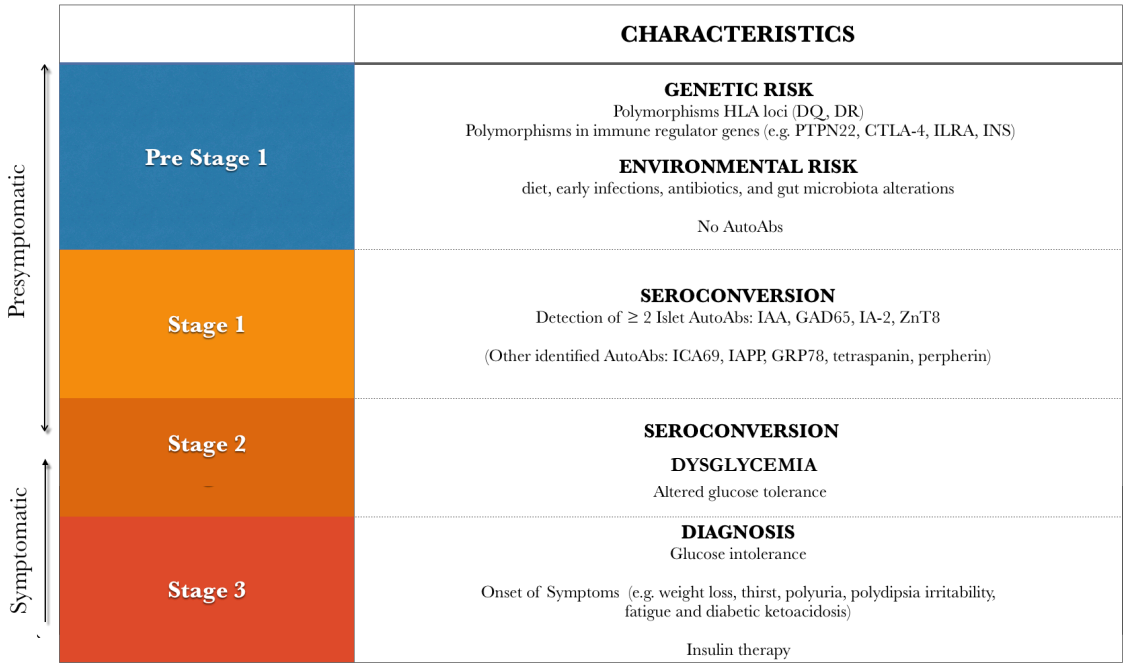


Table 1. Stages in the natural history of T1D. *Main Features of T1D stages: immune and metabolic markers distinguish pre-stage 1, stage 1, stage 2 and stage 3.*

In November 2022, teplizumab (a non-Fc receptor-binding humanized anti-CD3 mAb) was approved by the Food and Drug Administration as the first disease-modifying therapy in the United States for individuals at high risk of T1D [31]. Treatment with teplizumab delayed progression from stage 2 to stage 3 T1D by an average of 2 years in adults and pediatric subjects. The clinical trial leading to approval was relatively small, enrolling 76 individuals older than 8 years, who were relatives of patients with T1D. The subjects received either teplizumab or placebo for a single course of 14 days and were followed up every 6 months for clinical T1D through an oral glucose tolerance test [32].

Among the placebo group, 23 out of 32 subjects developed clinical T1D after 24 months (22% remained disease-free), while 19 out of 44 subjects treated with teplizumab developed clinical T1D after 48 months (50% remained disease-free). The mechanism of action of teplizumab is only partially understood; it appears to enhance regulatory T cell activity and promote immune tolerance by increasing the frequency of $CD4^+$ $FoxP3^+$ T cells and $CD8^+$ $KLRG1^+TIGIT^+$ T cells.

For years, the scientific community has focused on translating anti-CD3 monoclonal antibodies into clinical settings. Muromonab (OKT3, a murine anti-CD3 mAb) was the first approved for human use to prevent graft rejection, but it was withdrawn due to side effects caused by the release of inflammatory cytokines by T cells triggered by crosslinking of TCR and CD3 [33]. Consequently, non-Fc receptor-binding antibodies, which are better tolerated, have been developed. Among these, otelixizumab has demonstrated to preserve beta cell function in a clinical trial for T1D, though its use led to transient Epstein Barr Virus (EBV) reactivation [34]. Also teplizumab showed to have similar side effects.

In the study conducted by Herold [32], 75% of the group treated with teplizumab experienced temporary lymphopenia (6-8 weeks) and hypersensitivity reactions that spontaneously resolved, while the infection rate was similar to the placebo group. The appearance of antibodies directed against teplizumab has not been investigated in this study, however, in previous trials, antidrug antibodies were detected in approximately 20% to 55% of teplizumab-treated participants following the first course [35, 36].

Overall, a course of teplizumab has been shown to delay progression from stage 2 to stage 3 T1D. Results from clinical trials with teplizumab in patients with recent onset stage 3 T1D showed a positive impact on preservation of residual insulin secretion. While these effects are considered beneficial, it is important to recognize that teplizumab impacts but does not halt completely the disease process. Moreover, additional agents (i.e. low dose ATG) may afford comparable benefits to teplizumab. Further research is needed to identify additional treatments that can be synergistic and provide longer term therapeutic benefits, with minimal side effects, especially if chronic administration is required.

1.1.3 Genetic susceptibility

Extensive research has been conducted on the genetic predisposition to T1D, particularly focusing on genes related to T cell function. The most significant genetic association is found within the HLA complex, located on chromosome 6. These genes play a crucial role in immune recognition by T cells and antigen presentation.

The HLA region is organized into class I, class II, and class III, with the HLA class II genes DRB1, DQA1, DQB1, DPA1 and DPB1, being the most associated with T1D susceptibility. Variants and combinations of alleles at these loci account for 40 to 50% of T1D familial clustering [37], particularly in the Caucasian population [38]. HLA class I antigens present antigenic peptides to $CD8^+$ T cells and are expressed on all nucleated cells, while HLA class II antigens present peptides derived from extracellular antigens to $CD4^+$ T cells and are expressed by antigen-presenting cells.

Three distinct groups of different HLA combinations genes allow for the discrimination of individuals into high-risk, moderate-risk, or low-risk groups for T1D susceptibility, and are summarized in **Table 2**.

RISK	***Phenotype***
High (x20)	DRB1*03, 04; DQB1*0201, 0302 (Haplotype HLA-DR3,4-HLA-DQ2,8)
Moderate (x7)	DRB1*04,04; DQB1*0302, 0302 (Haplotype HLA-DR4,4 HLA-DQ8,8)
Moderate (x4)	DRB1*03, 03; DQB1*0302,Y (haplotype HLA-DR3,3 HLA-DQ8,Y) DRB1*04, X; DQB1*0302,Y (haplotype HLA-DR4,X HLA-DQ8,Y) (Where X is neither DRB1*2,3 nor 4 and Y is not DQB1*0302)
Average (x1)	HLA-DRB1*03,X

Table 2*. Genotypic combinations of DRB1-DQB1 haplotypes associated with T1D susceptibility*

Conversely, some HLA alleles, such as DRB1*1501, DQA1*0102, and DQB1*0602 protect against diabetes [39]. Over the past decades, natural history studies enrolling newborns and young children, such as DAISY, TEDDY, DIPP and BABYDIAB have

played a pivotal role in understanding genetic, environmental and other factors that are predictive or otherwise associated with the onset of T1D in newborns. Even though HLA-DR*03, 04 were known as genotypes with the highest risk for T1D development, using HLA alone for T1D prediction is not sufficient. In fact, approximately 50% of people in the United States have HLA-DR3 or HLA-DR4, but less than 1% of these people develop T1D [40]. Birth cohort studies suggested that in addition to HLA genotypes, the timing of islet AABs seroconversion could be informative about the progress of the disease. However, the detailed knowledge of autoantigenic T cell epitopes and their HLA restriction elements has empowered the development of assays to detect and characterize autoantigen specific T cell populations, which we have employed in this study.

Genome-wide association studies (GWAS) have also identified over 60 non-HLA loci associated with T1D risk. Some of these genes include *PTPN22*, *INS*, *NRP1, ERBB3*, IL2RA, *PTPN2, IFIH1*, CTLA-4 and *FUT2* [41-44]. These studies have contributed to our understanding of key pathways involved in disease development, which are impacted by genetic variants and other mechanisms, which include self-antigen and insulin expression in the thymus, regulation of T cell activation, and responses to viral exposures. In many cases T1D susceptibility loci overlap with immune- and autoimmune-related loci associated with other autoimmune diseases such as rheumatoid arthritis, systemic erythema lupus, ulcerative colitis, and asthma [45]. Although there are still more genetic loci to be discovered using larger datasets and diverse populations, the main genetic regions associated with T1D are now well-established and it appears that the existence of additional loci with major impact on risk can be excluded.

A number of genetic risk scores (GRS), which incorporate information from both HLA haplotypes and non-HLA variants, have been developed as a tool to support screening efforts as well as aiding in different diagnosis across various forms of diabetes [46].
GRS1 could distinguish T1D from T2D and, integrated with another GRS that included 12 non-HLA loci, was employed in TEDDY study [46, 47]. The latest GRS2 combines the genetic complexity of the HLA region with non-HLA variants, using 67 single nucleotide polymorphisms (SNPs) and it has shown promise for newborn T1D-risk screening, with a set of 36 GRS2 [48]. This tool has recently been improved with the

inclusion of four additional targeted SNPs, to increase the precision and expand the use of T1D-GRS2 to a larger population [49]. However, the predictive accuracy of GRS models is impacted by the specific ethnicity from which they are derived and may exhibit variability when applied to individuals of diverse ancestral backgrounds.

1.1.4 Environmental factors

Mounting evidence suggests that environmental factors significantly contribute to the disease risk. These findings involve patterns of increasing incidence, since the rapid growth of T1D prevalence observed in recent decades cannot be solely attributed to genetic changes [50], but also the variations across different geographic latitudes, with higher rates in Nordic countries compared to other regions [51]. Moreover, changes over time have been noted, since countries with previously low T1D incidence and those experiencing rapid economic growth tend to exhibit the highest rates now [50]. Another key observation is that the concordance rates of T1D among monozygotic twins, who share identical genetic makeup, are less than 50%, suggesting that non-genetic factors play a significant role [52, 53].

To date, the environmental factors that have the most supporting evidence of being associated with T1D are enterovirus infection, rapid weight gain in early life, and altered gut microbiota.

Enteroviruses belong to a viral family that includes various strains, such as coxsackievirus B, and are believed to trigger or accelerate islet autoimmunity in subjects at high risk of T1D. This hypothesis is supported by the presence of high titers of neutralizing antibodies to coxsackievirus B which cause persistent infection in human pancreatic islets [54, 55]. Particularly relevant in this context is the large prospective T1D study TEDDY (The Environmental Determinants of Diabetes in the Young), which collected data from over 8500 newborns and followed them from the age of 3 months to 15 years. This study found that the presence of persistent coxsackievirus B infection, was associated with a significantly high risk of islet autoimmunity. Conversely, multiple independent short-term enterovirus infections were not correlated with either autoimmunity or the

progression of T1D [56]. Animal studies have also demonstrated that infection with specific enterovirus strains, which cause a prolonged presence of viral RNA in the mice pancreas, can initiate an autoimmune response against pancreatic beta cells, resulting in T1D-like symptoms, such as chronic inflammation and an increase in islet AABs. In light of this evidence, antiviral strategies aimed at preventing or delaying destruction of beta cells are currently under investigation. There are no approved antiviral drugs currently available for enteroviruses, making the study of repurposed drugs an effective strategy for accelerating their entry into clinical trials [57]. Two such drugs, pleconaril and ribavirin, originally developed for the common cold and hepatitis C virus, have demonstrated antiviral efficacy against enteroviruses *in vitro* and *in vivo.* A recent clinical trial involving 96 children aged 6 to 15 years, who had been recently diagnosed with T1D, revealed that a 6-month treatment with pleconaril and ribavirin had the ability to preserve residual insulin production in these patients [58].

Obesity-induced insulin resistance has been shown to contribute to the progression of T1D. In individuals with a genetic predisposition to T1D, obesity imposes an increased burden on the pancreatic islet cells, due to an increased demand for insulin production, which can potentially initiate and accelerate autoimmune processes [59]. Also elevated birth weight and infant growth rate have emerged as potential contributors to T1D pathogenesis. Studies have shown that children born with a birth weight over 4kg have an increased risk of T1D by 17% [60].

The gut microbiome has been an area of active interest as well. Studies have revealed distinctive patterns in the gut microbiota of individuals who are genetically predisposed to T1D, as well as in those who have newly developed the disease. These patterns include a decrease in bacterial diversity, indicating a less varied microbial composition, and reduced stability, suggesting a less balanced and resilient ecosystem [61, 62]. Alterations in the gut microbiota may lead to enhanced intestinal inflammation, associated with intestinal permeability and a loss of barrier function. Recent findings have shown that the breakage of gut barrier integrity leads to the activation of islet-reactive T cells and triggers T1D. However, promising results in preclinical models demonstrated that restoring gut

barrier integrity, through administration of tolerogenic autoimmune drugs (AID), has been found to prevent T1D [63].

The sequencing of microbial proteins has led to discovery that a common gut bacterium, *Parabacteroides distasonis*, exhibits a sequence that mimic the B:9-23 peptide (SHLVEALYLVCGERG). InsB:9-23 is an epitope located in the B-chain of insulin which is recognized by autoantibodies and autoreactive T cells in patients with T1D. *Parabacteroides distasonis* was found to activate both human T cell clones isolated from T1D patients and T cell hybridomas derived from NOD mice [64]. Thus, gut microbiome may trigger the development of T1D through molecular mimicry. These findings may have implications for the development of new treatments for T1D, such as the use of probiotics or other interventions to modulate the gut microbiome and prevent the development of T1D in at-risk individuals.

Recently, associations between COVID-19 and T1D have been reported, including reports of increased incidence of autoimmune disease during the pandemic and a higher susceptibility of individuals with T1D to severe COVID-19 infection. The underlying mechanisms behind these associations are not fully understood and require further investigation [65-67].

1.1.5 Complications of T1D

Despite the significant advancements in diabetes management through the introduction of exogenous insulin, achieving complete normalization of metabolic control remains elusive. Many patients still face acute complications like ketoacidosis and long-term complications including neuropathy, retinopathy, nephropathy, atherosclerosis, and lipid disorders.

Diabetic retinopathy (DR) specifically exemplifies the impact of both hyperglycemia and high blood pressure on the microvasculature supplying oxygen and nutrients to the retina. Multiple mechanisms and pathways, such as oxidative stress, advanced glycation end-products (AGEs), and inflammation, contribute to the damage. This damage manifests as the formation of micro-aneurysms, abnormal microvascularization, and microbleeds

within the retina. Although laser treatment can mitigate the progression of DR, it does not provide a definitive cure [68].

Despite regular monitoring of kidney health, a significant proportion of patients with T1D - up to one-third - develop diabetic nephropathy (DN) [69, 70]. In some cases, DN progresses to kidney failure, also known as end-stage renal disease (ESRD), characterized by a severe decline in kidney function and impaired blood filtration. At this stage, patients require dialysis treatment or kidney transplantation. ESRD is associated with a significantly higher risk of premature mortality, up to 18 times higher compared to individuals without diabetes of the same age and sex [71]. Genetic factors also play a significant role in the development of diabetic kidney disease, with estimates suggesting a heritability rate ranging from 35% to 59% [72].

Atherosclerosis, characterized by the gradual build-up of lipid-containing plaques inside the arteries, is one of the most common complications of T1D. This inflammatory disorder leads to the narrowing and hardening of the arteries, ultimately obstructing normal blood flow. Atherosclerosis is a major cause of premature mortality in patients with diabetes, particularly those with DN [73, 74].

Neuropathy is another significant long-term complication of diabetes. Sustained exposure to high blood glucose levels can damage the small blood vessels, impairing the supply of nutrients and oxygen to the nerves. Patients with neuropathy often experience varying degrees of numbness, tingling, and/or burning in the extremities [75]. Peripheral neuropathy often leads to infection, ulceration, and, in severe cases, amputation. While less than a third of patients achieve satisfactory pain control, improved glycemic control can result in a reduction in paresthesia and dysesthesias within a year.

Diabetes ketoacidosis (DKA) is a severe and potentially life-threatening complication of T1D. This condition is characterized by uncontrolled hyperglycemia and the accumulation of high levels of ketones due to severe insulin deficiency. Major causes of DKA are lack of compliance with insulin treatment and infections. Early symptoms include excessive thirst and urination leading patients to have signs of dehydration,

followed by more severe symptoms such as fast and fruity-smelling breath, fatigue, stomach pain, nausea and vomiting. Commonly accepted criteria for DKA are blood glucose greater than 250 mg/dl, arterial pH less than 7.3, serum bicarbonate less than 15 mEq/l, and the presence of ketonemia or ketonuria [76].

Hypoglycemia is a common consequence of T1D, which manifests when the blood glucose level drops below 70 mg/dL. Hypoglycemia in people with T1D is usually a result of glucose lowering therapies, including insulin or other insulin secretagogue medications, not eating enough, or engaging in excessive physical activity without adjusting insulin doses or carbohydrate intake. Over 50% of severe hypoglycemic episodes in T1D patients treated with insulin occur at night [77]. Acute episodes may have serious health implications, including cognitive impairment, affection of memory and attention, and overall cognitive functions [78]. Severe hypoglycemia can lead to coma and death.

1.2 Autoreactive T cells

1.2.1 Origin of autoreactive T cells in patients with T1D

During T cell development, T cell receptors (TCRs) are generated randomly and undergo a selection process in the thymus. This process involves positive selection, to ensure recognition of MHC molecules/peptide complexes, and negative selection, to eliminate T cells that recognize self-MHC molecules/peptide complexes with high avidity [79].

However, the selection of a T cell repertoire that is completely devoid of self-reactive T cells is not achieved, and the immune system has evolved various checkpoints to control autoreactivity. Autoreactive lymphocytes that do not strongly recognize self-antigens due to modest affinity of their TCRs for specific self-antigen/HLA complexes may escape negative selection in the thymus; however, those may be inactivated or regulated in the periphery. Breakdowns in these checkpoints can promote the activation of autoreactive T cells into circulation, which exist in all individuals [80]. Autoimmune diseases develop when enough safeguards are overcome, resulting in a sustained reaction to self.

In the context of T1D, MHC-peptide complexes in individuals with risk genotypes are inefficient at deleting autoreactive T cells in the thymus or effective in activating and expanding autoreactive T cells in the periphery. For example, $CD4^+$ T cells specific for proinsulin and GAD65 beta cell antigens have been detected in cord blood samples from healthy newborns, suggesting the presence of an autoreactive T cell pool at birth. These autoreactive T cells in cord blood display a naïve phenotype and exhibit a proliferative response to beta cell antigens [81].

The diversity of TCRs plays a significant role in the origin of autoreactive T cells in patients with T1D. TCR contains a constant region (C region) and a variable region (V region), whose structure may acquire significant diversity. With the advent of next-generation sequencing (NGS) technology, it has become feasible to identify the gene sequences of TCRs expressed within a specific cell population at the level of individual clone. For example, over 1000 different TCRs have been classified within the pool of GAD65-reactive T cells from a cohort of 6 patients with T1D and 10 AABs positive before the disease onset. Interestingly, only a few TCR sequences were observed in the

same individual at different time points, and no shared TCRs were found among different subjects [82]. More recently, a study involving healthy, new-onset T1D and established T1D donors, a prominent population of autoreactive $CD4^+$ T cells was identified. These cells had different specificities characterized by TCRs with restricted TCRα junctions and germline-constrained antigen recognition properties [83].

In addition to the peripheral blood samples, which have been traditionally the main source of study samples, the Network for Pancreatic Organ Donors with Diabetes (nPOD) has provided pancreas and other tissue samples across a wide range of donors, allowing studies of the autoreactive T cell repertoire from insulitic lesions [84, 85]. An extensive analysis of the T cell repertoire from pancreatic LNs, spleen, peripheral blood and inguinal or mesenteric LNs from 18 patients with T1D and 9 control organ donors have reported a limited clonal overlap between islets and other samples, suggesting that studies restricted to circulating T cells were inadequate to deeply characterize the autoreactive T cells in patients with T1D [86].

Collectively, these findings indicated that the autoimmune T cell repertoire exhibited high interindividual variability and a wide range of TCR diversity and affinity towards beta cell antigens. This has significant implications for antigen-specific immune therapies, as they should be tailored to the individual characteristics of the autoreactive T cell pool.

1.2.2 Development of memory autoreactive T cells in T1D

The presence of autoreactive T cells in healthy subjects has prompted the scientific community to investigate whether and how these cells differ between healthy individuals and patients with T1D. Substantial efforts have been made to analyze differences in TCR repertoire, activation status, functional properties, cytokine production profiles and the interplay between autoreactive T cells and other components of the immune system.
We demonstrated that autoreactive T cells develop a memory phenotype only in T1D patients, by measuring proliferative T cell responses in naïve ($CD45RO^-$) and memory ($CD45RO^+$) $CD4^+$ T cells, following *in vivo* priming [87, 88]. However, other studies have demonstrated that autoreactive T cells with a memory phenotype are also present in

healthy subjects [82]. While autoimmune T cells are typically associated with the disease, a state of "benign" autoimmunity exists in healthy individuals. These findings highlight the complexity of autoimmune responses because neither the blood frequency nor the phenotype of autoreactive T cells differentiate between benign and pathogenic cells. However, authors found that, in contrast to their frequencies in the blood, the frequencies of ZnT8-reactive $CD8^+$ T cells were significantly higher in the pancreas of T1D patients compared to healthy donors and this can serve to distinguish T1D patients from healthy donors.

Several studies also focused on the differences in frequencies of autoreactive T cells between healthy individuals and patients with T1D. Some of them revealed higher frequencies of beta cell-specific $CD8^+$ T cells in T1D patients compared to healthy subjects, particularly in those with HLA-A*0201 or HLA-A*2402 alleles; this is in part due to the availability of tetramer reagents to interrogate T cells restricted to those HLA types. For example, $CD8^+$ T cells reactive to pre-proinsulin (PPI) have been more frequently detected in T1D patients than in healthy individuals [82, 89, 90]. However, other studies have reported no significant differences in the frequencies of circulating autoreactive $CD8^+$ T cells.

It has also been observed that islet-specific $CD8^+$ T cells in patients with newly diagnosed T1D exhibited a higher degree of differentiation compared to those found in healthy controls. Within the $PPI_{5\text{-}12}$-specific $CD8^+$ T cell populations, children with HLA-B*3906 newly diagnosed T1D, had an enrichment of memory T cell subsets, that was not observed in healthy controls [91, 92]. The analysis of telomere length to determine the history of T cell divisions has shown that the autoreactive memory T cells in T1D patients had undergone extensive proliferation *in vivo* [87]. Therefore, the characterization of memory subsets has sparked an increasing interest in immunologists to find the hallmarks of autoreactive T cells in T1D patients.

In addition to the well-established T cell memory subsets, namely T central memory (Tcm) and T effector memory (Tem) cells, a more recent discovery has identified an additional subset known as T stem cell memory (Tscm) [93]. Tscm have emerged as a

distinct population characterized by their stem cell-like properties, such as long-lived potential, self-renewal ability, multipotency, and robust proliferative capacity upon re-exposure to antigenic stimuli. Tscm can play a role in the initiation and progression of autoimmune diseases by serving as a reservoir for autoreactive effector T cells. These T cell reservoir can perpetuate the chronic destruction of targeted tissues [94].

Notably, an elegant study conducted by Schietinger's group identified a stem-like autoimmune progenitor population in the pancreatic draining LN in NOD mice. The stem-like autoimmune progenitors are characterized by high expression levels of T Cell Factor 1 (TCF-1), a transcription factor critical for T cell memory, longevity and self-renewal. These cells have the ability to self-renew over the long term and originate the terminally differentiated pancreatic autoimmune mediators, which express low levels of TCF-1 and play a crucial role in the destruction of beta cells in T1D. The stem-like autoimmune progenitors are responsible for the generation and replenishment of the pool of pancreatic autoimmune mediators, providing an explanation for the long-live memory autoreactive T cells in patients with T1D [95].

1.2.3 Persistence of autoreactive memory T cells is associated with recurrence of autoimmunity after pancreas or islet transplantation

Despite the estimated half-life of naïve $CD4^+$ and $CD8^+$ T cells being 4.2 and 6.5 years respectively, with effector memory $CD4^+$ and $CD8^+$ T cells lasting only a few months, the autoimmune memory persists for decades following the disease onset in T1D [96].
We previously characterized circulating autoreactive $CD8^+$ Tscm cells specific for islet antigens in the peripheral blood of recent onset (<6 months; n=6) and long-standing (≈27 years; n=8) T1D [97]. While autoreactive Tscm were already detectable in recent onset patients, at a frequency of 2-5%, they were significantly increased (8-15%) in long-standing patients, suggesting a role of autoreactive Tscm in the long-term persistence of autoimmunity.

The prolonged persistence of autoimmune memory is a challenge for patients undergoing islet or pancreas transplantation as beta cell replacement therapy; once activated,

autoreactive memory T cell responses are difficult to inhibit through standard immunosuppression [98, 99]. Although islet and whole-pancreas transplantation are currently the only clinically recognized treatments for the replacement of beta cell in patients with T1D, they represent an immunological challenge characterized by the coexistence of alloreactive T cell activation and the potential reactivation of autoreactive memory T cells.

Allo-transplants are susceptible to both acute and chronic rejection that may lead to early and late loss of islet graft function. Rejection rates for pancreatic transplants typically fall within a range of 5% to 25%, and these rates can vary based on the specific immunosuppression regimen used. Acute rejection is a risk factor for the development of chronic rejection, with a reported occurrence of 10% for pancreas transplant alone and 4% for simultaneous pancreas-kidney transplant (SPK) [100]. The diagnosis of pancreatic rejection is based on laboratory markers. An increase in serum creatinine levels can be indicative of a suspicion of kidney rejection, while elevated levels of serum amylase and lipase, or a decline in urine amylase, can serve as initial indicators of pancreas graft rejection. Later, the diagnostic protocol for rejection has been implemented with pancreas graft biopsies and the identification of islet cell AABs [101].

Among the different causes of graft loss, growing evidence suggests that persistent autoimmune responses can lead to the recurrence of diabetes. Sutherland et al. [102] described Type 1 Diabetes Recurrence (T1DR) in 1984, in recipients of segmental pancreas grafts from living identical twins or HLA-matched siblings; the recipients received no or mild immunosuppression, and in this setting, recurrence of diabetes was observed within a few weeks from the transplant. This observation is also critical to the concept that islet autoimmune responses have a critical role in the pathogenesis of T1D. Subsequent studies have provided further evidence that T1DR occurred in transplant recipients despite being HLA-mismatched and treated with standard immunosuppressive protocols [103-105].

Most recently, Burke et al. [106] and Vendrame et al. [107] have documented the occurrence of T1DR in 7–8% of patients undergoing SPK transplantation, despite

immunosuppression and in the absence of clear signs of rejection. This was observed in a large cohort from University of Miami (n=223), for which there was an extensive follow-up time (6.2 years on average), as T1DR typically takes years to manifest following transplantation. Features of T1DR include hyperglycemia and diabetes symptoms requiring exogenous insulin therapy, without evidence of exocrine pancreas or kidney rejection (unchanged serum amylase, lipase, urine amylase and serum creatinine levels) and biopsies demonstrating insulitis and/or beta cell loss [108]. Moreover, T1DR is preceded by the reappearance of AABs to pancreatic beta cell autoantigens and the presence of circulating autoreactive T cells, including memory T cells [99], near the time of diagnosis.

A potential explanation for the above is that the chronic immunosuppression regimen, necessary for preventing graft rejection, may over time promote the emergence of autoreactive T cells through homeostatic proliferation. Homeostatic proliferation of T cells following transplantation is primarily driven by the IL-7/IL-7R axis, which remains unaffected by standard immunosuppressive therapies. Autoreactive $CD4^+$ and $CD8^+$ T cells that undergo homeostatic expansion after transplantation exhibit a memory phenotype [109]. $CD4^+$ T cells expressing the chemokine receptor CXCR3 have been detected in circulation in pancreas recipients with T1DR [110]. Since CXCR3 is considered a marker of Tscm [111], the characterization of CXCR3 memory T cells infiltrating islets [112] can support the hypothesis that homeostatic cytokines can contribute to the expansion and reactivation of autoreactive Tscm.

1.3 Beta cell replacement in T1D

1.3.1 Pancreas and islet transplantation

Beta cell replacement is presently achievable through pancreas or islets transplantation, and it is a viable option especially for patients who experience ESRD or suffer from brittle diabetes, with inability to adequately control diabetes via conventional insulin therapy or with pumps. Limitations include the need for organ donations, invasive abdominal surgery (for pancreas transplantation), and the requirement for chronic immunosuppression. Recent progress with stem cell-based approaches to obtain transplantable beta cells are now being tested in clinical trials [113] (ClinicalTrials.gov NCT02239354; NCT03163511) and should address the concern about beta cells supply, however, the need for immunosuppression remains. Some efforts are advancing that focus on generating transplantable islets that are hypoimmune and cannot be detected by the immune system [114]. At present, however, beta cell replacement therapies need to rely on immunosuppression to prevent rejection, and yet antigenic re-exposure post-transplant can reactivate over time autoreactive memory clones that are less susceptible to standard immunosuppressive regimens [98]. Thus, controlling the autoreactive memory T cell response remains a therapeutic challenge.

1.3.2 Pancreas transplantation

Pancreas transplantation procedures have been performed since the 1960s. Over the past six decades, advancements in surgical techniques and the development of more effective immunosuppressive agents, in combination with careful patient selection, have yielded significant improvements in the outcomes of pancreas transplantation. The 5-year survival for patients is now 88%, and graft survival has reached 71%. Moreover, reports indicate that actual insulin independence rates at 10-year exceed 80% in SPK transplantation [115, 116]. Pancreas transplantation provides extended and stable control of blood glucose levels over long term, limiting wide glycemia fluctuations and offering the ability to halt or even reverse secondary microvascular complications associated with diabetes, such as retinopathy, nephropathy, and neuropathy [117].

There are three types of whole-pancreas transplantation: (1) Simultaneous pancreas-kidney transplant (SPK), (2) Pancreas after kidney transplant (PAK), (3) Pancreas transplant alone (PTA), and are summarized in **Figure 2**. Among these, SPK accounts for the majority of pancreas transplants (nearly 70% of cases) and is usually performed in patients with ESRD, as a consequence of pancreatic nephropathy [118].

The PTA is typically proposed to patients without significant renal disease but with life-threatening complications of diabetes, such as hypoglycemic unawareness or uncontrolled body mass index, whilst PAK transplantation is performed following a living donor kidney transplantation. The latter two types of transplants account for only 10% and 20%, respectively, of the total pancreas transplantations [118].

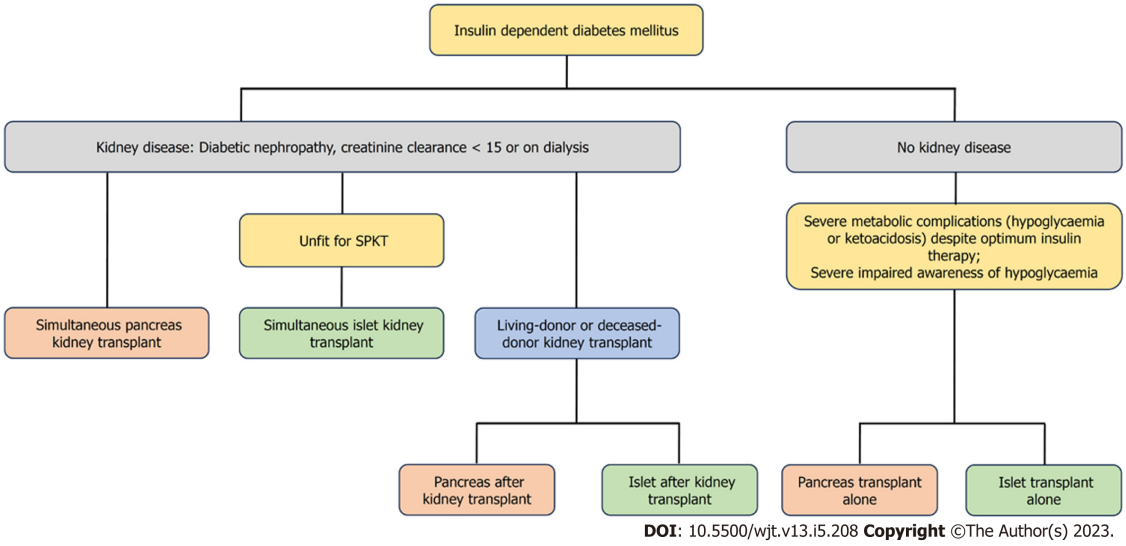


Figure 2. Overview of therapeutic options for T1D patients receiving intensive insulin therapy. *Modified by L Nagendra, CJ Fernandez and JM Pappachan [119]*

The ideal candidate for pancreas transplantation is a patient with T1D with no detectable C-peptide, poor metabolic control, and/or progressive secondary complications of diabetes. Another requirement is the failure of conventional therapy, the exogenous insulin administration, so usually most recipients have a 20- to 25-year history of diabetes [120]. The pancreas is recovered from a brain-dead donor, whose heart is beating, or from a controlled cardiac death deceased donor. In this category of donors, the process involves the waiting for cardiac arrest to occur following the withdrawal of ventilatory support in patients with fatal brain injuries who are not expected to progress to brain death [121].

In the surgical procedure, the donor pancreas with a small segment of the donor's small intestine is connected to a segment of the recipient's small intestine or the bladder. When a SPK transplant is performed, the kidney graft is implanted in the recipient’s left iliac vessels and is extraperitoneal, to facilitate biopsy. The primary differences in pancreas transplantation techniques regard two key aspects: the site for venous drainage, which can be either systemic or portal, and the site for exocrine drainage, which can be either urinary or enteric. In cases of enterically drained grafts, additional significant variations include the choice between a Roux-en-Y isolated loop or establishing a direct anastomosis between the donor duodenum and the recipient small bowel [122], duodenum [123] or stomach [124]. In all modifications, the recipient’s native pancreas is left untouched unless it is causing complications.

Several studies comparing enteric and bladder drainage methods have shown that, in contrast to enteric drainage, bladder drainage does not lead to an increase in early surgical complications. However, it carried higher rates of late reintervention, mostly for cases necessitating a conversion to an enteric drainage method [125, 126].

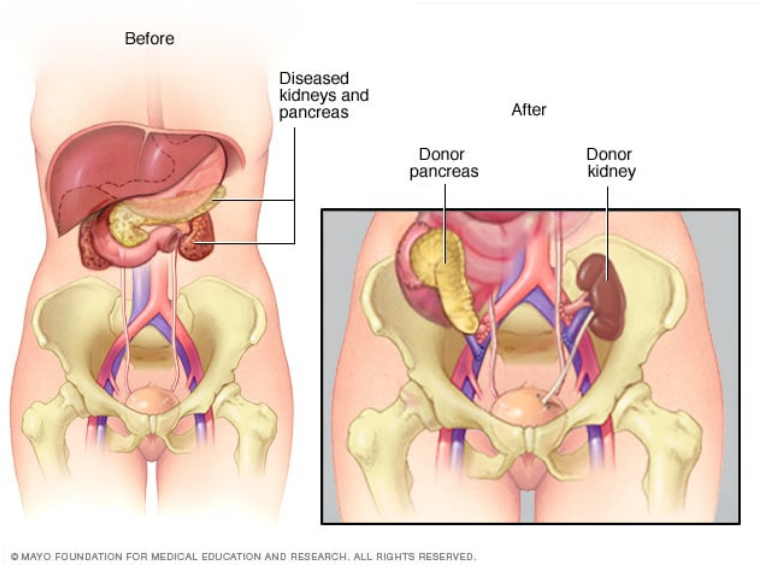


Figure 3. Graphical representation of pancreas and kidney transplant procedure.

1.3.3 Islet transplantation

Compared to whole-organ transplantation, islet transplantation is associated with lower surgical risks and is highly effective at eliminating severe hypoglycemia and glucose lability. However, the insulin independence rate at 10-year is only 28% [127]. The selection of the optimal therapeutic option between whole-pancreas or islet transplant is influenced by patient characteristics, clinical accessibility and the goals of transplantation.

The criteria for considering islet transplantation instead of whole-pancreas transplantation in patients requiring kidney replacement are well-established and include the following scenarios: (i) the recipient is dealing with significant cardiovascular disease; (ii) SPK transplantation is considered too risky; (iii) there is a risk of re-transplantation in the event of pancreas loss following an SPK; (iv) the surgical incompatibility of a pancreas from a kidney donor [128]. The main criterion of patients' eligibility for islet transplantation is severe hypoglycemia, patients with this condition have a 3.4-fold increased risk of death at five years [129].

The surgical procedure for islet transplantation is an intraportal infusion that can be performed either through surgical catheterization of a small mesenteric vein via mini-laparotomy or percutaneous catheterization of a peripheral portal branch under radiological guidance. The latter is considered the safest procedural method, with a low complication rate [130].

Advances in the techniques over the years, with the development of the "Edmonton Protocol" for islet preparation and post-transplant treatment, have allowed to achieve insulin independence rates up to 60–90% [130, 131]. In 2000, Shapiro et al. [132] from the University of Alberta, Edmonton, published the result of 7 consecutive subjects treated with glucocorticoid-free immunosuppressive therapy (rapamycin, low-dose FK506 and anti-IL-2R antibody, as induction therapy) combined with multiple islets infusions from two or more pancreases from deceased donors. All these patients achieved insulin independence with excellent metabolic control.

Although the Edmonton Protocol showed less nephrotoxic effect than standard cyclosporine and higher-dose FK506-based regimens, even low-dose FK506 should be

used with caution in the face of significant impairment in baseline renal reserve [133]. The life-long immunosuppression, required to prevent islet rejection, has nephrotoxic effects and is associated with a higher risk of opportunistic infections [134].
Many efforts have been made to induce islet transplantation tolerance without the need for systemic immunosuppression. The establishment of mixed chimerism through bone marrow transplantation is widely recognized as a robust method for inducing tolerance. However, the clinical application of this method has been hindered due to the associated toxicities related to recipient preconditioning and the risk of graft-versus-host disease (GvHD) [135-138].

In the setting of islet transplants more than one pancreas donor is required to reach insulin independence and the number of cadaveric donors of pancreases or islets is not sufficient, thus there is the need for a renewable source of islets. Porcine islet xenotransplantation and stem cell- derived islet transplantation have been explored as therapeutic strategies to address the issue of donors shortage. Trials in non-human primate has demonstrated that porcine islets xenografts can lead to insulin independence and long-lasting islet graft function, efficiently reverting diabetes. However, xenotransplantation poses a significant immunological challenge due to the risk of immune response against pig-specific antigens expressed by islets [139].
As for stem cell-derived islets, the company Vertex, has recently presented initial data from two ongoing clinical trials in humans. One trial involves the transplantation of embryonic stem cell (ESC)-derived islet along with functional beta cells into the portal circulation with immunosuppressive therapy. Results showed a reduction in glycated hemoglobin (HbA1c) and an increase in C-peptide, accompanied by a decrease in the required daily insulin dose. The other trial involves pancreatic endoderm cells (PEC) encapsulated into a device developed for subcutaneous placement. PEC-cells are programmed to mature into insulin-producing islet cells and the device is designed to enable the engrafted cells to be directly vascularized. Results showed effective maturation of PEC-cells into matured insulin-producing islet cells and increase in C-peptide levels during 12-24 months post-transplantation. However, in these patients insulin secretion levels were not therapeutic and there were no apparent clinical benefits for those who increased C-peptide levels [140].

1.4 Glucose transporter GLUT1 as a potential target

1.4.1 Overview on T cell metabolism

Several studies have identified memory autoreactive $CD4^+$ and $CD8^+$ T cells as the main barrier to the treatment of islet autoimmunity in T1DR after transplantation:

- Lymphopenia associated with immunosuppression and T cell depletion may trigger a paradoxical expansion of lymphocyte subsets. This response is driven by the phenomenon of homeostatic proliferation, which aims to restore the depleted lymphocyte populations and maintain immune system equilibrium [109];
- GAD65-specific $CD4^+$ T cells from patients with autoimmunity recurrence directly mediated beta cell damage in *in vivo* transplantation immunodeficient mouse model [98];
- GAD65-reactive $CD8^+$ T cells were enriched up to 34-fold in T1D patients and expressed markers of memory cells (CD45RO) [109];
- The presence of autoreactive $CD8^+$ T cells pre-transplant was associated with graft failure in islet transplant recipients treated with anti-thymocyte globulin (ATG), FK506 and mycophenolate mofetil (MMF) therapy [141].

These findings have led diabetes research to investigate cellular and molecular pathways involved in the activation of autoreactive T cells that can be targeted to improve the control of autoimmunity.

Memory T cells exhibit an increased mitochondrial cell mass and spare respiratory capacity compared to naïve T cells. Once activated, T cells shift their metabolism towards glycolysis. This enhanced mitochondrial capacity enables memory T cells to rapidly activate their metabolism upon re-exposure to antigens [141]. The metabolic differences between resting and activated T cells present an opportunity to develop therapeutic strategies that specifically affect the metabolism of activated T cells without impacting the resting T cell population. Targeting metabolic pathways to modulate T cell responses has become a prominent area of research.

1.4.2 Immunometabolism-based therapies

The modulation of the metabolism of immune cells implicated in autoimmune diseases is emerging as an area of strong interest.

In 2016 Garyu et al. explored the small molecule 2-deoxy-D-glucose (2DG), a glycolysis inhibitor, as a metabolic blocker in NOD mice [142]. In this mouse model, a subset of $CD8^+$ T cells, which are specific to a peptide derived from the diabetes antigen IGRP (NRPV7-reactive), exhibit features resembling those of activated memory T cells. These features include elevated glycolytic rates and active oxidative phosphorylation. When these mice were treated with 2DG, there was a reduction in the frequency of IGRP-specific activated T cells, a decrease in immune cell infiltration in the pancreatic islets, and an enhancement in the granularity of beta cells, which is beneficial for diabetes management.

Another molecule, PFK15, a competitive inhibitor of the rate-limiting enzyme 6-phosphofructo-2-kinase (PFKFB3), suppresses the utilization of glycolysis in $CD4^+$ T cells. Studies in NOD mice demonstrated that treatment with PFK15 can delay the onset of T1D, by inducing metabolic and functional exhaustion of T cells. Essentially, the inhibition of glycolysis in $CD4^+$ T cells by PFK15 leads to a state of metabolic exhaustion, being a potential therapeutic strategy for managing islet autoimmunity [143].

Metformin, a drug that is widely used to treat Type 2 Diabetes (T2D), acts as an inhibitor of the electron transport chain and has shown promise in attenuating clinical symptoms in a mouse model of experimental autoimmune encephalomyelitis (EAE) [144]. Metformin treatment was associated with a decrease in pathogenic Th17 cells and an increase in $Foxp3^+$ regulatory T cells that secrete TGF-β and IL-10.

Although the field of immunotherapy based on metabolic targeting is relatively new, a growing body of evidence suggests that efficient control of autoimmunity can be achieved by selectively modulating specific metabolic pathways in memory and effector T cells. These findings offer promising insights for developing novel therapeutic strategies to regulate immune responses and manage autoimmune disorders.

1.4.3 GLUT1 receptor

In order to initiate glycolysis, cells must first uptake glucose from their surroundings. Glucose transport across cell membranes is facilitated by specific transport proteins called glucose transporters (GLUTs). The GLUT family consists of 14 different proteins that regulate the movement of glucose by concentration gradients, making the transport process bi-directional.

One prominent member of the GLUT family is GLUT1, which is found in nearly all mammalian cells. It exhibits a Michaelis-Menten constant (Km) of approximately 3mM. This value is lower than the normal blood glucose level, which is 5.5mM, resulting in a continuous transport of glucose into cells at a relatively constant rate.

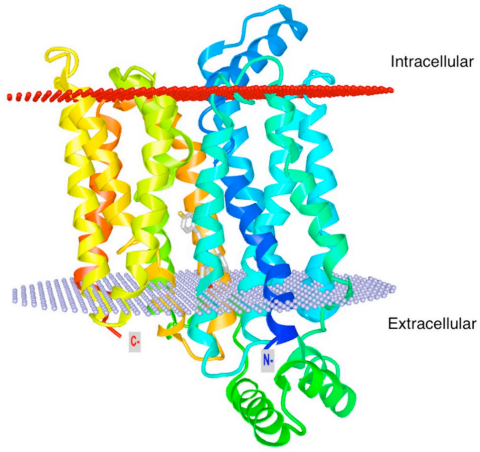


Figure 4. *Predicted conformation of the crystal structure of GLUT1 (PDB: 4PYP; https://www.rcsb.org/structure/4PYP) C Di Dedda, D Vignali, L Piemonti and P Monti [145]*

GLUT1 is encoded by the *Slc2a1* gene on chromosome 1 and consists of a sugar-binding pocket that faces the outer side of the cell in its outward open conformation (**Fig. 4**). When glucose molecules bind to this pocket, GLUT1 undergoes a conformational change, shifting towards the cytoplasmic side of the cell and releasing glucose molecules inside the cell. T cells express various members of the glucose transporter family, including GLUT1, GLUT2, GLUT3, GLUT6, and GLUT8. Despite this diverse range of expression, the conditional deletion of the *Slc2a1* gene has revealed that GLUT1 plays a non-redundant role in the expansion of activated effector T cells. The group of Macintyre demonstrated that in the absence of GLUT1, activated effector T cells experience

significant impairment in their expansion process, highlighting the critical reliance of these cells on GLUT1-mediated glucose uptake for their metabolic needs [146].

GLUT1 and GLUT3 have similar affinities for glucose [147] but play different roles in T cell metabolism and function. GLUT1 is selectively essential for $CD4^+$ T cell activation and effector function, while $CD8^+$ T cells preferentially increase GLUT3 and GLUT2 proteins levels over GLUT1, under specific conditions. Conditional deletion of GLUT3 in $CD4^+$ T cells has been shown to reduce lactate production in Th17 cells, this was accompanied by a significant reduction of IL-17, IL-2 and GM-CSF, while IFNγ production remained unaffected. The deletion of GLUT3 in a mouse model of EAE showed a dramatic attenuation of the disease, highlighting the critical role of GLUT3 in Th17 cell- mediate autoimmune responses [148, 149].

Like GLUT4 and GLUT2, the localization of GLUT1 on the cell surface is controlled by external signals, such as glucose and oxygen availability and extracellular pH [150]. Activation of T cells through TCR signaling, combined with co-stimulation via CD28 engagement, along with the autocrine production of the cytokine IL-2 and the up-regulation of CD25, triggers the GLUT1 translocation to the cell surface from a cytoplasmic pool. This process is mediated by the activation of the phosphoinositide 3-kinase (PI3K)-Akt pathway [151]. Additionally, the homeostatic cytokine IL-7 can also induce GLUT1 translocation in resting T cells, independent of antigenic or co-stimulatory signals, maintaining basal levels of the transporter expression necessary for T cell survival [152].

The trafficking of GLUT1 to the cell membrane is facilitated by gamma chain (γc) signaling: when IL-7 binds its high-affinity receptor, IL-7Rα and γc, the intracellular domains of these chains activate the tyrosine kinases Janus kinase 1 (JAK1) and JAK3, which phosphorylate each other and the intracellular domain of IL-7Rα, allowing the recruitment of the signaling molecule signal transducer and activator of transcription 5 (STAT5). Phosphorylated STAT5 dimerizes, activates Akt and translocates to the nucleus, where it promotes the transcription of the gene encoding GLUT1 [153].

This intricate regulatory network involving TCR signaling, co-stimulation, cytokine production, and downstream signaling pathways (**Fig. 5**) ensures the increasing of GLUT1 surface expression and consequent uptake of glucose by activated T cells, supporting their metabolic demands during immune responses.

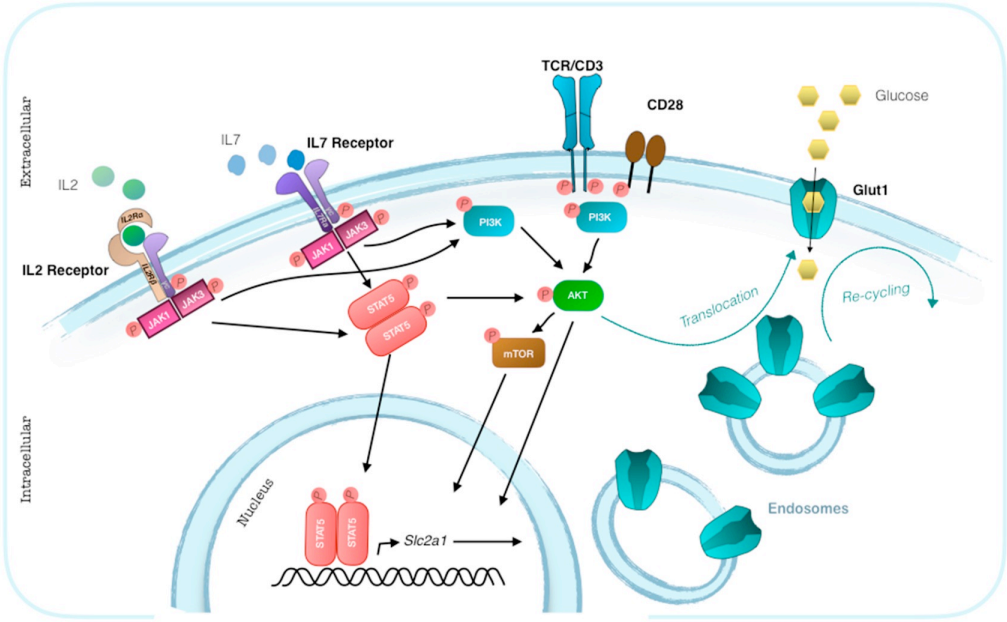


Figure 5. Signaling transduction cascade that regulates the surface expression of GLUT1 in T cells*. C Di Dedda, D Vignali, L Piemonti and P Monti [145]*

1.4.4 GLUT1 inhibitors

Over the years, several natural or synthetic inhibitors of GLUT1 have been developed, three of them have gained attention in recent years: STF-31, BAY876 and WZB117. These drugs have been developed in the context of tumors due to their ability to inhibit glycolysis. Cancer cells often exhibit increased glycolytic activity as part of the Warburg effect, which provides them with metabolic advantages for rapid growth and survival. The Warburg effect, described in the 1920s by Otto Warburg, is a metabolic alteration characterized by the increased reliance of cancer cells on glycolysis for energy production, even under normal oxygen levels. GLUT1 upregulation in tumors is associated with more aggressive phenotypes and poor survival [154].

STF-31 was initially described by Chan et al. [155] as a specific inhibitor of GLUT1 and was reported to inhibit glucose uptake in von Hippel-Lindau (VHL)-deficient renal carcinoma cells, causing necrosis. Inactivation of VHL led to increased expression of the hypoxia-inducible factor (HIF), a transcription factor that induce the transcription of glucose metabolism genes, including the GLUT1 gene. Normal kidney cells, which do not strictly depend on glycolysis and utilize GLUT2 for glucose uptake, are not sensitive to the toxic effects of STF-31[156]. STF-31 has also been demonstrated to quell glycolysis in human T cells overexpressing GLUT1, by inhibiting GLUT1-dependent glucose uptake [157]. *In vivo* studies showed the ability of STF-31 to reduce microglial activation in a mouse model of light-induced retinal degeneration, without affecting normal mice's body weight, behavior, and the electrical responses of the retina [158].
A study aimed to evaluate the impact of STF-31 and WZB117, along with other GLUTs inhibitors on glucose uptake, tumor cell proliferation and survival, demonstrated that STF-31 affected tumor cell metabolism at a relatively lower concentration than WZB117, but while WZB117 was well tolerated at a concentration of up to 10μM by all tumor cells tested, STF-31 showed a critical cytotoxic effect already at a concentration of 1μM [156].

The small molecule WZB117 reversibly binds the sugar-binding site of GLUT1 on the cell surface [159], and it induces cell death in multiple solid tumors while sparing normal cells [160, 161]. Other studies have demonstrated that WZB117 in combination therapy with other anticancer drugs may represent a promising option for further development in the context of antitumor therapies [162]. In combination with apatinib, an inhibitor of the VEGFR2/Akt1 pathway, WZB117 synergistically suppressed the migration, invasion and proliferation and induces apoptosis of melanoma cells by blocking the STAT3/PKM2 axis [163]. In combination with imatinib, a tyrosine kinase inhibitor, WZB117 synergistically inhibited growth and induced apoptosis in imatinib-resistant cells [161].

The favorable toxicity profile of WZB117, given its minimal toxicity on normal cells, makes it an interesting candidate for development as a therapeutic agent. We previously showed that WZB117, at a concentration of 10μM, induced a 75% inhibition of the uptake of 2NBDG (a fluorescent glucose analog) and strongly suppressed Tscm division without

causing significant apoptosis [97]. Moreover, WZB117 effectively prevented the differentiation of autoreactive Tscm from naïve T cell precursors.

BAY876 has recently emerged as a promising molecule. It is an orally bioavailable, highly selective GLUT1 inhibitor characterized by an IC_{50} of 2nM and showed good metabolic stability *in vitro* [164, 165]. Originally developed as an anti-cancer drug, BAY876 has been the subject of expanded studies involving immune cells, including T cells and macrophages. The investigation of the effect of BAY876 on activated $CD4^+$ T cells and macrophages *in vitro* has shown that it significantly reduces the secretion of pro-inflammatory cytokines IFN-γ and TNF, downregulates the glucose consumption and decreases the cell proliferation, without affecting cell viability [166]. Recently, a novel microcrystalline BAY876 formulation has been developed and studied for hepatocellular carcinoma (HCC). To overcome the challenges associated with oral administration of the drug, this new formulation is injected directly into the tumor tissue and is slowly released, remaining detectable for long time. While the concentration of BAY876 in tumor tissue was no longer detectable at 3 days following the oral administration of the drug solution, the microcrystalline injection assured 12 days of BAY876 release in the tumor tissue [167].

The last year, a new small molecule, SMI277, has been investigated *in vitro* and *in vivo* in the context of tumors. SMI277 showed desirable effects when used against different cancer cell lines: it reduced cell proliferation and tumor cell colony formation, at a concentration of 25μM, and this was associated with a significant decrease in lactate release. The molecule also reduced the tumor growth in a C57BL/6 mouse xenograft tumor model by 50% without affecting the heart, liver, spleen, lung and kidney of mice or their body weight [168].

Chapter 2

AIM OF THE WORK

AIM OF THE WORK

Replacement of lost beta cell mass, through whole pancreas or islet transplantation, represents a therapeutic option for patients with severe T1D. However, its long-term success is limited by the host immune response. Broadly speaking, the donor's beta cells or other cells in a solid organ graft, express both allogeneic major and minor histocompatibility antigens, which are the conventional targets for the recipient's immune response in the context of organ transplantation. Moreover, the donor's pancreatic beta cells also express a complete array of autoantigens targeted by islet autoimmunity. Beta cell replacement in a patient with preexisting autoimmunity is essentially a double immunological challenge, where two immune responses - transplant rejection and tissue-specific immunity - coexist. The possible reactivation of autoreactive effector memory cells poses an additional obstacle; while allo-rejection can be efficiently controlled by immunosuppressive regimens, long-lived autoreactive memory T cells are less susceptible to immunosuppression. The need for novel immune-modulatory approaches that effectively target autoreactive T cell clones has prompted us to explore GLUT1 inhibitors. GLUT1 is primarily involved in the uptake of glucose to fuel the glycolytic pathway and plays a fundamental and non-redundant role in T cell activation.

Based on these premises, I investigated pharmacological GLUT1 blockade, as a potential strategy to control T cell activation and hence autoimmune response. I focused my work on a small molecule that acts as a GLUT1 inhibitor, named WZB117.
The specific aims of this thesis were:

i) to determine the dynamic of GLUT1 expression *ex-vivo* in immune cell subsets associated with recurrence of T1D in SPK patients, and investigate their phenotypes.
ii) to examine the impact of WZB117 *in vitro* on T cell activation, proliferation, apoptosis and phenotype in $CD4^+$ and $CD8^+$ T cells;

A thorough understanding of mechanisms that are involved in the recurrence of T1D in transplanted patients is expected to define molecular and cellular targets of GLUT1 blockade, to open new avenues for utilizing WZB117 as a therapeutic intervention.

Chapter 3

RESULTS

3.1 Determining immune subsets and phenotypic features associated with recurrence of T1D in SPK patients

This part of the study was performed in the laboratory of Prof. Pugliese at the Arthur Riggs Diabetes and Metabolism Research Institute, City of Hope, Duarte California. Samples were again provided by Prof. George W. Burke III from the University of Miami.

3.1.1 Ex-vivo analysis of $CD4^+$, $CD8^+$ and Tregs and their subpopulations among HD, T1D and SPK patients

This part of my thesis focused on the study of peripheral blood samples from SPK recipients that have been collected over several years by Dr. Pugliese and Dr. Burke. Besides the SPK recipients, who are autoantibody-negative (SPK AAB-Neg), which we also studied in the *in vitro* experiments, the cohort included SPK recipients who experienced autoantibody conversions on follow-up (SPK AAB-Conv) but have remained normoglycemic and SPK recipients who were diagnosed with T1D recurrence (T1DR, 8 out of 9 patients following autoantibody conversion). All SPK patients were chronically immunosuppressed.

Groups	n Samples	Mean Age	SD	Sex
Healthy donors	10	50	9	10 males
Type 1 Diabetes*	11	13.6	2.4	7 males, 4 females
SPK AAb-Neg	15	48.4	10	9 males, 6 females
SPK AAb-Conv	10	50.1	11.4	4 males, 6 females
T1DR	9	49.2	8.8	7 males, 2 females

**For T1D patients the mean disease duration was 4 ± SD 2.87 years; the range was 0.6-8 years.*

Table 3. *Main demographic features of studied subjects*

Overall, I studied samples from 55 individuals, examining a wide range of 22 immune cell populations using a 37-parameter flow cytometry panel (**Table 4**).

1	SSC	
2	FSC	
	Surface markers	Fluorophores
3	**Live/Dead**	Zombie UV
4	**Cell Proliferation**	CellTrace™ Violet
5	**CD122**	BV786
6	**CD127 (IL-7Ra)**	BV421
7	**CD183 (CXCR3)**	BUV496
8	**CD194 (CCR4)**	PE-Fire810
9	**CD195 (CCR5)**	FITC
10	**CD196 (CCR6)**	BV510
11	**CD197 (CCR7)**	BV711
12	**CD223 (LAG-3)**	PE-Dazzle 594
13	**CD25**	BUV737
14	**CD27**	BV570
15	**CD278 (ICOS)**	BUV563
16	**CD279 (PD1)**	APC-Fire 750
17	**CD28**	BUV395
18	**CD3**	APC-Fire 810
19	**CD38**	PE-Fire 700
20	**CD4**	Spark Blue™ 574
21	**CD45RA**	PerCP
22	**CD62L**	Spark NIR™ 685
23	**CD69**	BUV805
24	**CD73**	BUV661
25	**CD8**	AlexaFluor532
26	**CD95 (FAS)**	BV650
27	**GLUT-1**	PE-Cy7
28	**HLA-DR**	BV605
29	**TIGIT**	BUV615
30	**CD272 (BTLA)**	BV750
31	**CD98 (LAT1)**	APC
32	**CD39**	BV750
	Intracellular markers	Fluorophores
33	**CD152 (CTLA-4)**	PE-Cy5
34	**Ki-67**	BV480
35	**GLUT-3**	Alexa Fluor 700
36	**HELIOS**	FITC
37	**FoxP3**	PE

Table 4. *37-parameters flow cytometry panel used for the functional phenotyping of T cells.*

Table 5 describes the combinations of markers used in flow cytometry for the identification of all main immune cell populations and their most important subsets among the 5 groups: HD, T1D, SPK-Neg, SPK-Conv and T1DR.

Population	Identification markers
CD3+ T cells	CD3+
CD4+ T cells	CD3+ CD4+
CD8+ T cells	CD3+CD8+
CD4+ T naïve cells	CD3+CD4+CD45RA+CD62L+
CD4+ Tscm cells	CD3+CD4+CD45RA+CD62L+CD95+
CD4+ Tcm cells	CD3+CD4+CD45RA-CD62L+
CD4+ Tem cells	CD3+CD4+CD45RA-CD62L-
CD4+ Temra cells	CD3+CD4+CD45RA+CD62L-
CD8+ T naïve cells	CD3+CD8+CD45RA+CD62L+
CD8+ Tscm cells	CD3+CD8+CD45RA+CD62L+ CD95+
CD8+ Tcm cells	CD3+CD8+CD45RA-CD62L+
CD8+ Tem cells	CD3+CD8+CD45RA-CD62L-
CD8+ Temra cells	CD3+CD4+CD45RA+CD62L-
CD4+ Th1 cells	CD3+CD8+CCR4-CCR6-
CD4+ Th2 cells	CD3+CD4+CCR4+CCR6-
CD4+ Th17 cells	CD3+CD4+CCR4+CCR6+
CD4+ Tfh cells	CD3+CD4+CCR4-CCR6-CCR5+PD-1+
CD4+ Treg	CD3+CD4+CD127^{lo}CD25^{hi} FoxP3+ Helios+
CD4+ Treg naive	CD3+CD4+CD127^{lo}CD25^{hi} FoxP3+ Helios+ CD45RA+CD62L+
CD4+ Treg Tcm	CD3+CD4+CD127^{lo}CD25^{hi} FoxP3+ Helios+ CD45RA-CD62L+
CD4+ Treg Tem	CD3+CD4+CD127^{lo}CD25^{hi} FoxP3+ Helios+ CD45RA-CD62L-
CD4+ Treg Temra	CD3+CD4+CD127^{lo}CD25^{hi} FoxP3+ Helios+ CD45RA+CD62L-

***Table 5**. Marker combinations for the identification of T cell subtypes in flow cytometry.*

My analyses compared cell frequencies (represented as percentage of lymphocytes) of various subsets, as well as the Mean Fluorescence Intensity (MFI) of GLUT1 and other markers within these populations across different groups. I observed overlapping differences in cell frequencies, which were also reflected in the analysis of cell numbers (not shown). As shown in **Figure 6**, T1D and SPK-Conv had a lower frequency of $CD3^+$ T cells compared to HD. SPK-Neg patients had reduced proportions of $CD4^+$ T cells compared to HD. The proportions of $CD8^+$ T cells trended higher in SPK-Neg, followed by SPK-Conv, without reaching significance. No differences in the proportion of Treg cells were noted across groups.

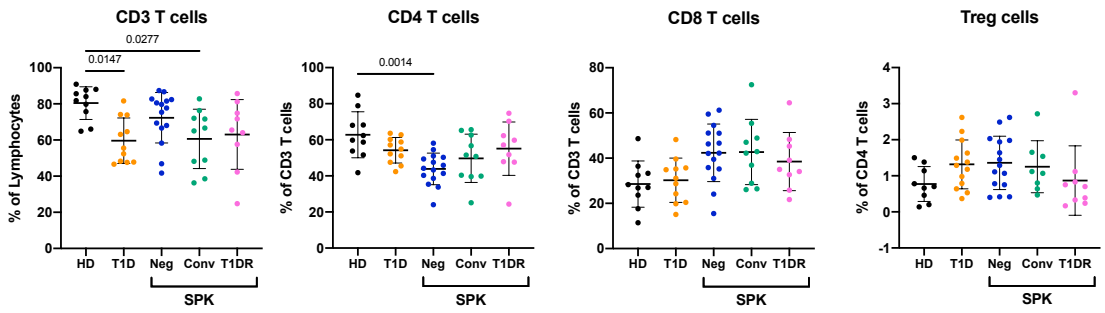


Figure 6. Ex-vivo analysis of major lymphocyte populations in the study cohorts. *Differences in cell frequencies (%) of CD3$^+$, CD4$^+$, CD8$^+$ T cells and CD4$^+$ Treg cells in the different groups. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

Figure 7 compares the frequencies of CD4$^+$ and CD8$^+$ T cell subsets across groups. Among the CD4$^+$ T cells subsets, categorized by the expression of CD45RA and CD62L (**Fig. 7A**), all SPK groups demonstrated higher frequencies of CD4$^+$ Tem cells compared to HD and T1D patients. CD8$^+$ Tem cells were also increased in frequency among SPK-T1DR patients compared to HD, T1D, and importantly compared to SPK-neg patients (**Fig. 7B**). Perhaps the higher frequency of Tem cells is a feature of transplanted patients under chronic immunosuppression is consistent with homeostatic proliferation of Tem cells, most notable in SPK-T1DR patients, which could be enriched in autoreactive T cells.

Conversely, the frequency of CD4$^+$ and CD8$^+$ Tcm cells trended lower among T1D and all SPK patients compared to HD. Interestingly, the frequency of CD4$^+$ TEMRA was reduced among SPK groups, and especially in SPK-T1DR subjects, compared to T1D patients, in whom this was higher than in HD. SPK-Conv patients had a higher frequency of CD8$^+$ Tscm cells compared to HD. There were no significant differences observed for the Th1, Th17 and Tfh populations (**Fig. 7C**). Substantial differences were observed in the Th2 population, with SPK patients showing the highest frequency of CD4$^+$ Th2 CCR4$^+$CCR6$^-$ cells compared to HD and T1D.

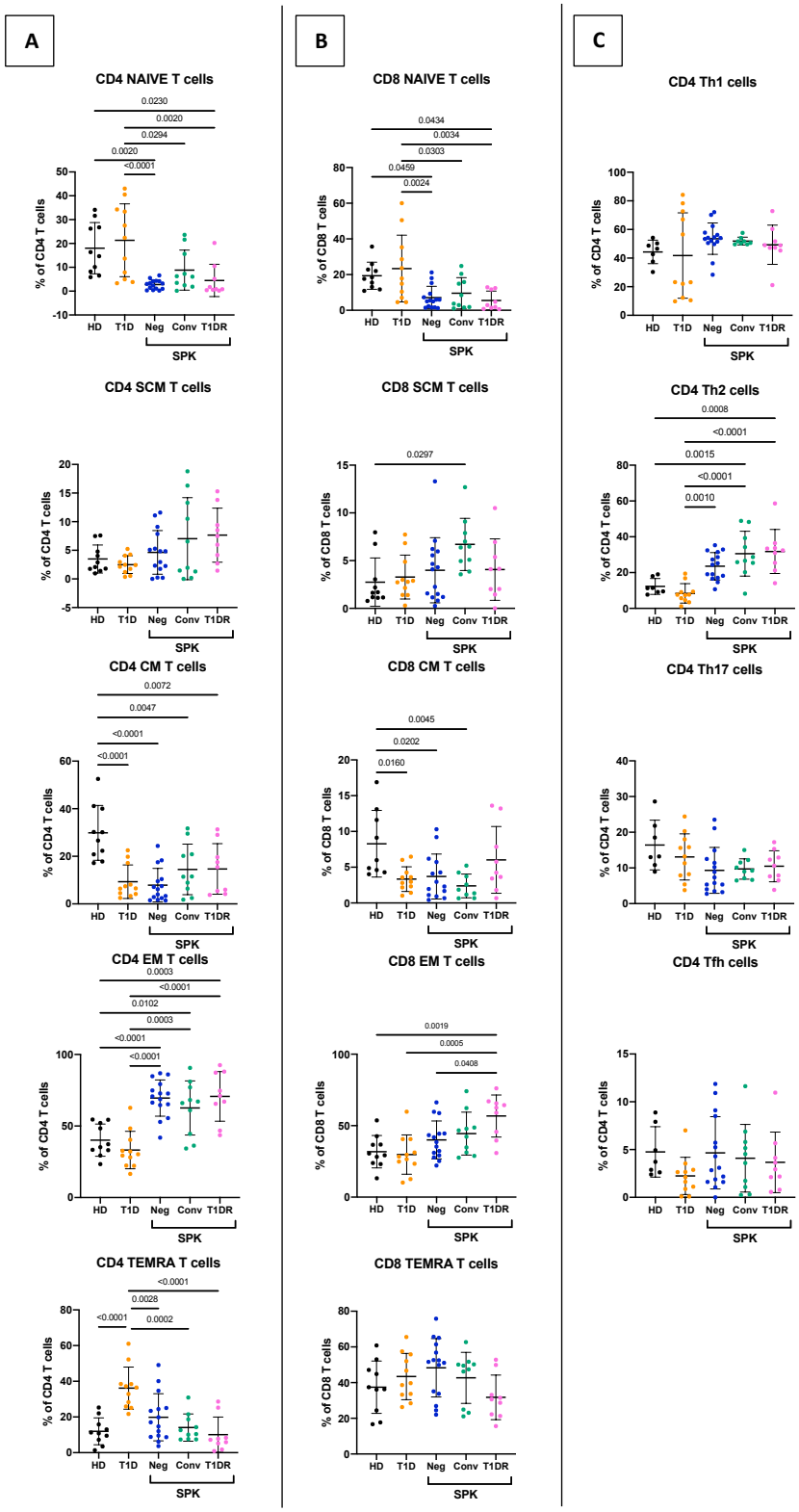


Figure 7. Ex-vivo analysis of CD4$^+$ and CD8$^+$ T cell subsets in the study cohorts. *Differences in cell frequencies (%) among memory and naïve subsets of CD4$^+$ (A) and CD8$^+$ (B)T cells, along with the differences of T helper subpopulations (C) in the different groups. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

3.1.2 Ex-vivo analysis of Treg cells and their subsets among HD, T1D and SPK patients

After evaluating the proportion of the total Treg cells (see **Fig. 6**), identified as $CD4^{+}CD25^{hi}CD127^{lo}Helios^{+}FoxP3^{+}$, I studied their memory/naïve subsets across groups (**Fig. 8**). While the frequencies of Treg cells were similar in the 5 groups, the distribution of Treg memory and naïve subsets varied. Naïve Tregs were higher in T1D group compared to SPK-Neg, HD had increased Treg central memory than T1D and SPK-Neg. The latter showed to have higher effector memory Treg subset compared to HD.

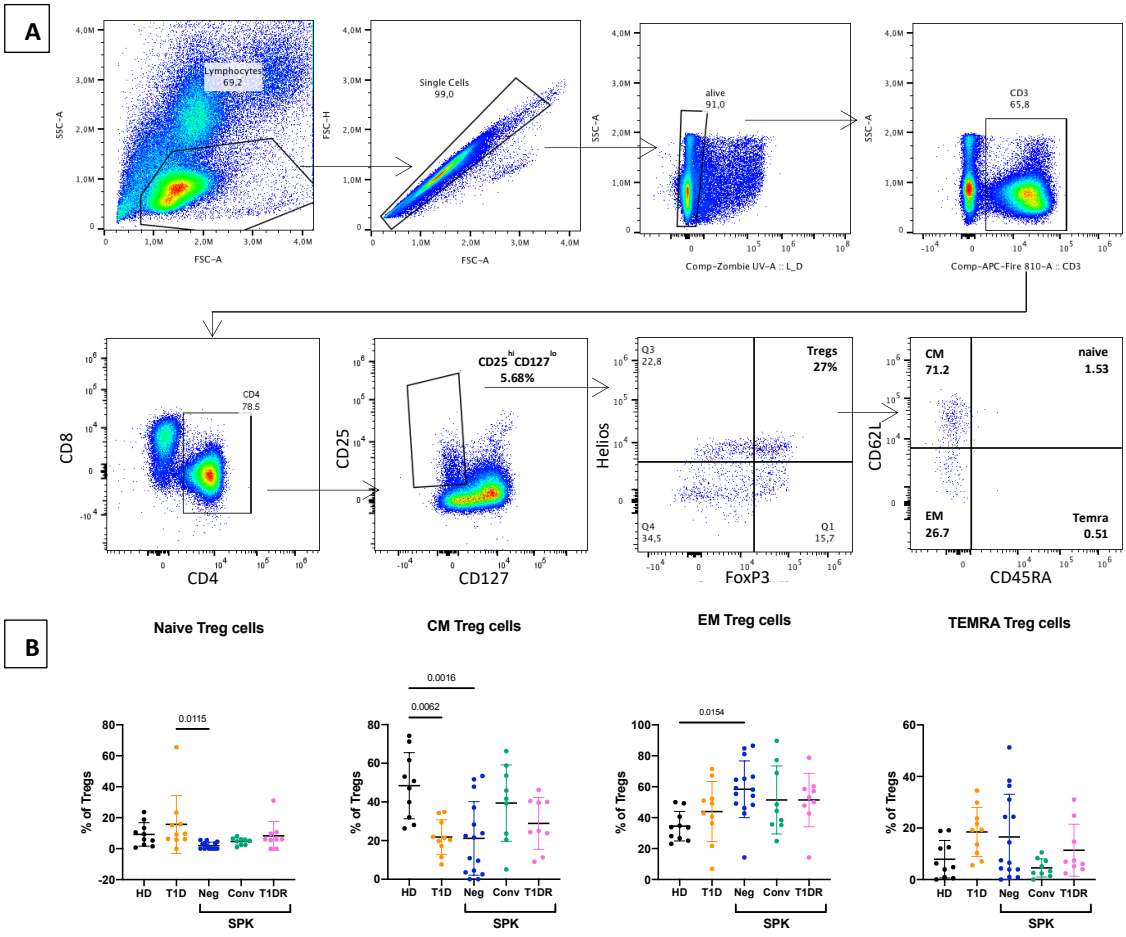


Figure 8. Ex-vivo analysis of $CD4^{+}$ Treg cell subsets in the study cohorts. *(A) Plots show the gating strategy used to identify memory and naïve subsets of Treg cells. The gating strategy is shown by the arrows: the lymphocytes were determined by the forward (FSC) and side scatter (SSC) profile, doublets were discarded and living cells were distinguished from dead cells by plotting SSC vs ZombieUV. $CD4^{+}$ T cells were gated on CD3 positive T cells and a population of $CD25^{hi}$ $CD127^{lo}$ was identified. From this gate, Treg cells were determined by FoxP3 and Helios expression; these can subsequently be divided into naïve and memory Tregs by using CD45RA and CD62L. (B) Differences in cell frequencies (%) of Treg cell subsets in the different groups. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

3.1.3 Phenotypic features of CD4$^+$, CD8$^+$ and Treg cells among HD, T1D and SPK patients

We then examined the expression levels (MFI) of several markers on CD4$^+$, CD8$^+$, and regulatory T cells. Among CD4$^+$ T cells (**Fig. 9**), we noted that both T1D and SPK patients had increased expression levels of some activation markers (HLA-DR, CD73) and chemokine receptors (CCR6, CCR5), compared to HD group. The exhaustion markers, LAG-3 and TIGIT, were also higher in T1D and SPK groups compared to HD, and PD-1 was increased in the SPK-Conv and SPK-Neg compared to HD. Also CTLA-4 and CD122 were increased in SPK-Neg compared to HD. SPK groups exhibited reduced expression levels of CD38 and CD28 compared to T1D or HD. Similar changes were noted for CD38 and CD28 on CD8$^+$ T cells (**Fig. 10**), and CCR7 was also reduced. Increased expression levels in CD8$^+$ T cells of SPK patients were noted for HLA-DR, ICOS, CD122, CD95, CCR5, PD-1 and LAG-3.

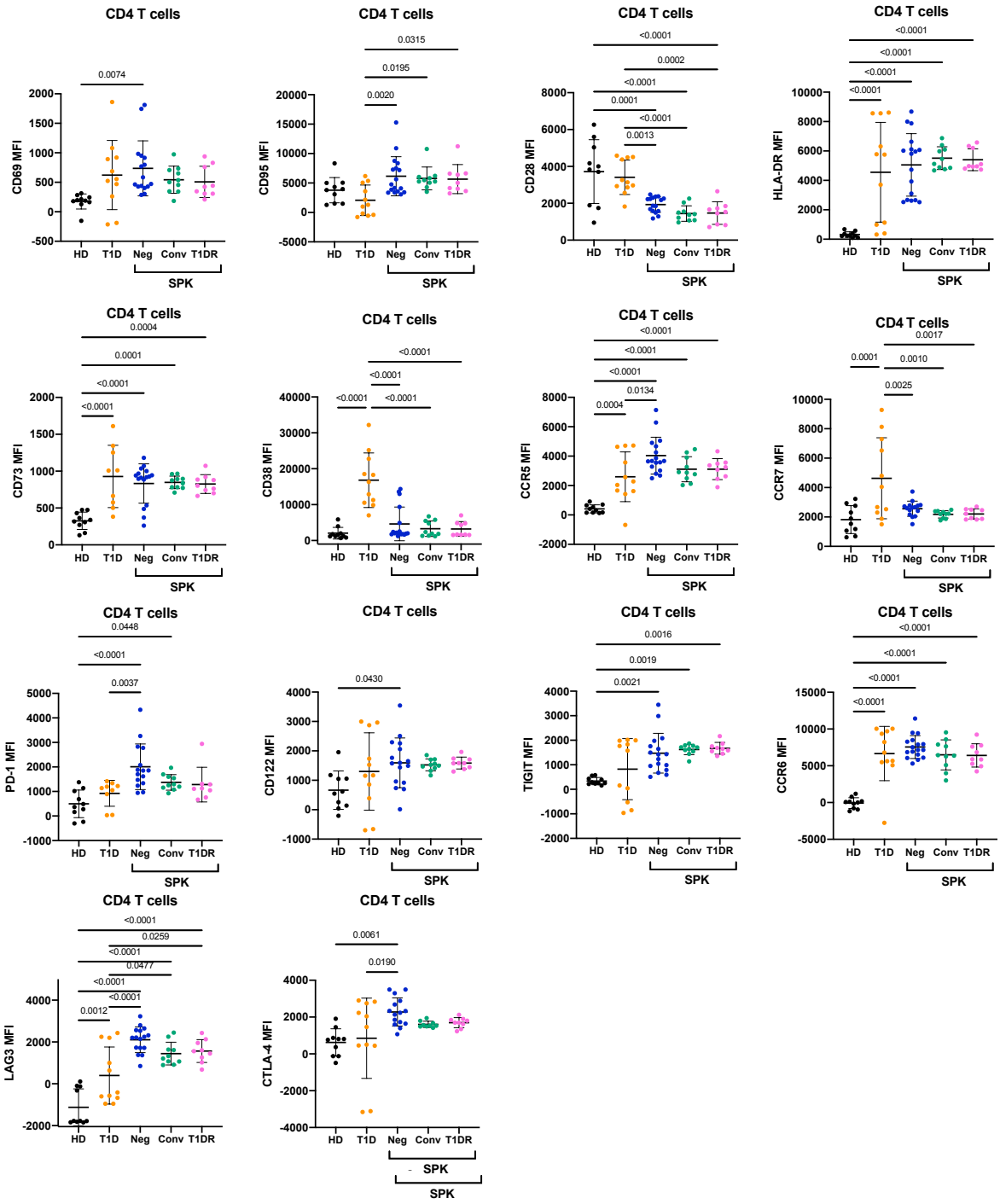


Figure 9. Phenotypic features of CD4⁺ T cells among the different groups. *Flow cytometry assessment of key phenotypic markers showing the most evident changes, from our 37-parameter panel. Only selected markers are shown. MFI of CD4-positive gate. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

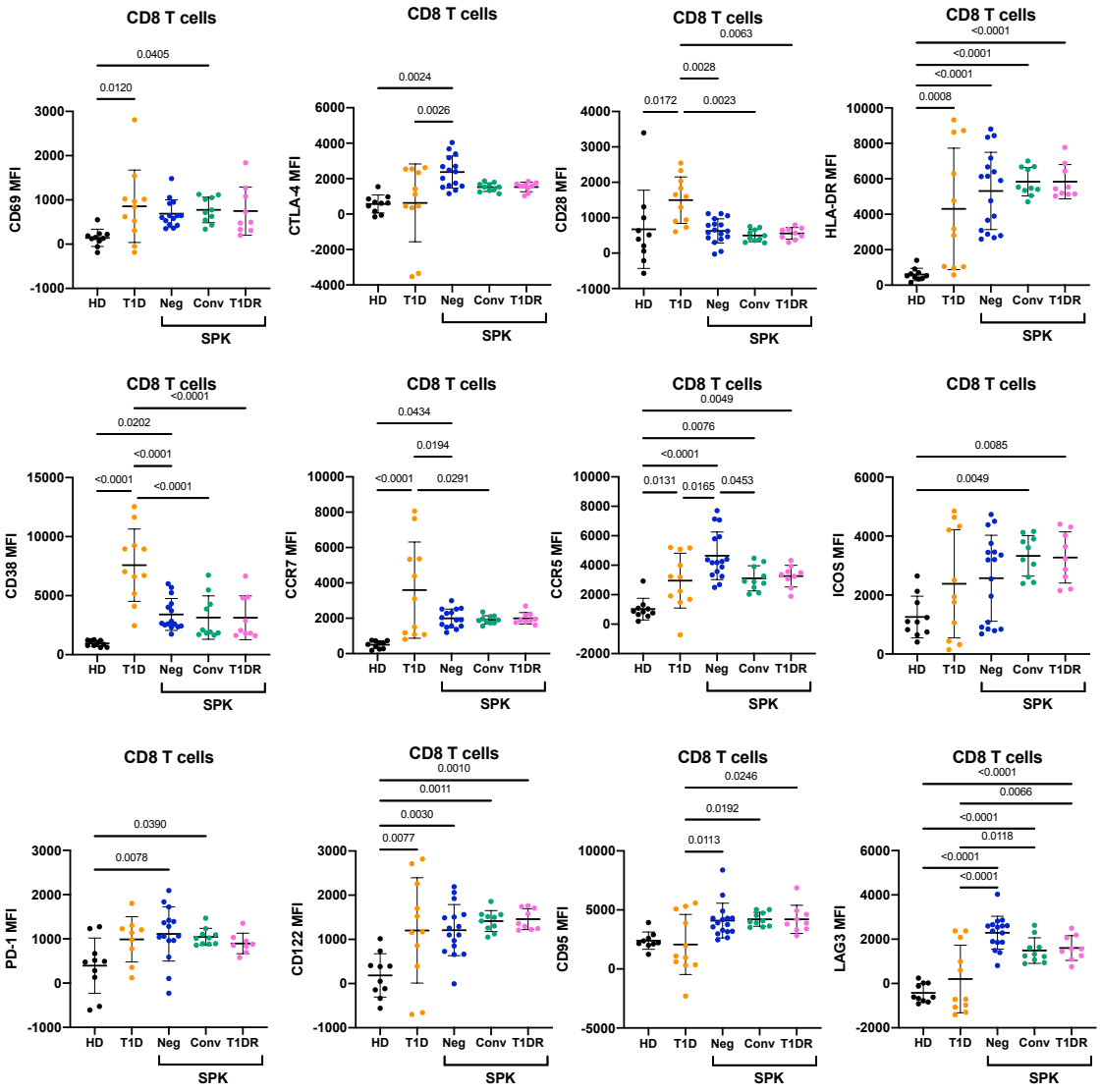


Figure 10. Phenotypic features of CD8$^+$ T cells among the different groups. *Flow cytometry assessment of key phenotypic markers showing the most evident changes, from our 37-parameter panel. Only selected markers are shown. MFI of CD8-positive gate Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

Among CD4$^+$ Treg cells (**Fig. 11**), we observed that SPK groups had increased expression of several molecules compared to T1D patients, including CD95, PD-1, ICOS, CD27, and TIGIT; however, TIGIT levels were lower in SPK groups than in HD subjects. CCR7, LAG-3, and HLA-DR were expressed at higher levels by Treg cells in SPK groups compared to HD. Notably, the SPK groups had lower expression of CD38 compared to HD. The Foxp3 MFI was reduced among T1D and SPK-Neg patients compared to HD,

and trended lower for SPK-Conv and T1DR. SPK groups also had lower Treg CD98 expression compared to T1D patients.

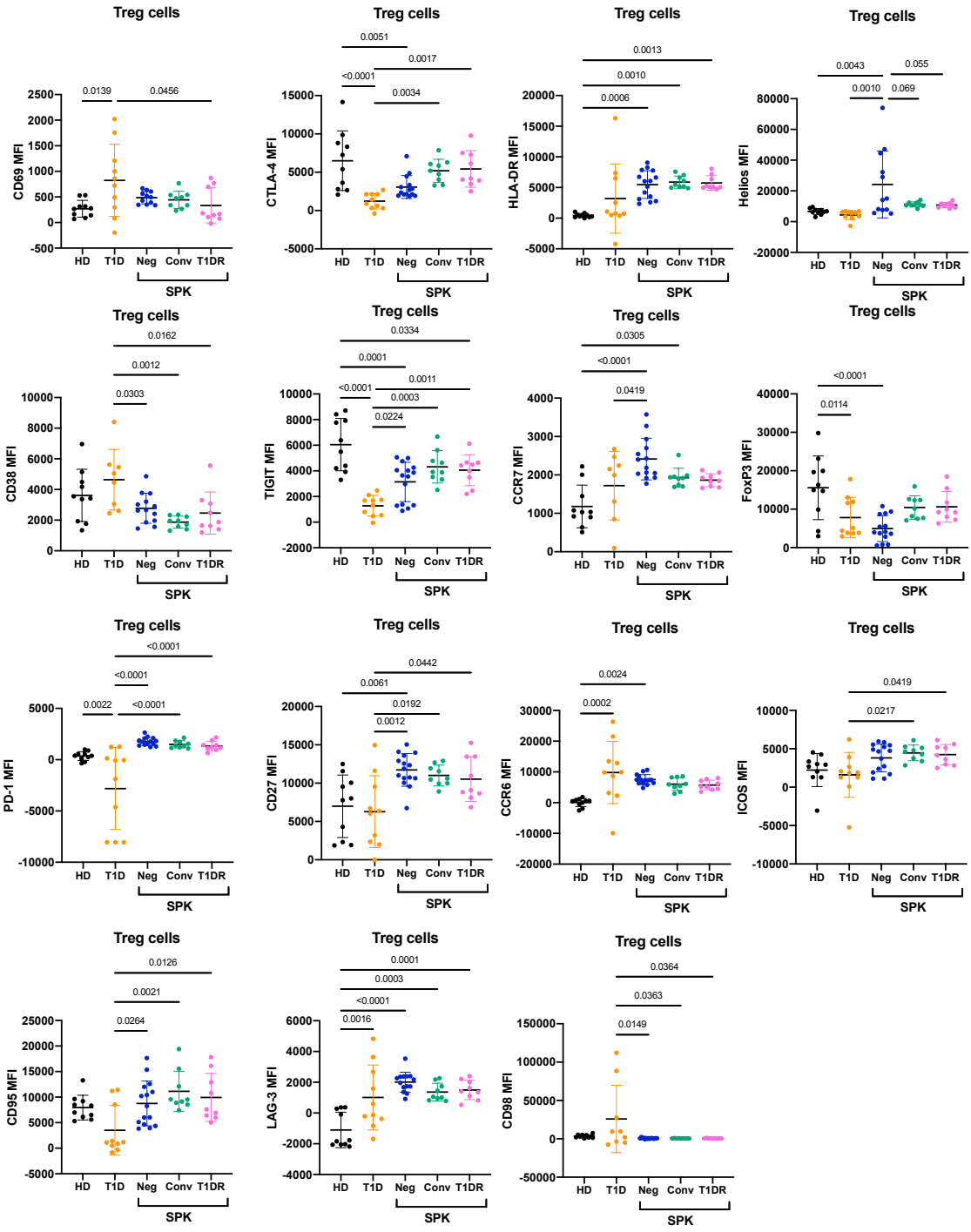


Figure 11. Phenotypic features of CD4⁺ Treg cells among the different groups. *Flow cytometry assessment of key phenotypic markers showing the most evident changes, from our 37-parameter panel. Only selected markers are shown. MFI of Treg-positive gate. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

3.1.4 Assessment of GLUT1 and GLUT3 cell surface expression on CD4⁺, CD8⁺ and Treg cells among HD, T1D and SPK patients

In addition to characterizing the subsets and subpopulations of T cells across the five different groups, I also examined the MFI levels of the glucose transporters GLUT1 and GLUT3. **Figure 12** illustrates these levels for $CD4^+$ and $CD8^+$ T cells, and for $CD4^+$ Treg cells. GLUT1 MFI levels were similar across groups in $CD4^+$ T cells and Treg cells. Among all T cell types, SPK-Neg patients had higher GLUT1 MFI levels than HD subjects (**Fig. 12A**). GLUT3 also distinguished SPK-neg patients (higher MFI) from the other SPK groups and T1D patients, in all three major T cell populations (**Fig. 12B**).

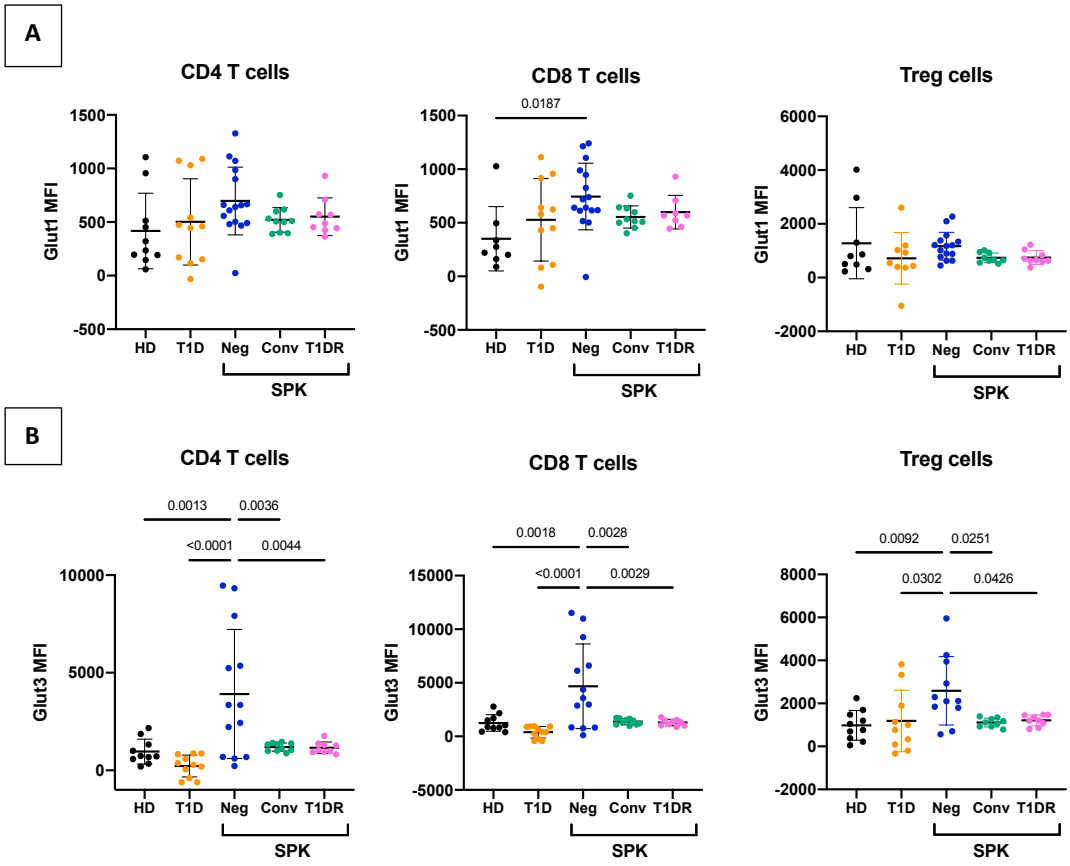


Figure 12. Expression levels of GLUT1 and GLUT3 in major T cell populations among the different groups. *Flow cytometry assessment of the expression levels (MFI) of GLUT1 (A) and GLUT3 (B) among the 5 different groups. MFI of GLUT1 and GLUT3 of CD4, CD8 or Treg-positive gate. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

For further characterization, I evaluated GLUT1 (**Fig. 13**) and GLUT3 (**Fig. 14**) expression in $CD4^+$ and $CD8^+$ subsets, Treg subsets, and T helper subsets. Across groups, GLUT1 levels were broadly similar, but some differences were noted: SPK-Neg patients had higher GLUT1 MFI than HD subjects in $CD4^+$ and $CD8^+$ naïve cells (**Fig. 13A,B**), among $CD4^+$ Temra, and $CD4^+$ Th17 cells (**Fig. 13C**). GLUT1 MFI in $CD4^+$ Th2 cells was higher in T1D, SPK-Neg, and T1DR patients compared to HD subjects.

With respect to GLUT3 expression (**Fig. 14**), I also observed increased MFI levels in SPK-neg patients compared to all the other groups. This was observed for all $CD4^+$, $CD8^+$ and Treg subsets examined. Overall, it appears that GLUT1 and especially GLUT3 levels were on average higher in SPK-neg patients, and that this distinguished this group from the SPK-Conv and SPK-T1DR.

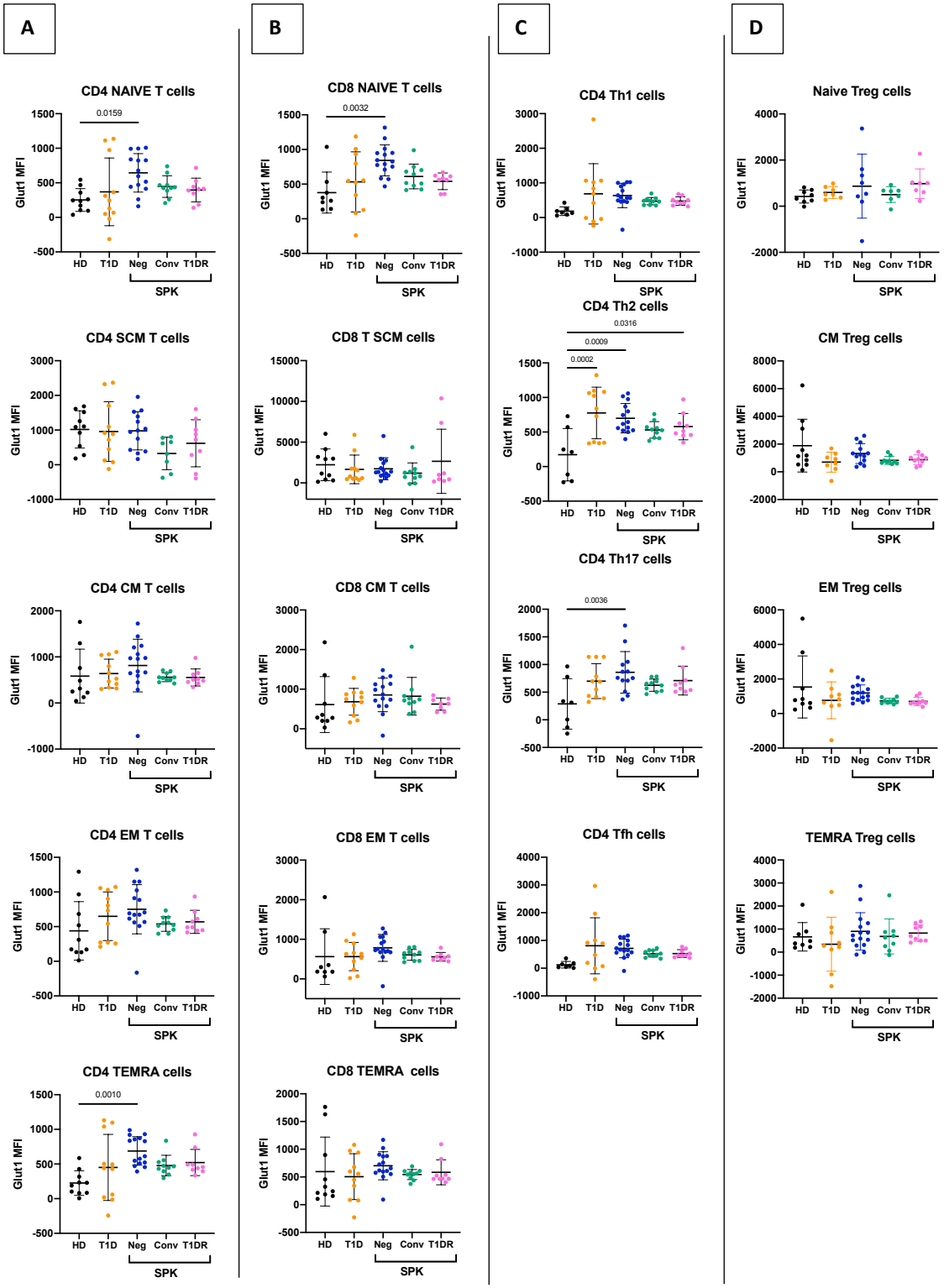


Figure 13. Ex-vivo flow cytometry assessment of cell surface GLUT1 expression for major T cell subsets, across the different groups. *(A) shows the expression (MFI) in $CD4^+$ subsets; (B) in $CD8^+$ subsets; (C) in T helper subpopulations and (D) in Treg cells subsets. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

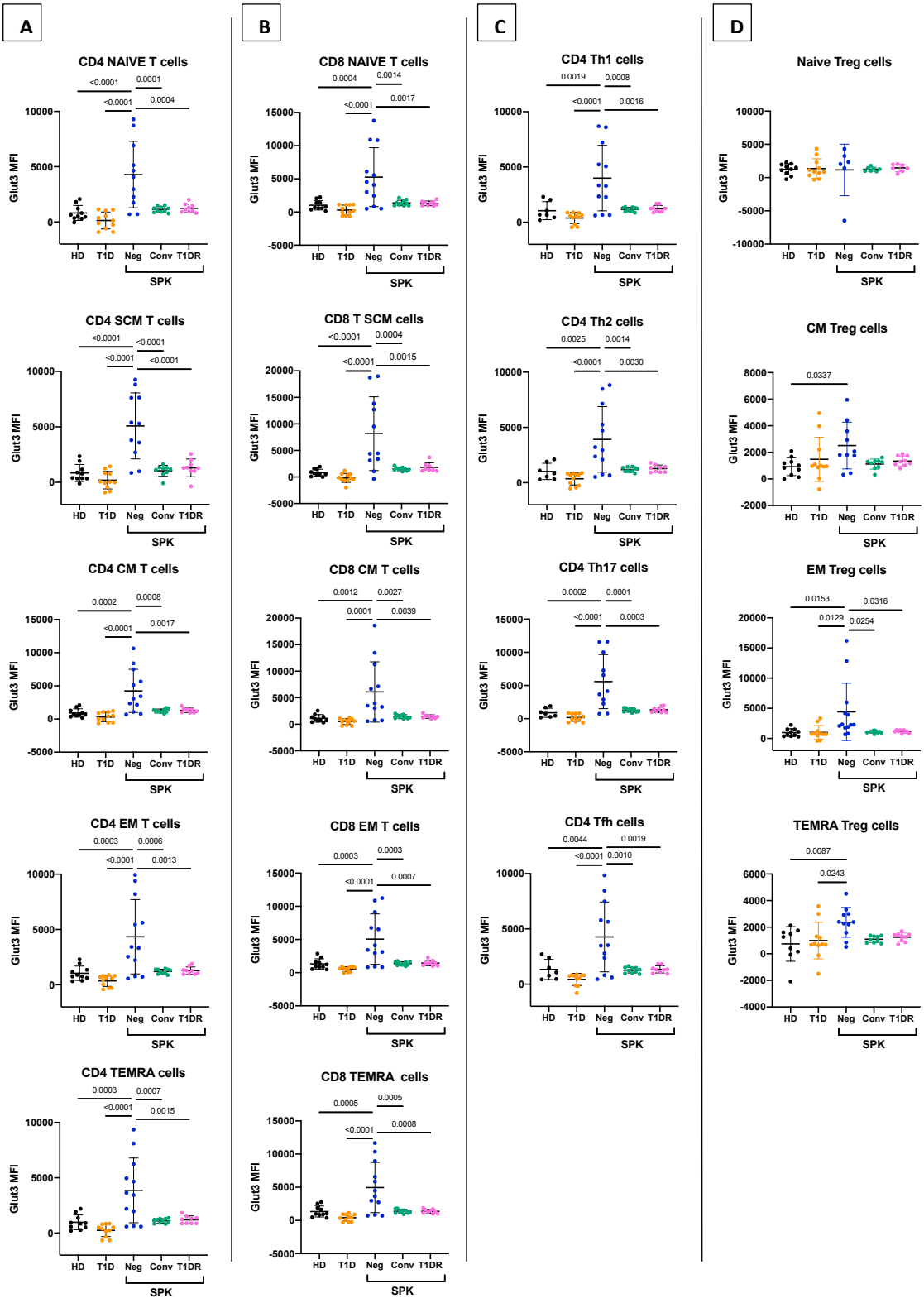


Figure 14. Ex-vivo flow cytometry assessment of cell surface GLUT3 expression for major T cell subsets, across the different groups. *(A) shows the expression (MFI) in $CD4^+$ subsets; (B) in $CD8^+$ subsets; (C) in T helper subpopulations and (D) in Treg cells subsets. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using One-way ANOVA test.*

3.2 Characterizing autoreactive T cells in SPK patients

This part of the study was performed in the laboratory of Prof. Pugliese at the Arthur Riggs Diabetes and Metabolism Research Institute, City of Hope, Duarte California. Samples were again provided by Prof. George W. Burke III from the University of Miami.

3.2.1 Dextramers testing for the detection of autoreactive T cells

Based on the clinical differences observed in the outcomes among patients who underwent SPK transplantation (some experienced autoantibody conversions, others developed recurrence of clinical T1D and others remain unaffected with a functional transplant and in the absence of AABs), I aimed to investigate the presence, abundance and key phenotypic features of autoreactive T cells targeting islet cell autoantigens in the three SPK groups and determine whether distinctive features were associated with T1DR. Moreover, I include in this analysis the evaluation of various T cell subsets, with emphasis on memory cells, and the assessment of GLUT1 and GLUT3.

Since $CD4^+$ and $CD8^+$ autoreactive T cells held promise as predictive and prognostic biomarkers in the natural history of diabetes, we hypothesized that the prevalence of autoreactive T cells would provide a hint of transplant outcome prediction in these patients. I used the following class I and class II dextramers for the detection of beta cell antigen-specific T cells, using flow cytometry:

Class I Dextramers used for the identification of antigen-specific $CD8^+$ T cells were:

- PE-conjugated HLA-A*0201 $GAD65_{114-123}$ VMNILLQYVV
- PE-conjugated HLA-A*0201 $Insulin_{10-19}$ HLVEALYLV
- PE-conjugated HLA-A*0201 $IGRP_{265-273}$ VLFGLGFAI

Class II Dextramers used for the identification of antigen-specific $CD4^+$ T cells were:

- PE-conjugated DRB1*0401 $GAD65_{555-567}$ NFIRMVISNPAAT
- PE-conjugated DRB1*0401 $Proinsulin_{73-90}$ GAGSLQPLALEGSLQKRG

As dextramers positive control, we used:

- APC-conjugated DRB1*0401 Flu $HA_{306-318}$ PKYVKQNTLKLAT

- APC-conjugated HLA-A*0201 CMV IE-$1_{316\text{-}324}$ VLEETSVML

As dextramers negative control, we used:

- PE-conjugated DRB1*0401 PVSKMRMATPLLMQA
- PE-conjugated HLA-A*0201ALIAPVHAV

To test MHC class II dextramers, we utilized a well-defined $CD4^+$ T cell clone BRI-4.13 specific for $GAD65_{555\text{-}567}$, stained with the class II dextramer specific for GAD65, and we took advantage of MHC class II dextramer negative control to accurately identify positive cell population (**Fig. 15A**). As negative control we used HLA-DRB1*0401 PVSKMRMATPLLMQA, specific for endogenous Class II associated Invariant Chain Peptide (CLIP). To test the MHC class I dextramers we used $CD8^+$ T cells transduced with HLA-A*0201- restricted IGRP-specific TCRs, stained with the PE conjugated HLA-A*0201 $IGRP_{265\text{-}273}$ VLFGLGFAI dextramer, and we compared it with the PE conjugated HLA-A*0201ALIAPVHAV (Neg. control) (**Fig. 15B**).

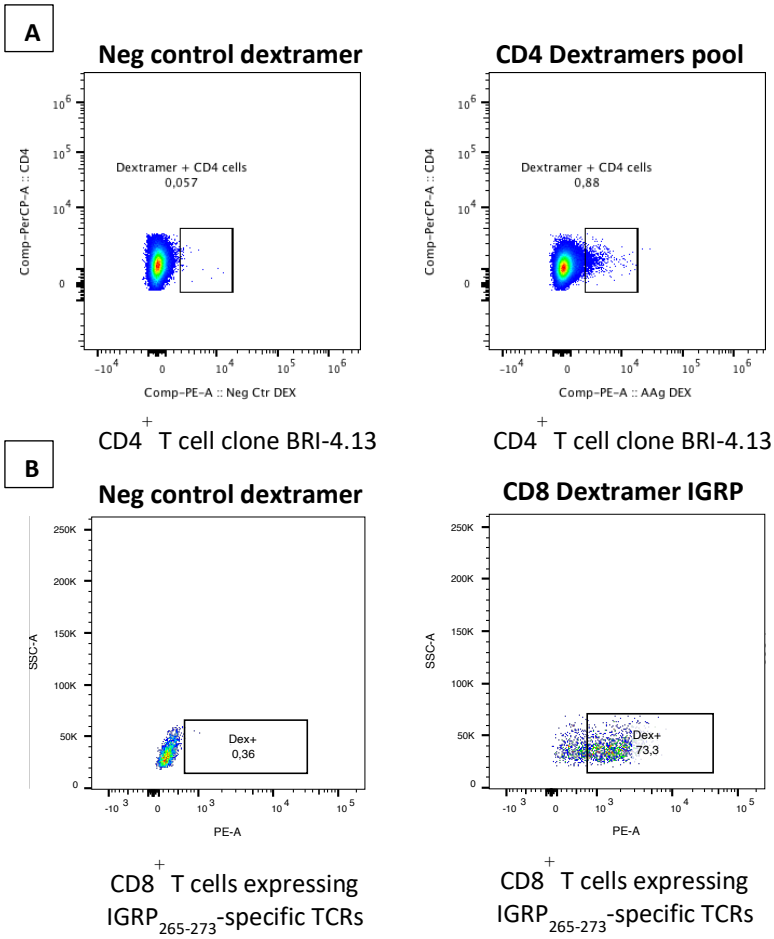


Figure 15. Validation of the MHC class I and class II dextramers staining. *(A) Detection of BRI-4.13 cells positive for GAD65. The plots compare the $CD4^+$ BRI-4.13 cells stained using negative control dextramers (left) and the CD4 dextramers pool containing $GAD65_{555-567}$ and $Proinsulin_{73-90}$ PE conjugated dextramers (right). (B) Detection of $CD8^+$ T cells positive for IGRP. The plots compare $CD8^+$ T cells expressing $IGRP_{265-273}$- specific TCRs stained using negative control dextramers (left) and the CD8 dextramer specific for $IGRP_{265-273}$ PE conjugated (right).*

3.2.2 Detection of beta cell antigens specific T cells in samples from SPK recipients using MHC dextramers

After testing dextramers with positive and negative controls, I studied samples from 9 SPK-Neg, 9 SPK-Conv and 8 T1DR patients, based on sample availability from those with appropriate HLA types. All dextramers for islet autoantigens were pooled to maximize detection of autoreactive T cells, which are rare in the circulation, and were included in the 37-parameter flow cytometry panel for in depth phenotypic analysis (see **Table 4**). This approach provides the opportunity to identify subsets/phenotypes associated with recurrent T1D, and, at the same time, to test the hypothesis that GLUT1 expression is a marker of recurrent T1D, by examining various immune cell populations.

Autoreactive T cells were present in most SPK patients, regardless of group. In *ex-vivo* analysis of PBMCs from SPK patients I detected low frequencies of $CD4^+$ and $CD8^+$ T cells specific for beta cells antigens (**Fig. 16A**). The frequency was similar across SPK-Neg, SPK-Conv and T1DR in $CD4^+$ T cells. The proportions of $CD8^+$ autoreactive T cells trended higher in SPK-Conv, followed by T1DR, compared to SPK-Neg, the latter without reaching statistical significance (**Fig. 16B**).

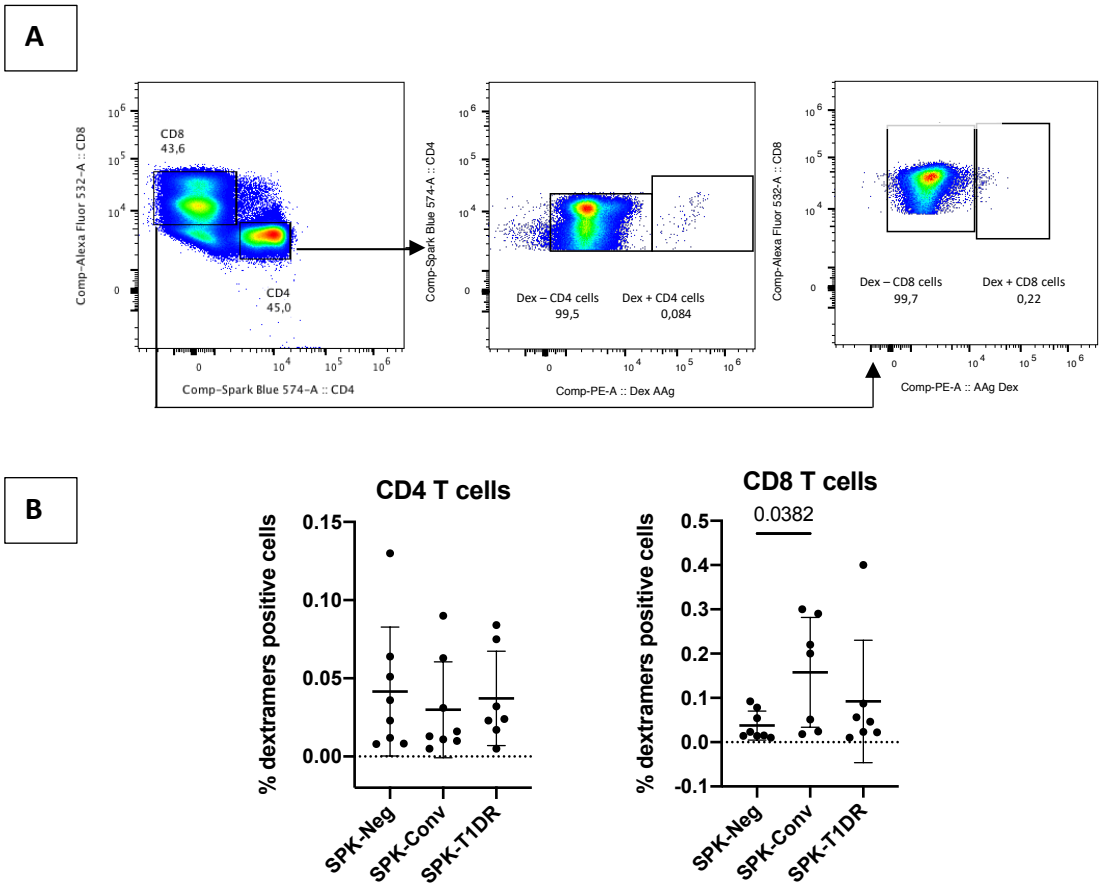


Figure 16. Detection and frequency of autoreactive $CD4^+$ and $CD8^+$ T cells using MHC dextramers. *(A) Plots show the expression of $CD4^+$ and $CD8^+$ cells, with the gated region showing the percentage of the population expressing beta cell antigens. (B) The proportion of cells positive for MHC I ($CD8^+$) and MHC II ($CD4^+$) dextramers pool. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using unpaired nonparametric Mann-Whitney test.*

3.2.3 Analysis of phenotype and subsets of autoreactive T cells in samples from SPK recipients.

Since the frequencies of autoantigens-specific T cells were not different across the SPK groups (except for the increase in $CD8^+$ dextramers positive cells in SPK-Conv), we investigated the phenotype of dextramer-positive T cells. We expected to identify phenotypic markers that characterize the function and better define populations of autoreactive T cells, which are likely to play a role in T1D recurrence.

Figure 17 illustrates heatmaps showing the MFI of CD4 or CD8 dextramer-positive gate in SPK-Conv and T1DR patients, measured as Log2fold change over SPK-Neg patients. All data were obtained by flow cytometry. Among $CD4^+$ T cells, we noted differences in the T1DR group, compared to SPK-Neg patients. T1DR patients had increased expression levels of activation markers HLA-DR and CD95, and decreased expression of some others, such as CD25 and CD28. The levels of chemokine receptors CCR5 and CCR6, which regulate trafficking and effector functions of T cells, were increased. The exhaustion markers PD-1, CD122 and BTLA were lower than in the SPK-Neg group, while TIGIT was increased (**Fig. 17A,B**). In $CD8^+$ T cells, similar changes were observed for CD95, CCR6, PD-1 and CTLA-4 in T1DR patients compared to SPK-neg patients (**Fig. 17A,B**).

Overall, these data suggest that in T1DR patients autoreactive T cells are more activated and less exhausted than in the other SPK recipients, which is consistent with an active autoimmune response against beta cells.

Remarkably, GLUT1 expression on autoreactive T cells was higher in the T1DR group compared to SPK-Neg and SPK-Conv groups in both $CD4^+$ and $CD8^+$ T cells.

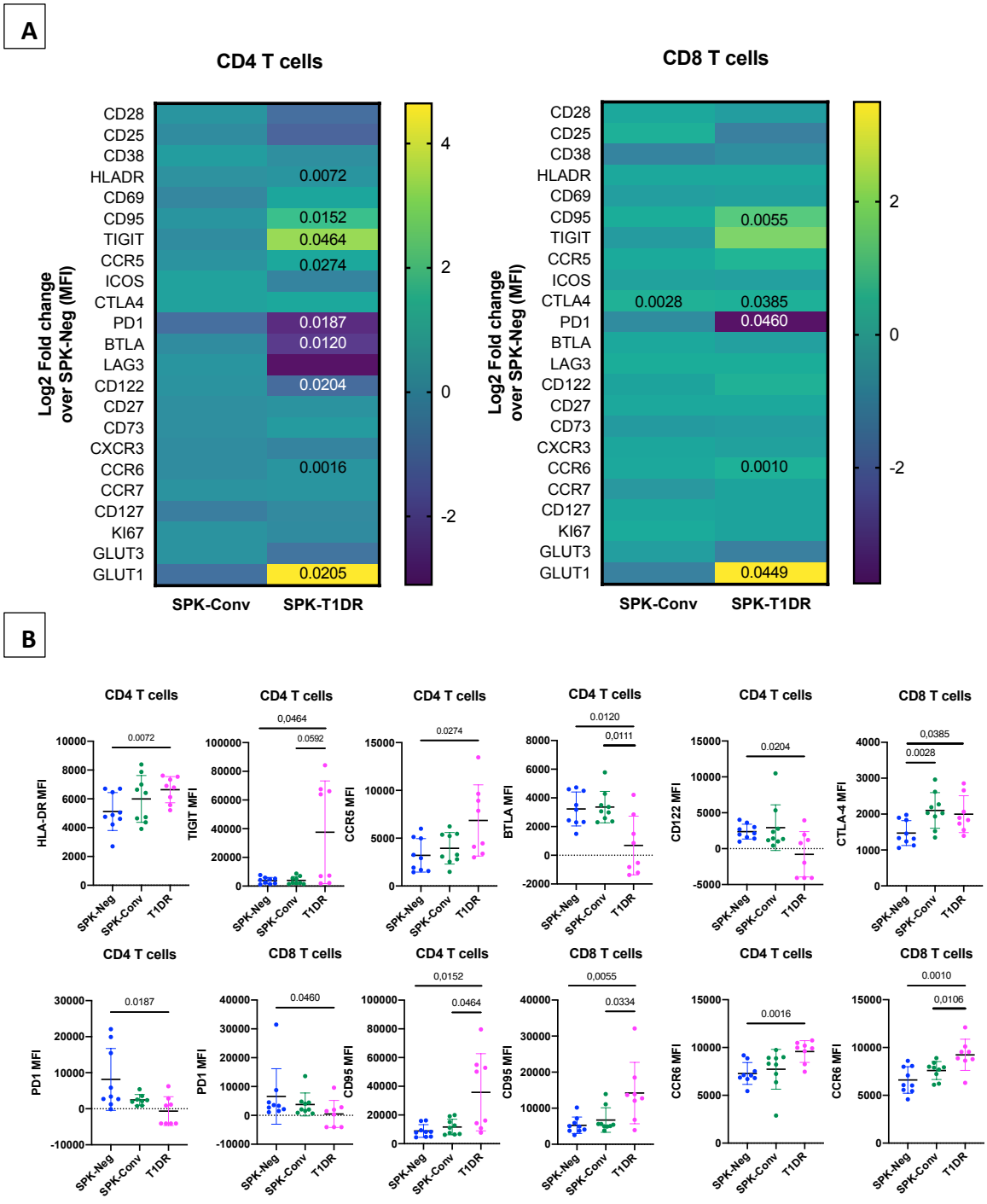


Figure 17. Heatmap visualization of protein expression profiles in SPK-Conv and T1DR groups. *(A) Differences in marker expression (expressed as MFI of CD4 and CD8 dextramer-positive gate) are calculated as the Log2 of the ratio between the MFI mean in SPK-Neg and SPK-Conv or SPK-Neg and T1DR. (B) Only selected markers are shown, those showing the most evident changes, from our 37-parameter panel. Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using Mann-Whitney test.*

While the expression of GLUT1 in autoreactive $CD4^+$ T cells was similar across SPK-Neg and SPK-Conv groups, in the T1DR group we observed a pronounced increase either in GLUT1 MFI or in the proportion of cells positive for the marker (**Fig. 18**). As shown in the graphs (**Fig. 18C,D**), despite not all patients exhibited high GLUT1 MFI, the overall average expression of GLUT1 was significantly elevated.
An intriguing observation emerges when examining these patients: those with high GLUT1 expression levels also demonstrated high levels of activation markers while exhibiting lower levels of exhaustion markers, and conversely, those with lower GLUT1 expression levels displayed the opposite pattern. This finding highlights a correlation between GLUT1 expression and the immune activation status of patients.

GLUT3 expression was found similar in SPK-Neg and SPK-Conv groups and lower in T1DR patients. $CD8^+$ T cells specific for beta cell antigens followed the same pattern of $CD4^+$ T cells. Taken together, these results suggest that in T1DR patients, autoreactive T cells upregulate GLUT1 and several activation markers, and this is accompanied by the downregulation of GLUT3 and exhaustion markers. These findings support the concept that glucose metabolism may be selectively altered in autoreactive T cells from patients who develop T1DR.

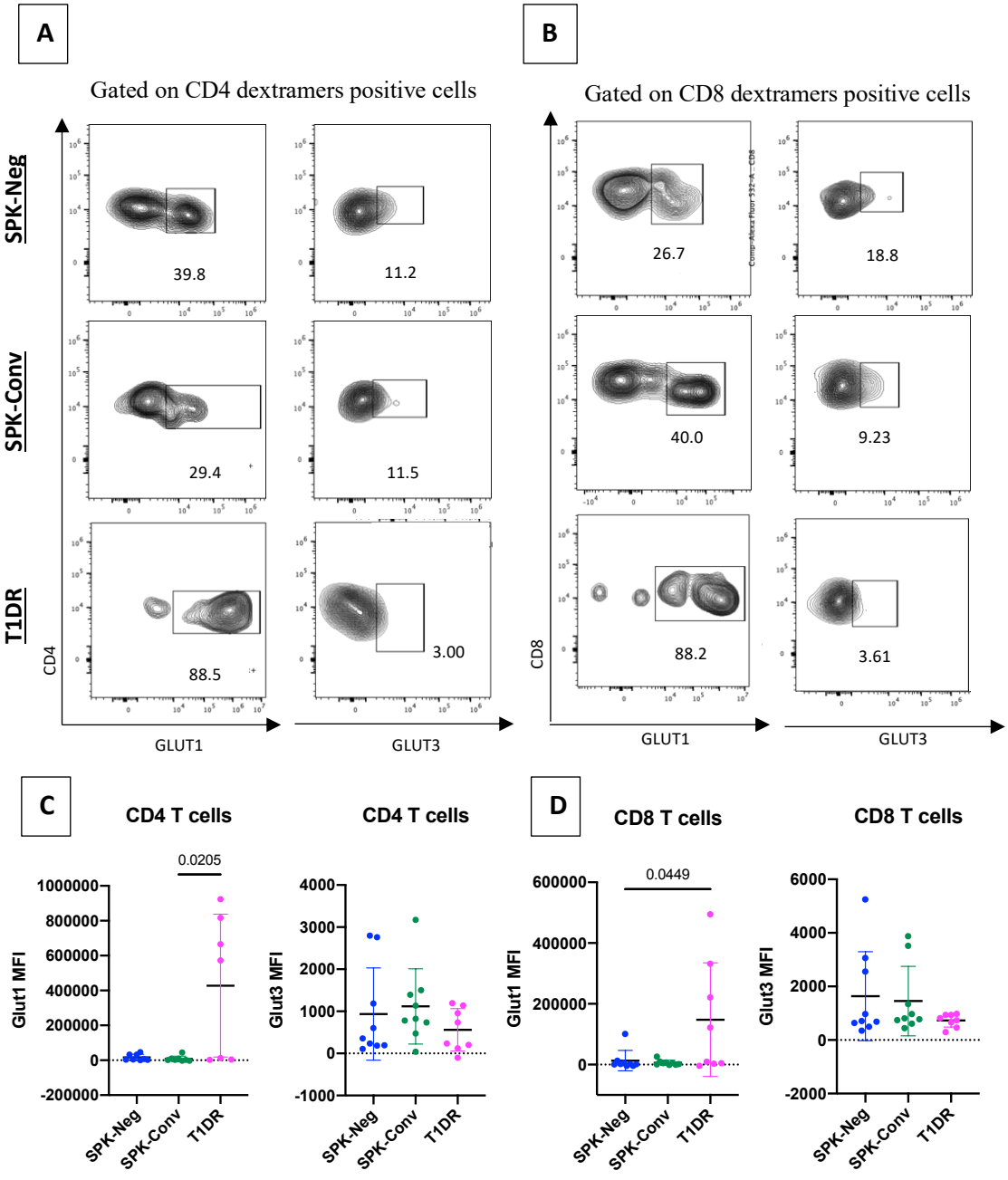


Figure 18. GLUT1 and GLUT3 expression in CD4$^+$ and CD8$^+$ autoreactive T cells. *Plots show the percentage of CD4$^+$ (A) and CD8$^+$ (B) T cells specific for dextramers expressing GLUT1 and GLUT3 in SPK-Neg (upper), SPK-Conv (middle) and T1DR (lower) patients. Graphs show GLUT1 and GLUT3 MFI in CD4$^+$ (C) and CD8$^+$ (D) T cells specific for dextramers (dextramer-positive gate). Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using Mann-Whitney test.*

To better characterize autoreactive T cells in these patients, I then evaluated changes in the memory/naïve subsets both for $CD4^+$ and $CD8^+$ T cells, identified through differential expression of CD45RA and CD62L. Since the number of detected autoreactive cells is low, a rout assessment of the Tscm population was not feasible. A future goal is to collect a larger number of events in flow cytometry or to pool more sample vials to overcome this technical limitation.

The data showed that autoreactive $CD4^+$ and $CD8^+$ T cells in SPK patients with T1DR had a higher proportion of Tem (**Fig. 19C**). Temra and naive subsets were dramatically decreased among the autoreactive $CD4^+$ T cells in SPK-Conv and T1DR (**Fig. 19B,D**); SPK-Conv had also a lower frequency of $CD4^+$ effector memory (**Fig. 19C**). While no differences were observed in the $CD8^+$ Tcm, both SPK-Conv and T1DR had increased proportion of $CD4^+$ Tcm (**Fig. 19A**). The distribution of the populations among SPK-Neg and SPK-Conv groups were similar for all the $CD8^+$ T cell subsets.

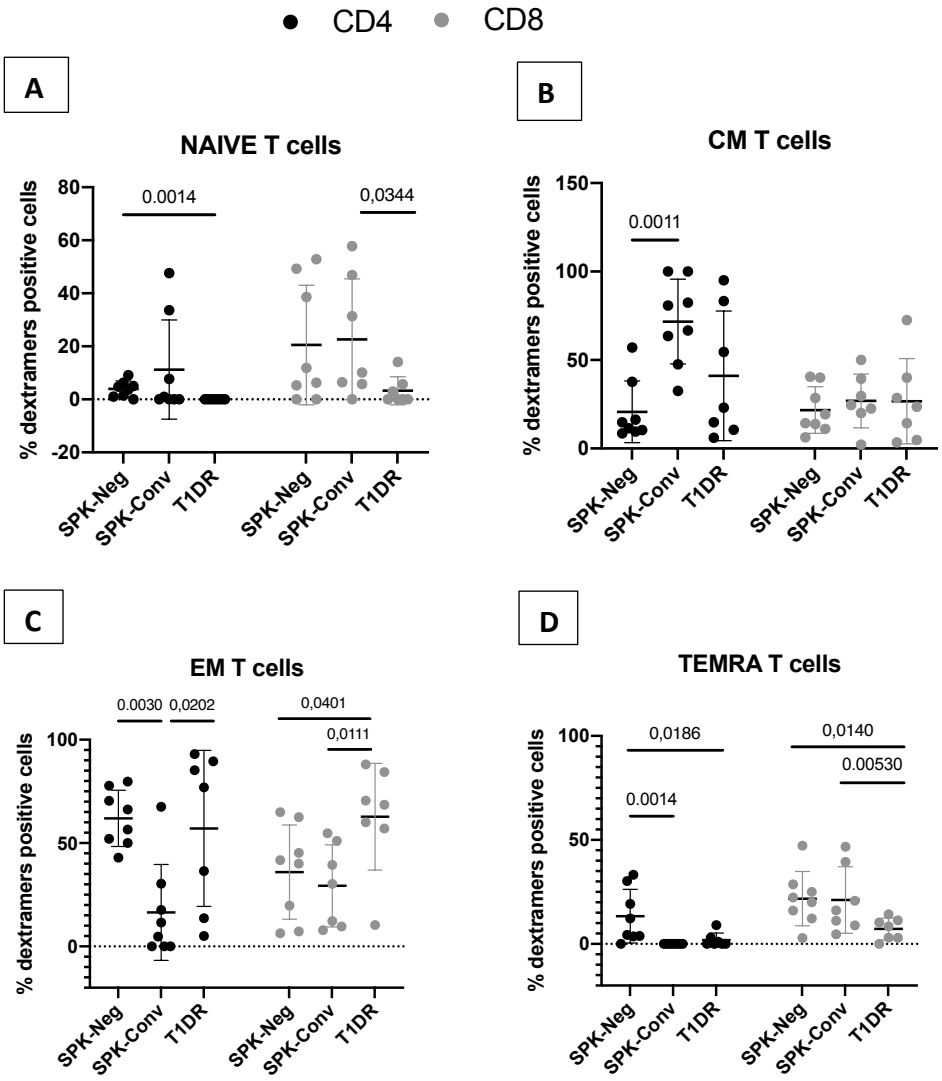


Figure 19. Analysis of major lymphocyte populations in the study cohorts on autoreactive T cells. *Differences in cell frequencies (% of lymphocytes) of $CD4^+$ and $CD8^+$ T cells positive for dextramers specific for beta cell antigens in the different groups. (A) Effector memory $CD4^+$ (black dots) and $CD8^+$ (grey dots) T cells; (B) naïve $CD4^+$ (black dots) and $CD8^+$ (grey dots) T cells; (C) central memory $CD4^+$ (black dots) and $CD8^+$ (grey dots) T cells; (D) terminally differentiated effector memory $CD4^+$ (black dots) and $CD8^+$ (grey dots) T cells Each data point represents an individual subject. Data shown as Mean ± SD. Comparisons were made using Mann-Whitney test.*

Subsequently, I determined the phenotypic signature of naïve/memory subsets of $CD4^+$ and $CD8^+$ autoreactive T cells within the T1DR cohort, focusing on the markers that showed significant changes in the total $CD4^+$ and $CD8^+$ T cell populations. The determination of marker expression levels in $CD4^+$ Temra and naïve subsets proved challenging due to their exceeding low frequency. However, in $CD4^+$ Tcm and Tem subsets, we observed a predominant upregulation of CD69 and CD95, accompanied by a concomitant downregulation of PD-1, CD122 and BTLA (**Fig. 20**). Notably, the expression of GLUT1 in T1DR was increased in both $CD4^+$ Tcm and Tem compared to the other SPK groups. Among $CD8^+$ autoreactive T cells, a differential pattern emerged, characterized by unchanged markers expression in Tcm, including GLUT1, and a corresponding increase in Tem and Temra subsets. Phenotypic markers remained relatively consistent between $CD8^+$ Tem and Temra, marked by a reduction in PD-1 expression and an increase in CD95 and TIGIT levels.

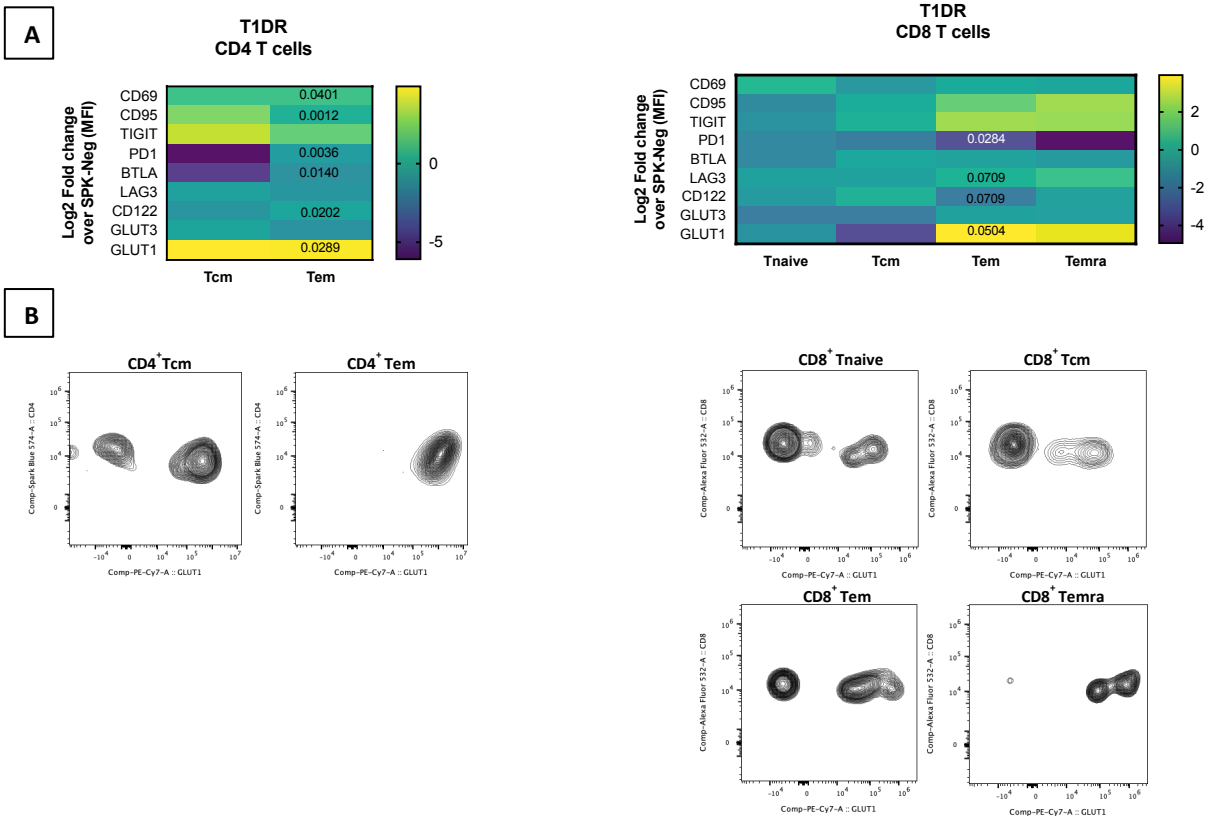


Figure 20. Heatmap visualization of protein expression profiles of memory/naïve $CD4^+$ and $CD8^+$ T cell subsets in the T1DR group. *(A) Differences in markers expression (MFI) are calculated as the Log2 of the ratio between the MFI mean in SPK-Neg and T1DR. (B) representative plots showing the expression of GLUT1 in the four subsets of $CD4^+$ and $CD8^+$ dextramer-positive cells, identified through the differential expression of CD62L and CD45RA.*

3.2.4 Unsupervised flow cytometry phenotypic analysis of autoreactive and non-autoreactive T cells in SPK recipients

We conducted an unsupervised analysis to identify T cell phenotypic clusters among populations of autoreactive and non-autoreactive T cells in the three SPK groups. We focused this analysis on the SPK patients to define phenotypic clusters and markers that may differentiate SPK recipients with T1DR from those with increased (SPK-Conv) or low (SPK-Neg) risk of T1DR.

For this purpose, we used the computer algorithm Uniform Manifold Approximation and Projection (UMAP). This algorithm better preserves the global structure in the data compared to t-distributed stochastic neighbor embedding (tSNE). We applied default parameter settings to generate UMAP using FlowJo v10. The dextramer-positive $CD4^+$ or $CD8^+$ T cells from each of the 26 SPK patients were clustered together to generate a single concatenated file encompassing 1,350 events for $CD4^+$ and 2,050 for $CD8^+$ T cells. The gating strategy is shown in **Figure 21A** and **Figure 22A.**

The UMAP visualization of our dataset spatially separated autoreactive T cells from the three SPK groups in distinct populations. Once the events were embedded, clusters were detected using the FlowSOM algorithm (v3.0.18), which initially defined 100 clusters using a Self-Organizing Map (SOM) [169]. These clusters were combined into 11 *meta*-clusters for $CD4^+$ T cells (**Fig. 21D**) and 13 for $CD8^+$ T cells (**Fig. 22D**) by hierarchical clustering, based on phenotypic similarity of the chosen markers. All markers except cell-lineage were included in the clustering (n=25 markers). To define the phenotypes of each population, we generated heatmaps showing the median intensity of each phenotypic marker we measured (**Figs. 21-22F**). In **Figure 21B** and **21C**, $CD4^+$ autoreactive T cells from T1DR patients mainly exist in a distinct cluster separated from autoreactive T cell populations from SPK recipients with a functional transplant (SPK-Neg and SPK-Conv). **Figure 22B** (right, red color) and **22C** shows also distinct populations of autoreactive $CD8^+$ T cells from T1DR patients. A second cluster of T1DR autoreactive $CD8^+$ T cells is less separated from the other groups.

Among $CD4^+$ T cells (**Fig. 21D, 21E**), clusters Pop0 and 3 were uniquely representative of autoreactive $CD4^+$ T cells in patients with T1DR; these were characterized by high

GLUT1 expression and low PD-1 expression. CD95, TIGIT and CD122 were also consistently different from the other subpopulations identified. Pop0 and 3 showed a mild reduction in BTLA and LAG-3 expression. Pop6, a small cluster of autoreactive CD4^{+} T cells also observed in T1DR patients, was characterized by high CD122 and BTLA expression. Moreover, autoreactive CD4+ T cell populations of the T1DR patients exhibited markedly lower expression of CD45RA and CD62L, consistent with an effector memory phenotype.

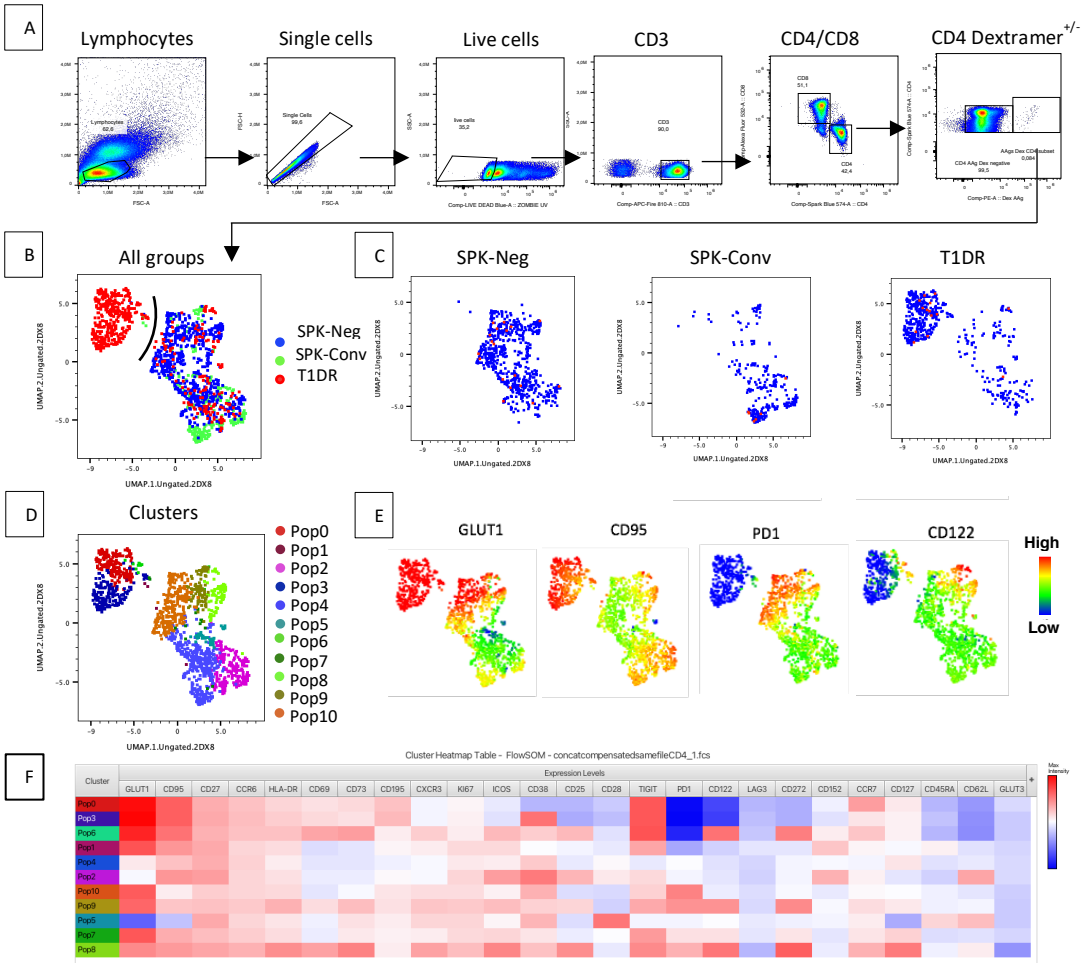


Figure 21. Unsupervised analysis of islet antigen specific CD4^{+}T cells among SPK groups. *(A) Gating strategy for the identification of autoreactive CD4^{+}T cells; (B) overall and (C) individual UMAP projections on concatenated files of autoreactive CD4^{+}T cells in SPK-Neg (n=9), SPK-Conv (n=9), and T1DR (n=8); (D) Distribution of the 11 identified FlowSOM clusters overlaid on the UMAP projection (E) MFI of selected surface markers in the UMAP described in B. (F) Heatmap showing the relative intensity of each parameter for a given cluster. The events in the gate of the dextramer-positive CD4^{+} cells from each of the 26 subjects were clustered together to generate a single concatenate file encompassing 1,350 events.*

Among CD8$^+$ autoreactive T cells, Pop0 was uniquely observed among T1DR patients, and this was spatially separated from other SPK groups (**Fig. 22B, 22D**).

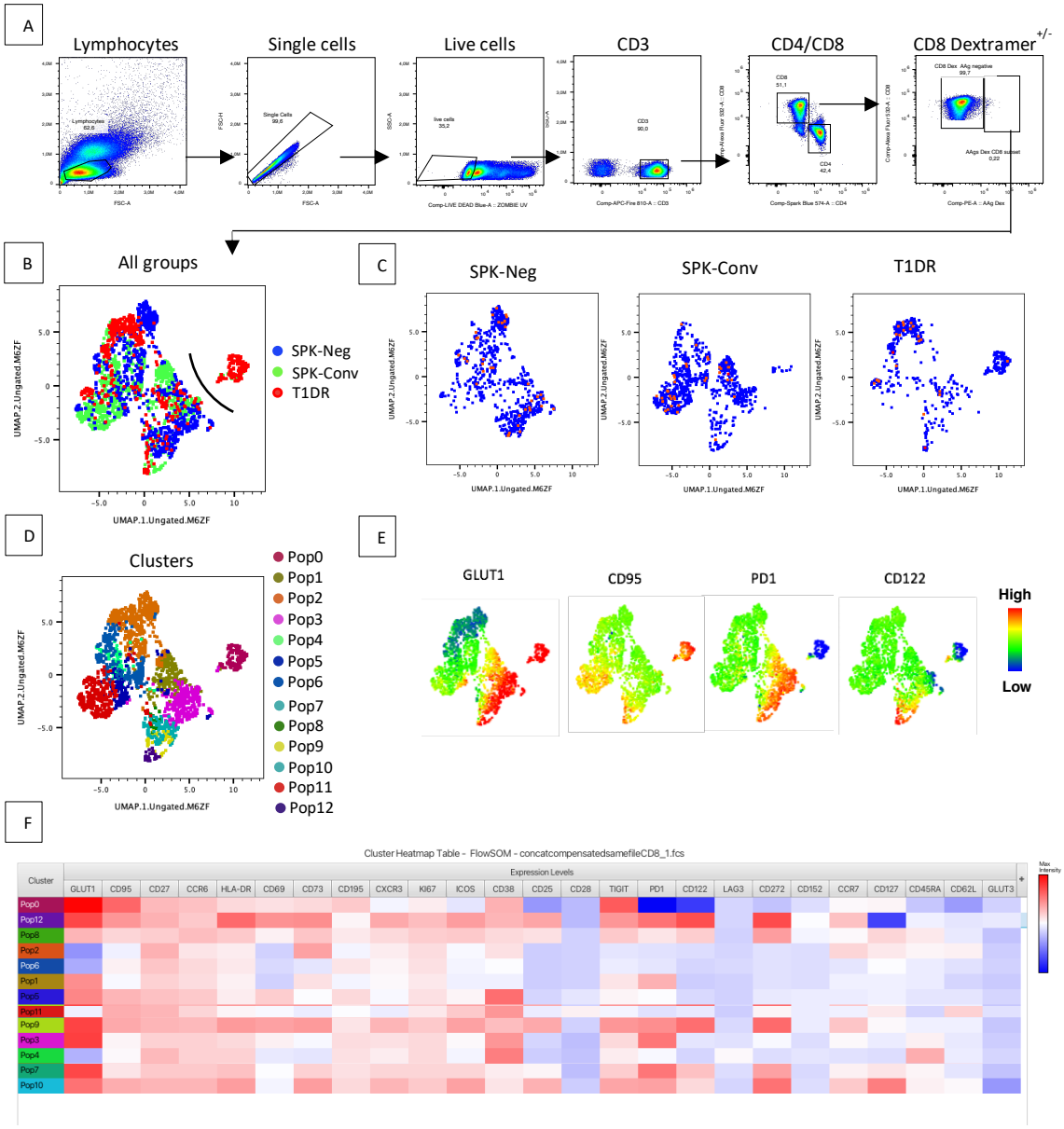


Figure 22. Unsupervised analysis of islet antigen specific CD8$^+$T cells among SPK groups. *(A) Gating strategy for the identification of autoreactive CD8$^+$T cells; (B) overall and (C) individual UMAP projections on concatenated files of autoreactive CD8$^+$T cells in SPK-Neg (n=9), SPK-Conv (n=9), and T1DR (n=8); (D) Distribution of the 13 identified FlowSOM clusters overlaid on the UMAP projection (E) MFI of selected surface markers in the UMAP described in B. (F) Heatmap showing the relative intensity of each parameter for a given cluster. The events in the gate of the dextramer-positive CD8$^+$ cells from each of the 26 subjects were clustered together to generate a single concatenate file encompassing 2,050 events.*

Pop0 showed increased expression of GLUT1 and the activation marker CD95, along with a decrease of exhaustion markers, such as PD-1, CD122 and BTLA. This phenotype is similar to that observed in autoreactive $CD4^+$ T cells of T1DR patients. Autoreactive T cells of T1DR patients were also observed in Pop2, 12 and 8, and these populations also existed in the SPK-Conv group, which is considered at increased risk for T1DR. The autoreactive $CD8^+$ T cell populations of T1DR patients had low expression of CD45RA and CD62L. This was particularly evident for Pop0.

Overall, this analysis demonstrated functional clustering of autoreactive T cells that are characterized by increased activation markers (CD95, HLA-DR) and reduced exhaustion markers (PD-1, CD122) among T1DR autoreactive $CD4^+$ and $CD8^+$ T cells, consistent with an aggressive phenotype. These populations were not evident in the SPK Neg and SPK Conv groups, in which only small clusters expressed activation markers, however in the presence of elevated PD-1, suggesting a less aggressive, exhausted phenotype. Importantly, this unbiased analysis and our targeted analysis concurred in the definition of these phenotypes and further identify elevated GLUT1 expression among the aggressive autoreactive T cell populations of T1DR patients.

To determine whether the observed differences in phenotype were exclusive to autoreactive T cells or extended to non-autoreactive ones, we applied the UMAP algorithm to $CD4^+$ and $CD8^+$ T cells with irrelevant reactivity. The dextramer-negative $CD4^+$ or $CD8^+$ T cells from each of the 26 SPK patients were clustered together to generate a single concatenate file encompassing 100,000 events for $CD4^+$ and 100,000 for $CD8^+$ T cells. The gating strategy is shown in **Figure 23A** and **Figure 24A**.

Unlike dextramer-positive cells, $CD4^+$ dextramer-negative T cells did not exhibit clear separation in the UMAP plots (**Fig. 23B**). There was substantial overlap among the three groups of SPK patients, with most FlowSOM clusters being comprised of a mixture of subjects from all groups. We did not observe any dextramer-negative clusters with phenotypic features consistent with those found among the autoreactive $CD4^+$ T cell populations. Elevated GLUT1 expression was noted in Pop3 and 4, which were detected in all three SPK groups; these were not large populations and were characterized by high PD-1 expression.

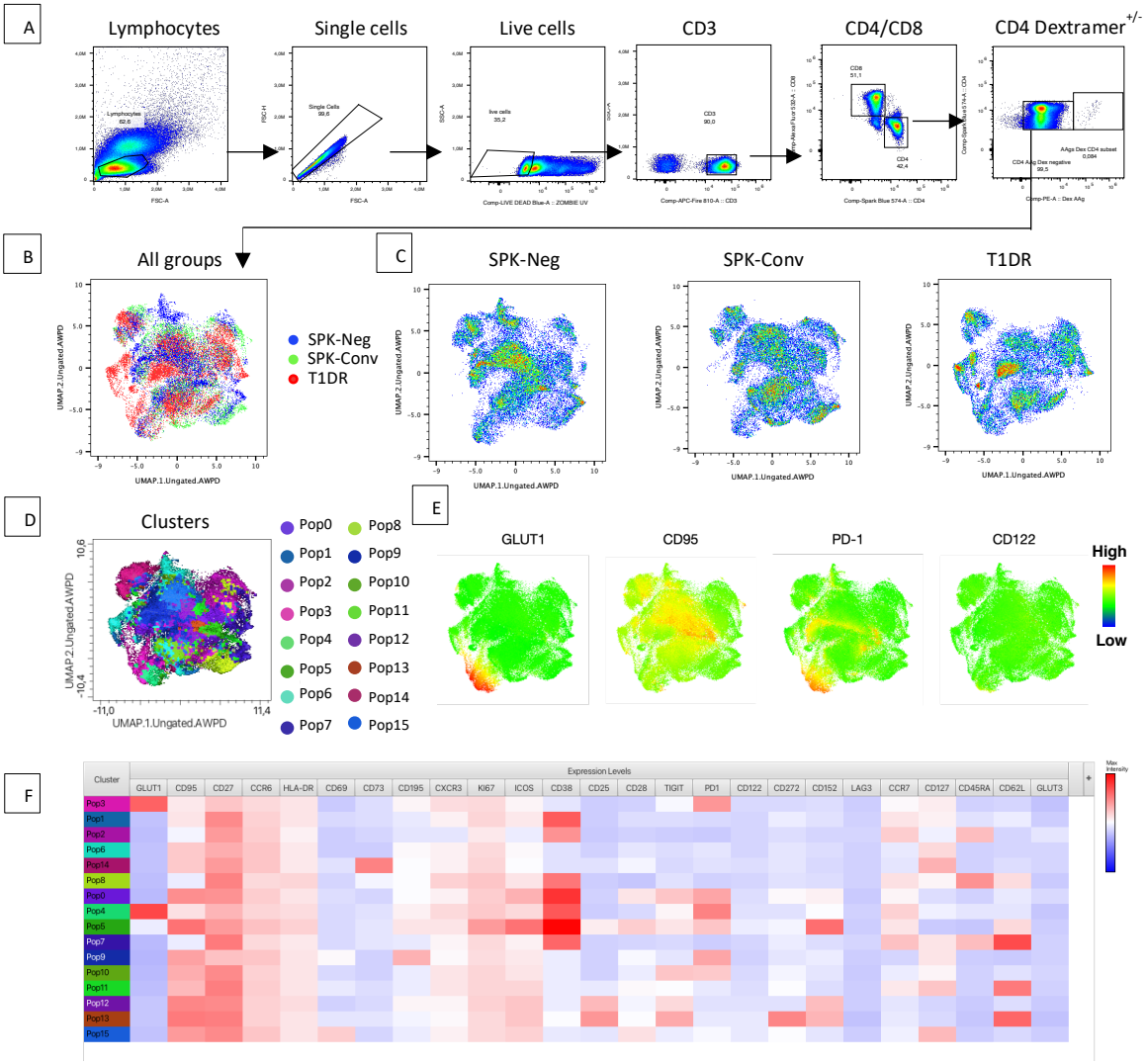


Figure 23. Unsupervised analysis of non-autoreactive CD4$^+$T cells among SPK groups. *(A) Gating strategy for the identification of non- autoreactive CD4$^+$T cells; (B) overall and (C) individual UMAP projections on concatenated files of non- autoreactive CD4$^+$T cells in SPK-Neg (n=9), SPK-Conv (n=9), and T1DR (n=8); (D) Distribution of the 16 identified FlowSOM clusters overlaid on the UMAP projection (E) MFI of selected surface markers in the UMAP described in B. (F) Heatmap showing the relative intensity of each parameter for a given cluster. The events in the gate of the dextramer-negative CD4$^+$ cells from each of the 26 subjects were clustered together to generate a single concatenate file encompassing 100,000 events.*

Clusters of non-autoreactive CD8$^+$ T cells (**Fig. 24B**) also exhibited considerable overlap among the three groups. We could discern clusters with higher abundance among SPK-Conv, specifically Pop3 and 4, which were characterized by high CD73, CD25, and CD62L expression. Pop14 and 9 predominantly included cells from T1DR patients, with Pop14 showing high GLUT1, PD-1, and CCR7 expression, and Pop9 displaying high CD73, CD25, CD45RA, and GLUT3 expression.

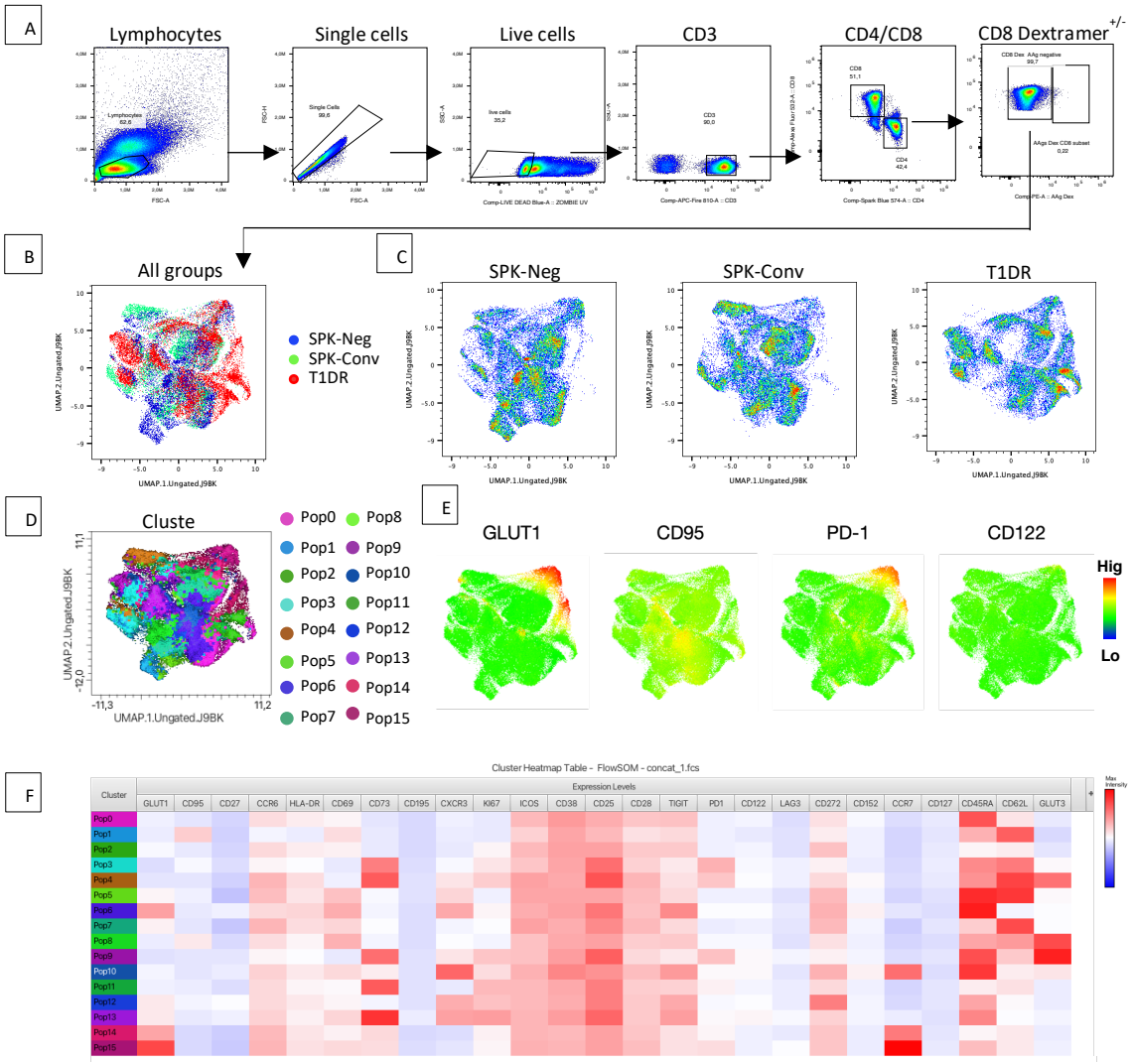


Figure 24. Unsupervised analysis of non-autoreactive CD8$^+$T cells among SPK groups. *(A) Gating strategy for the identification of non- autoreactive CD8$^+$T cells; (B) overall and (C) individual UMAP projections on concatenated files of non- autoreactive CD8$^+$T cells in SPK-Neg (n=9), SPK-Conv (n=9), and T1DR (n=8); (D) Distribution of the 16 identified FlowSOM clusters overlaid on the UMAP projection; (E) MFI of selected surface markers in the UMAP described in B. (F) Heatmap showing the relative intensity of each parameter for a given cluster. The events in the gate of the dextramer-negative CD8$^+$ cells from each of the 26 subjects were clustered together to generate a single concatenate file encompassing 100,000 events.*

Pop1 was enriched in cells from SPK-Neg patients, exhibiting high CD95 and CD62L expression (**Fig. 24D, 5F**). All other clusters represented a mixture of cells from all patient groups. Similar to the observations in $CD4^+$ T cells, analysis of key phenotypic markers in non-autoreactive $CD8^+$ T cells revealed that high GLUT1 expression was associated with high PD-1 and low CD95 expression, contrasting with data on autoreactive T cells where T1DR cells had a clearly distinct phenotype. Of note, most of the non-autoreactive $CD8^+$ T cell populations exhibited a naïve phenotype characterized by higher levels of CD45RA and CD62L.

The distinct functional clusters of autoreactive T cell populations that distinguish T1DR patients from the other SPK groups were not identified in the analysis of non-autoreactive T cells, in which T cell populations were not well separated and did not distinguish the three groups.

3.3 Effect of WZB117 on survival and proliferation of T cells from healthy donors

The experiments in this section were performed at the Diabetes Research Institute in Milan.

3.3.1 Glucose availability affected survival of activated T cells

To determine the role of glucose availability on T cells, I tested the effect of glucose deprivation on T cells survival, during activation. PBMCs from healthy donors were stimulated with anti-CD3/CD28 beads (bead-to-cell ratio 1:25) and cultured for 3 days in RPMI 1640 with different glucose concentrations:

- 5.5mM (corresponding to 110 mg/dL) represents the physiological blood glucose concentration and was used as the baseline;
- 1mM (corresponding to 20 mg/dL) represents a low glucose concentration that in the body can be found during severe hypoglycemia or in tumor tissues;
- 0mM, represents a complete glucose deprivation. To achieve this condition in the experimental setting we used dialyzed FBS.

Cell viability was evaluated using Annexin V and Propidium Iodide (PI) staining, at day 3 when activated T cells showed the highest surface expression of GLUT1 [170]. As compared to the 5.5mM glucose baseline condition, no significant changes in T cell viability were observed when T cells were exposed to glucose 1mM (**Fig. 25**). Since FBS contains glucose, to set up the 0mM glucose conditions we used dialyzed FBS, which does not contain glucose yet it provides growth factors essential to T cell survival. When cultured with dialyzed FBS, $CD4^+$ and $CD8^+$ T cells showed 27% and 47% reductions in viability, respectively.

These findings suggested that T cells can adapt to very low glucose levels (1mM), but their survival is severely affected by complete glucose deprivation.

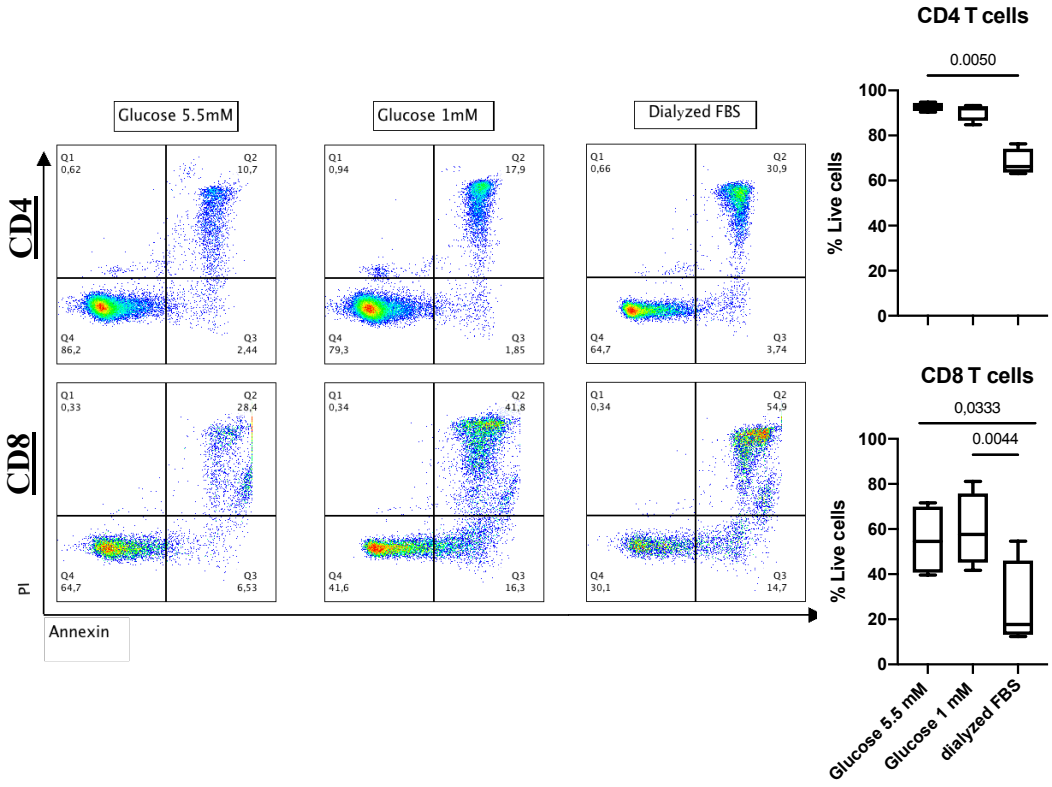


Figure 25. CD3/CD28-stimulated cells survival is not affected at glucose levels as low as 1mM. *The percentage of live cells harvested after 72 h of culture under the indicated conditions was determined by flow cytometry using Annexin/PI staining. For annexin/PI experiments the gating strategy used was different from that used in the rest of the experiments included in the dissertation. All the lymphocytes, determined by the FSC and SSC profile, were included, debris and doublets were discarded and $CD3^+$ T cells were distinguished by plotting SSC vs CD3. From CD3, gate $CD4^+$ and $CD8^+$ T cells were identified. Data shown as Mean ± SD. Comparisons were made using a paired T test.*

Experiments culturing T cells in low glucose conditions were performed before using the GLUT1 inhibitor, to determine whether glucose starvation and GLUT1 inhibition have similar effects on T cell survival. We had previously determined that the GLUT1 inhibitor WZB117 at 10μM reduced glucose uptake in T cells by 75% [97].

Cells cultured for 3 days in glucose 5.5mM RPMI stimulated with anti-CD3/CD28 beads (bead-to-cell ratio 1:25) in the presence of 10μM of WZB117 showed a reduction in T cell viability of 13% (+8% of late apoptotic cells; +5% of early apoptotic cells) compared to the T cells cultured in the same conditions but without WZB117 (**Fig. 26**). This result suggested that the survival of T cells was not considerably compromised by the decreased glucose uptake, caused by WZB117 treatment, aligning with their ability to survive in a glucose-limited environment, such as glucose 1mM concentration.

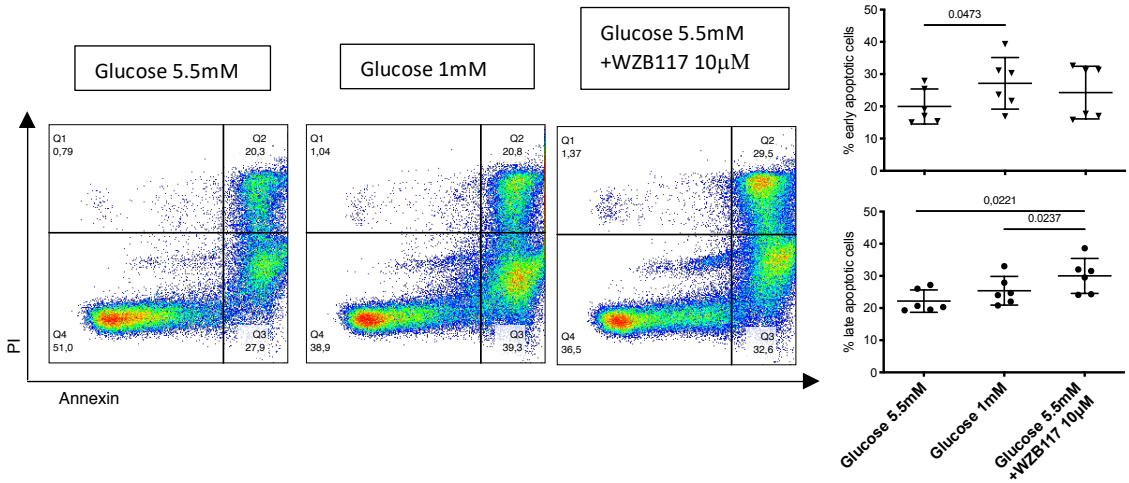


Figure 26. CD3/CD28-stimulated cells survival is not severely affected by the presence of the GLUT1 inhibitor WZB117 at a concentration of 10µM*. The percentage of live cells harvested after 3 days of culture under the indicated conditions was determined by flow cytometry using Annexin/PI staining. For annexin/PI experiments the gating strategy used was different from that used in the rest of the experiments included in the dissertation. All the lymphocytes, determined by the FSC and SSC profile were included, debris and doublets were discarded and $CD3^+$ T cells were distinguished by plotting SSC vs CD3. From CD3 gate $CD4^+$ and $CD8^+$ T cells were identified. Data shown as Mean ± SD. Comparisons were made using a paired T test.*

3.3.2 WZB117 inhibited T cell proliferation, and the effect persisted after WZB117 removal

As glucose is essential to support T cell proliferation, I determined whether proliferation was affected by WZB117. T cell proliferation was evaluated using a CFSE dilution assay in a 6-day culture system in the presence of anti-CD3/CD28 beads (bead-to-cell ratio 1:75) using flow cytometry analysis. CFSE is a fluorescent compound that undergoes intracellular enzymatic conversion into a fluorescent dye, enabling the labeling of cytoplasmic proteins. CFSE fluorescence intensity diminishes by half with each subsequent cell division, allowing the identification and quantification of proliferating cells.

As for the viability assay, I compared the low glucose concentration condition (1mM) to the physiological one (5.5mM) and with the addition of WZB117 on PBMCs from healthy donors. Compared to the glucose concentration of 5.5mM, I observed a reduction in T cell proliferation by 50% in the presence of glucose 1mM and by 20% when T cells were cultured in the presence of WZB117 (**Fig. 27**). A similar trend was observed both in $CD4^+$ and $CD8^+$ T cells.

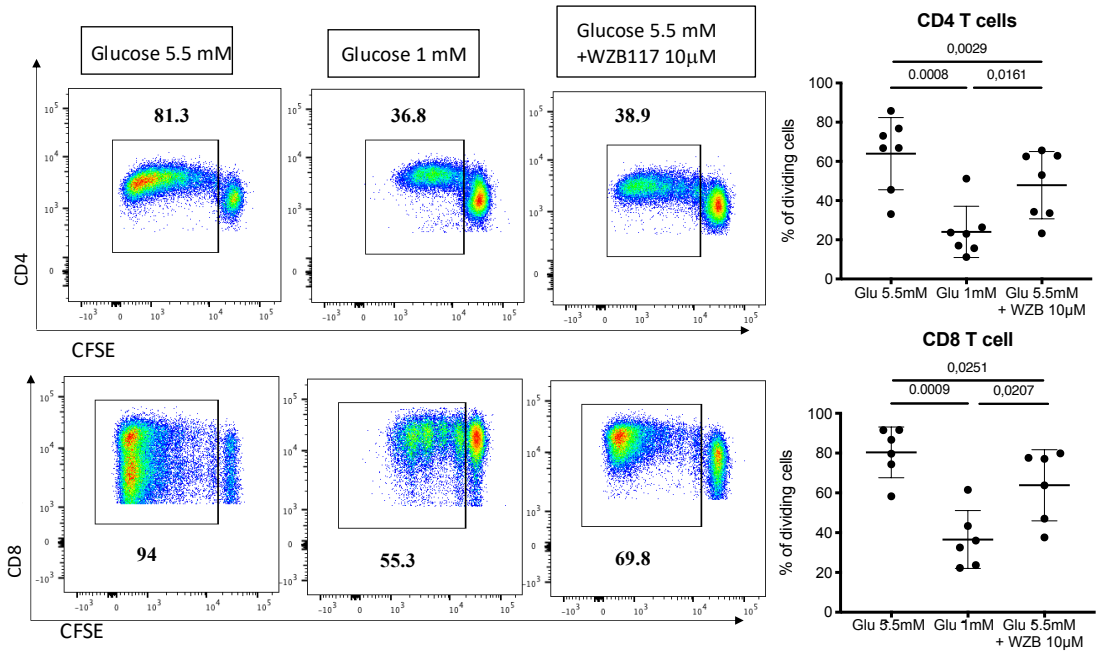


Figure 27. Analysis of proliferation using a CFSE dilution assay by flow cytometry*. The graphs show the fraction of cells diluting CFSE over 6 days of culture under the indicated conditions. Data shown as Mean ± SD. Comparisons were made using a paired T test.*

To determine whether the impact of glucose deprivation and the treatment with WZB117 on T cell proliferation were reversible or imparted a permanent state of hypo-responsiveness, I conducted long-term culture experiments in which, after stimulation of T cells with low glucose or WZB117 treatment, T cells were further cultured in 5.5mM glucose drug-free medium. PBMCs from healthy donors were stimulated with anti-CD3/CD28 beads and initially cultured in either glucose 1mM or 5.5mM plus WZB117 for 6 days, followed by an additional 6 days of culture in 5.5mM glucose medium without WZB117, as illustrated in **Figure 28A.**

The results revealed that T cells cultured initially in glucose 1mM exhibited a complete recovery of proliferation in the subsequent culture in 5.5mM glucose, with approximately 60% of the cells recovering their proliferative capacity. Conversely, T cells cultured in the presence of WZB117 during the first round remained hypo-responsive even after the removal of WZB117 and culture in 5.5mM glucose, showing a further 30% reduction in proliferation (**Fig. 28C**). Our data suggested that WZB117 induced a state of hypo-responsiveness in T cells that persists upon its removal and subsequent re-stimulation. As

T cells cultured in low glucose concentration completely recovered proliferation when moved into a 5.5mM glucose medium, this suggested that the effect of WZB117 on T cell proliferation involves mechanisms other than the mere reduction of glucose uptake.

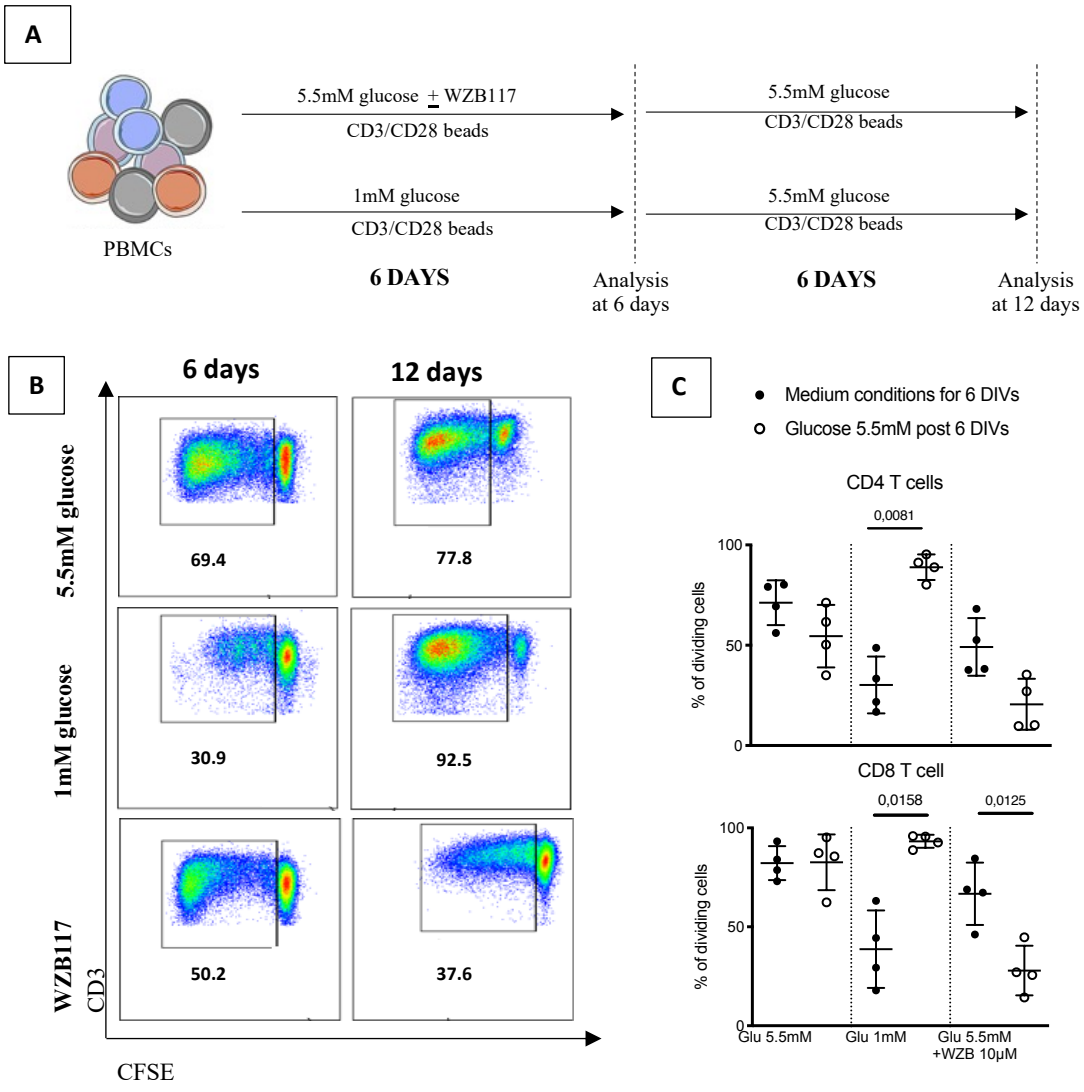


Figure 28. Effect of WZB117 on proliferation in 6 and 12 days culture experiment*. (A) In vitro culture system. Cells were activated with CD3/CD28 beads and maintained for 6 days in a medium containing glucose 5.5mM + 10µM WZB117 or 1mM glucose. Then cells were transferred to a medium containing glucose 5.5mM for an additional 6 days. Analysis was performed on days 6 and 12. (B) Representative plots showing the expression of CFSE and CD3 positive cells, with the gated region showing the percentage of the population expressing proliferating cells. (C) The graphs show the fraction of cells diluting CFSE over 6 and 12 days of culture under the indicated conditions. Data shown as Mean ± SD. Comparisons were made using a paired T test.*

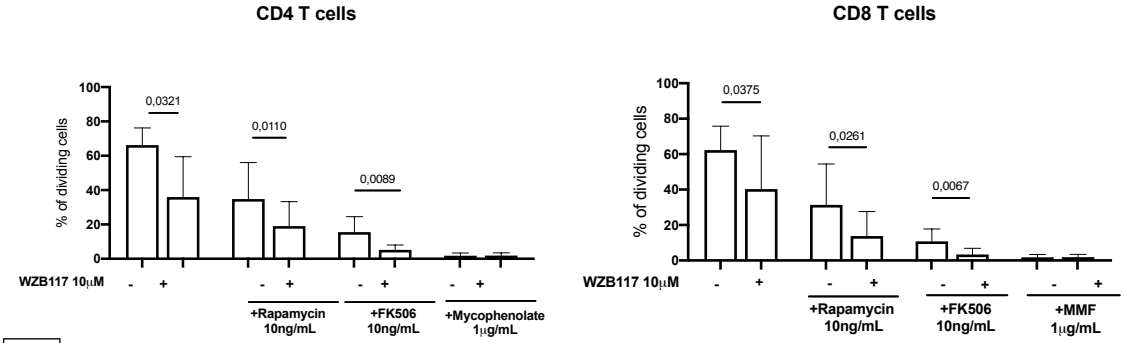
3.3.3 WZB117 displayed additive effect with immunosuppressive drugs

Since we envisage a potential application of GLUT1 inhibitors as part of the induction therapy in pancreas or pancreatic islet transplantation, we investigated the effect of WZB117 when administered along with commonly used immunosuppressive drugs, such as rapamycin, MMF, and FK506. To test the effect of GLUT1 inhibitor in combination with immunosuppressive drugs, CFSE-labeled PBMCs from healthy donors were stimulated with anti-CD3/CD28 beads for 6 days in the presence of different drug combinations. We used concentrations of rapamycin, FK506 and MMF that resemble the plasma target concentration of these drugs in the clinical settings [109].

WZB117 alone induced 35% and 45% inhibition on $CD8^+$ and $CD4^+$ T cells respectively (**Fig. 29**). The combination of WZB117 with rapamycin resulted in 78% and 71% of inhibition of proliferation on $CD8^+$ and $CD4^+$ T cells respectively, while the combination of WZB117 with FK506 resulted in 94% and 92% inhibition of proliferation on $CD8^+$ and $CD4^+$ T cells respectively. Despite not inducing substantial cell apoptosis, MMF alone showed a nearly 100% of inhibition of T cell proliferation, making it impossible to determine a potential combinatory effect with WZB117. We already reported MMF-induced complete inhibition of proliferation in our previous work [109].

These results demonstrated an additive inhibitory effect on immune activation when WZB117 was used in combination with both rapamycin and FK506, at concentrations relevant to clinical application (10ng/mL). These findings suggest that combining WZB117 with rapamycin or FK506 could represent a promising approach to enhance the immunosuppressive effects of these widely used drugs.

A



B

CD4+ T cells	reduction of proliferaton (%)*	p value*	p value**	CD8 + T cells	reduction of proliferaton (%)*	p value*	p value**
WZB117	45.7	0,0321	\	WZB117	35.3	0,0375	\
Rapamycin	47.5	0,0272	0,4674	Rapamycin	49.6	0,0132	0,1221
WZB117 + rapamycin	71.3	0,0013	0,0242	WZB117 + rapamycin	77.8	<0,0001	0,0486
FK506	76.6	0,0026	0,0492	FK506	83.7	0,001	0,0258
WZB117 + FK506	92.1	<0,0001	0,0189	WZB117 + FK506	94.5	0,0016	0,0212
MMF	96.1	<0,0001	0,0111	MMF	97.3	0,0031	0,0257
MMF + FK506	96.2	0,0001	0,0149	MMF + FK506	97.2	0,0022	0,0234
*Compared to Control culture							
** compared to WZB117 alone culture							

***Figure 29**. **WZB117 exerted an additive effect on T cell proliferation when used in combination with rapamycin or FK506 on CD4+ and CD8+ T cells**. (A) The graphs show the fraction of cells diluting CFSE over 6 days of culture with different drug combinations. (B) The table shows the percentage of reduction of T cell proliferation compared to the control condition. Data shown as Mean ± SD. Comparisons were made using a paired T test.*

3.4 WZB117 effect on proliferation and phenotype of T cells from healthy donors, T1D patients and transplanted patients

The experiments that belong to this part of the thesis were performed in the lab of Prof. Alberto Pugliese at the Diabetes Research Institute, Miller School of Medicine, University of Miami. Blood samples from SPK patients were kindly made available by Dr. George Burke III, Department of Surgery, Miller School of Medicine, University of Miami.

3.4.1 WZB117 exhibited enhanced reduction of T Cell Proliferation in T1D and SPK patients compared to healthy donors

To broaden the investigation and further characterize the influence of GLUT1 inhibition on human T cells, I extended my research beyond the previous observations made on PBMCs from healthy donors. In this phase of the study, I examined three distinct groups of subjects:

(i) SPK patients who are autoantibody-negative (SPK AAB-Neg), with a functional transplant supporting normal glucose tolerance and kidney function, under chronic immunosuppression (n=6)

(ii) patients with T1D (n=8)

(iii) healthy donors (HD) (n=10).

My goal was to further explore the effectiveness of GLUT1 inhibition to patient populations, and to potentially identify differences among the three groups, where diabetes and immunosuppression are important factors that need to be considered.

PBMCs were cultured in RPMI 5.5mM glucose, stimulated with anti-CD3/CD28 beads (bead-to-cell ratio 1:75), with or without 10μM of the GLUT1 inhibitor WZB117, for 6 days. After 6 days cells were collected and analyzed using a comprehensive 37 parameters flow cytometry panel (**Table 4**). In all groups, on day 6, death cells were excluded using ZombieUV.

Culturing T cells in the presence of WZB117 decreased cell viability in the HD group (91 ± SD 3% Ctrl vs 89 ± SD 3% WZB117, n=10, P=0.0352), T1D group (75 ± SD 12% Ctrl vs 63 ± SD 22% WZB117, n=8, p=0.0156) and in SPK group (75 ± SD 10% Ctrl vs 62 ±

SD 17% WZB117, n=6, p=0.0312). All groups were compared to the initial cell viability right after thawing, indicated as 0 days *in vitro* (0 DIVs) (**Fig. 30A**).

Although WZB117 showed only a minor effect on T cell viability, it strongly affected T cell proliferation. On day 6, WZB117 reduced proliferation in $CD4^+$ (55 ± SD 24% Ctrl vs 24 ± SD 12% WZB117, n=8, p=0.0078) and in $CD8^+$ T cells (60 ± SD 28% Ctrl vs 30 ± SD 17% WZB117, n=8, p=0.0078) in T1D patients (**Fig. 30B**). Also in the SPK group, WZB117 reduced proliferation both in $CD4^+$ (81 ± SD 4% Ctrl vs 49 ± SD 16% WZB117, n=6, p=0.0312) and in $CD8^+$ T cells (81 ± SD 11% Ctrl vs 51 ± SD 16% WZB117, n=6, p=0.0312). In the HD group the reduction of proliferation was less profound (49 ± SD 13% Ctrl vs 40 ± SD 16% WZB117, n=10, p=0.0195 for $CD4^+$ T cells) (71 ± SD 10% Ctrl vs 61 ± SD 15% WZB117, n=10, p=0.059 for $CD8^+$ T cells) (**Fig. 30B, C**).

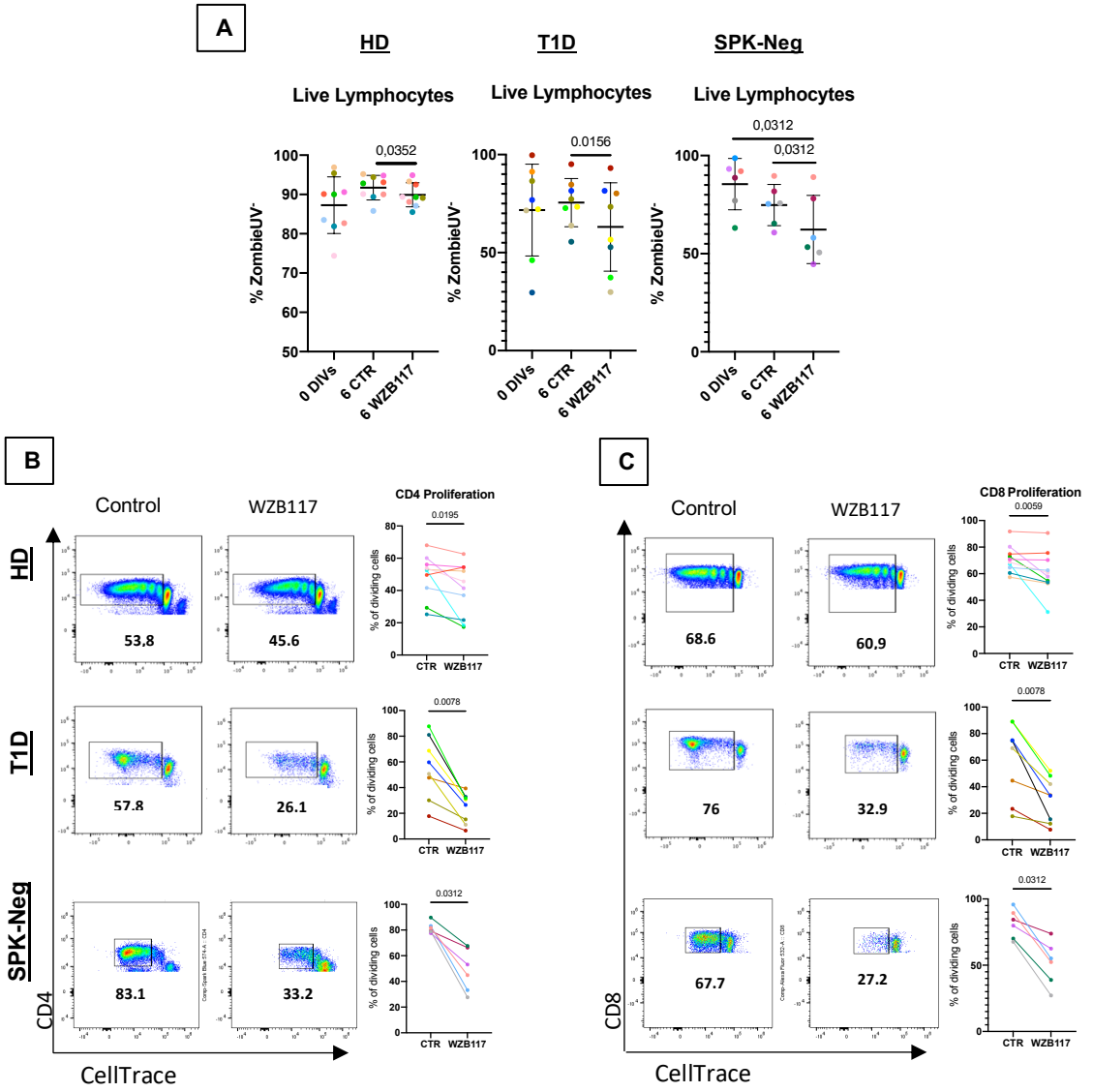


Figure 30. GLUT1 inhibition reduces the proliferation of CD4$^+$ and CD8$^+$ T cells. *(A) In vitro T cell viability after anti-CD3/CD28 stimulation, at day 6, in the presence or absence of the GLUT1 inhibitor WZB117. Inhibition of CD4$^+$ (B) and CD8$^+$ (C) T cell proliferation at 6 days in the three groups, showed as representative plots and graphs expressing means of percentage. The inhibition of proliferation was statistically significantly higher in the T1D and SPK-Neg groups compared to HD. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

I subsequently investigated whether cell numbers for the main T cell subsets of CD4$^+$ and CD8$^+$ were impacted by WZB117 treatment. Cell subsets were identified by the expression of cell markers as indicated in **Table 5**. Cell numbers were calculated as counts per µL for control and WZB117 cultures. Notable observations were reductions in the numbers of CD4$^+$ naïve, Tscm and Tcm cells among T1D and SPK-Neg patients (**Fig. 31A**). CD8$^+$ naïve, Tscm, Tcm and Tem cells also showed lower counts after WZB117 treatment (**Fig. 31B**).

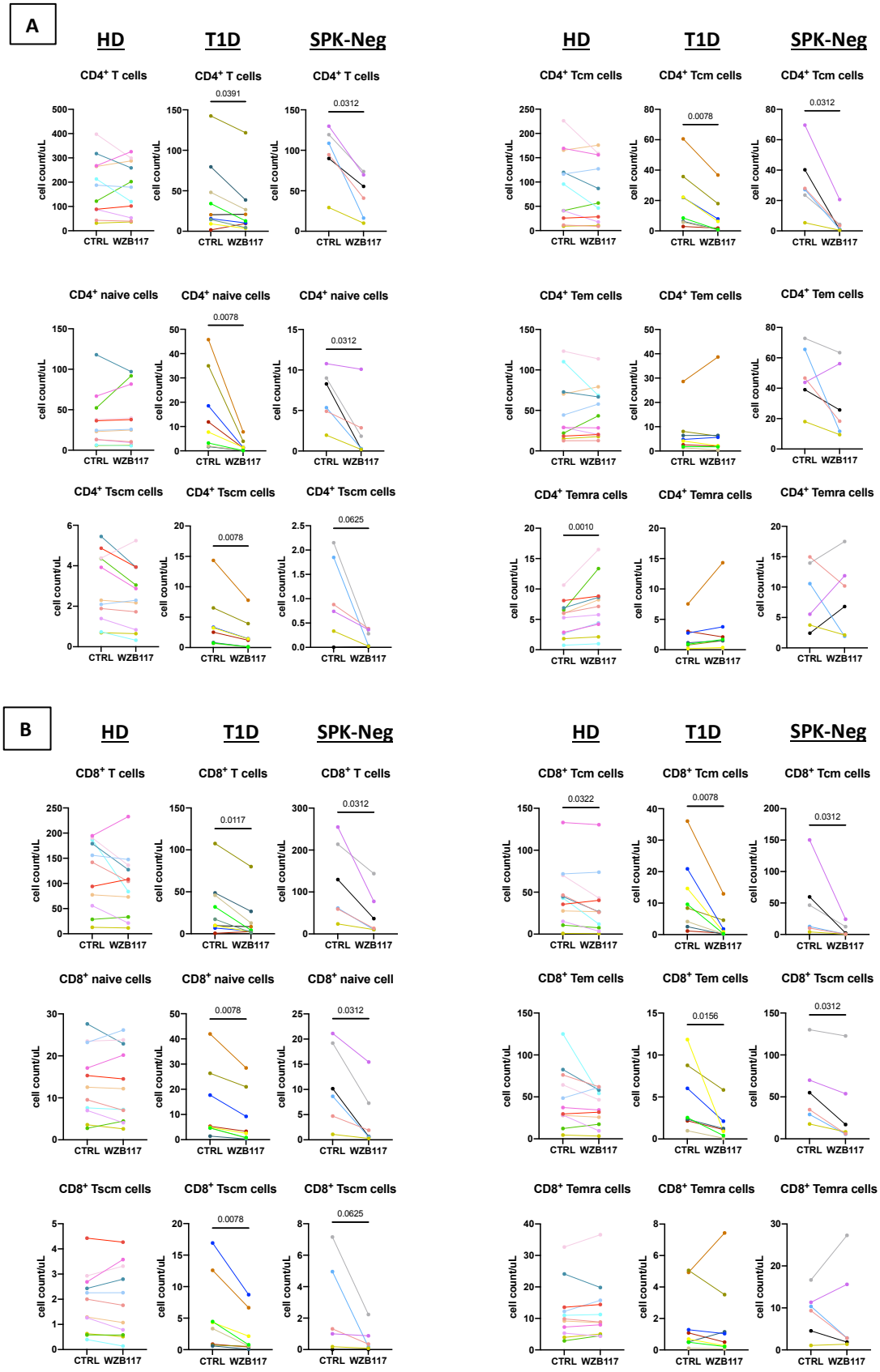

A
HD
T1D
SPK-Neg
CD4+ T cells
CD4+ Tcm cells
CD4+ naive cells
CD4+ Tem cells
CD4+ Tscm cells
CD4+ Temra cells
B
CD8+ T cells
CD8+ Tcm cells
CD8+ naive cells
CD8+ Tem cells
CD8+ Tscm cells
CD8+ Temra cells
cell count/uL
CTRL WZB117
0.0391
0.0312
0.0078
0.0010
0.0322
0.0117
0.0156
0.0625

Figure 31. Impact of in vitro GLUT1 inhibition on cell counts in CD4$^+$ and CD8$^+$ T cell naïve and memory subsets*. Assessment after 6 days in culture, with/without WZB117, of CD4$^+$ (A) and CD8$^+$T cells (B). Data shown are the absolute cell counts (cells/µL) for control and WZB117 cultures, which are compared using a paired Wilcoxon test. Data shown as Mean ± SD.*

3.4.2 GLUT1 inhibition reduced proliferation of Treg cells

CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells (Treg cells) also express GLUT1 but, like quiescent T cells, they primarily utilize oxidative phosphorylation and fatty acid oxidation to meet their metabolic requirements. Recently it has become increasingly evident that Treg cells also rely on glycolysis for activation and proliferation, but to a lesser extent than T helper cells. Using the experimental setting described above, I investigated the effect of the GLUT1 inhibitor WZB117 on Treg cells. Although Treg cells are expected to be less dependent on glycolysis during activation and proliferation, we did observe reductions in proliferation (**Fig. 32**). As previously noted, also in CD4$^+$ Treg cells the effect of WZB117 on proliferation is more pronounced in T1D (78 ± SD 8% Ctrl vs 48 ± SD 26% WZB117, n=8, p=0.0078) and SPK groups (78 ± SD 8% Ctrl vs SD 51 ± 19% WZB117, n=6, p=0.0313), compared to HD (60 ± SD 17% Ctrl vs 50 ± SD 18% WZB117, n=10, p=0.0098).

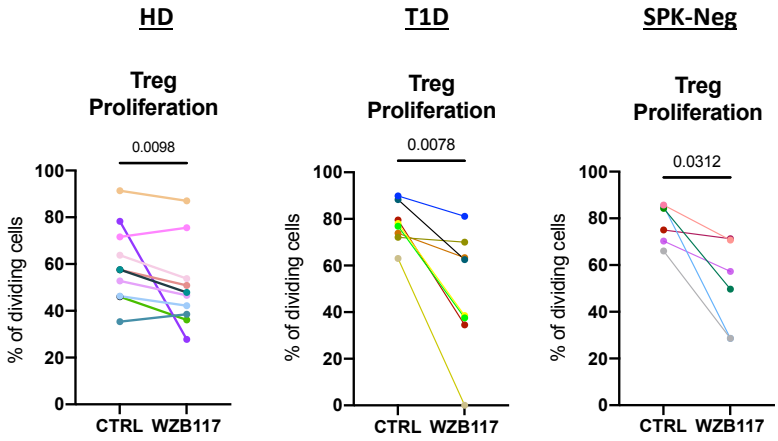


Figure 32. In vitro proliferation of Treg cells after CD3/CD28 stimulation, at 6 days, in the presence or absence of the GLUT1 inhibitor WZB117*. Percent inhibition of Treg cells proliferation in the three groups, at 6 days, relative to the control culture. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

The study of cell numbers for all Treg cell subsets, calculated as absolute numbers (counts per µL) for control and WZB117 cultures, showed reductions in Tcm and naïve Treg cells after WZB117 treatment in T1D and SPK-Neg patients.

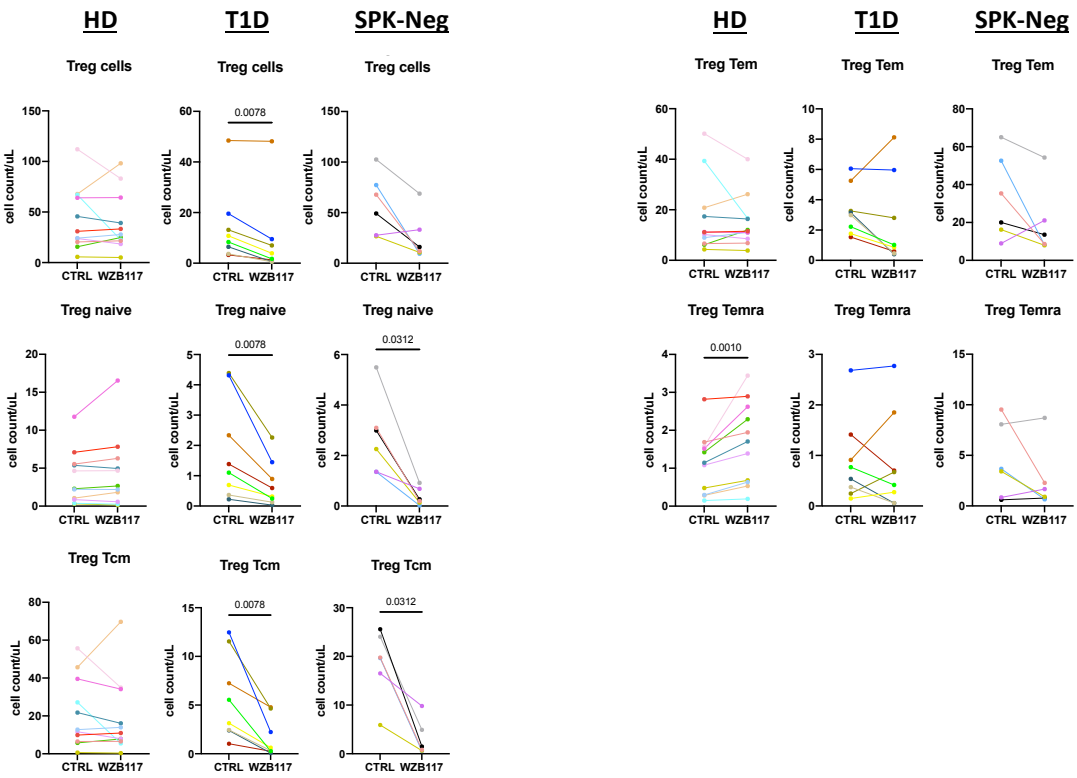


Figure 33. Impact of in vitro GLUT1 inhibition on cell counts for CD4$^+$ Treg cell subsets. *Flow cytometry assessment after 6 days in culture, with/without WZB117, of CD4$^+$ Treg cells. The data shown are the absolute counts (cells/µL) for control and WZB117 cultures. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

3.4.3 The T cell expression of key activation markers was reduced after treatment with WZB117

After 6 days of *in vitro* culture with or without 10μM of WZB117 and anti-CD3/CD28 beads, I determined phenotypic changes at the protein levels, using flow cytometry to examine both surface and intracellular markers (see **Table 4**). This approach allowed me to unravel the alterations caused by GLUT1 inhibition during T cell activation.

Overall, when examining $CD4^+$ (**Fig. 34**) and $CD8^+$ (**Fig. 35**) T cell populations, I noted MFI reductions that consistently occurred in samples from T1D and SPK-neg patients but were rarely noted in samples from HD. These reductions impacted key activation markers such as CD25, CD38, and ICOS. In addition, the MFI values of TIGIT and PD-1 were reduced in $CD4^+$ T cells, and those of HLA-DR were reduced in $CD8^+$ T cells. The MFI of CTLA-4 was also reduced by WZB117 treatment in $CD4^+$ and $CD8^+$ T cells from HD and T1D patients, but not among the SPK-Neg patients (not shown).

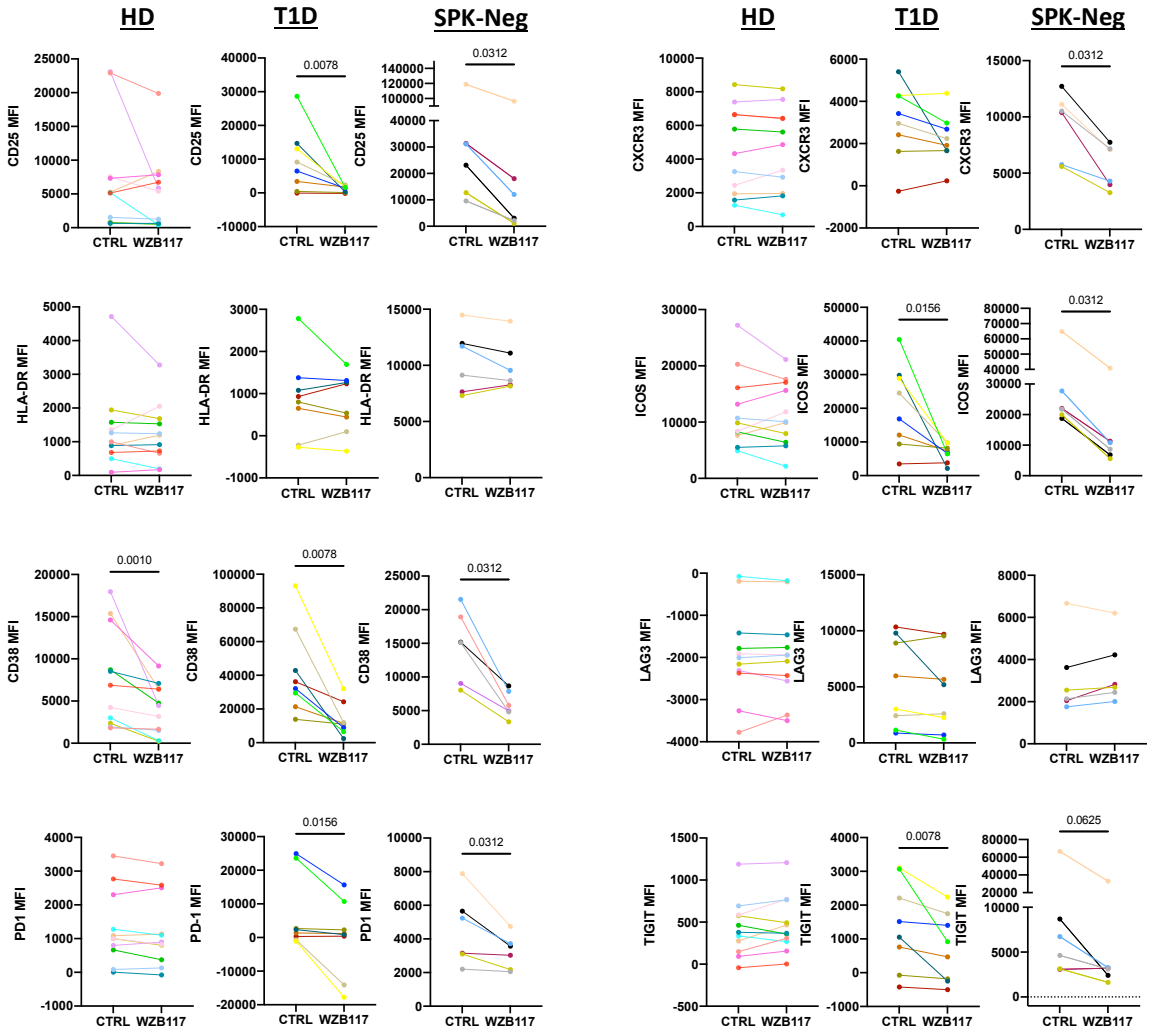


Figure 34. Phenotypic changes associated with GLUT1 inhibition in vitro in CD4$^+$ T cells. *Flow cytometry assessment of key phenotypic markers after 6 days in culture, with/without WZB117. Only selected markers are shown, those showing the most evident changes, from our 37-parameter panel. MFI of CD4-positive gate. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

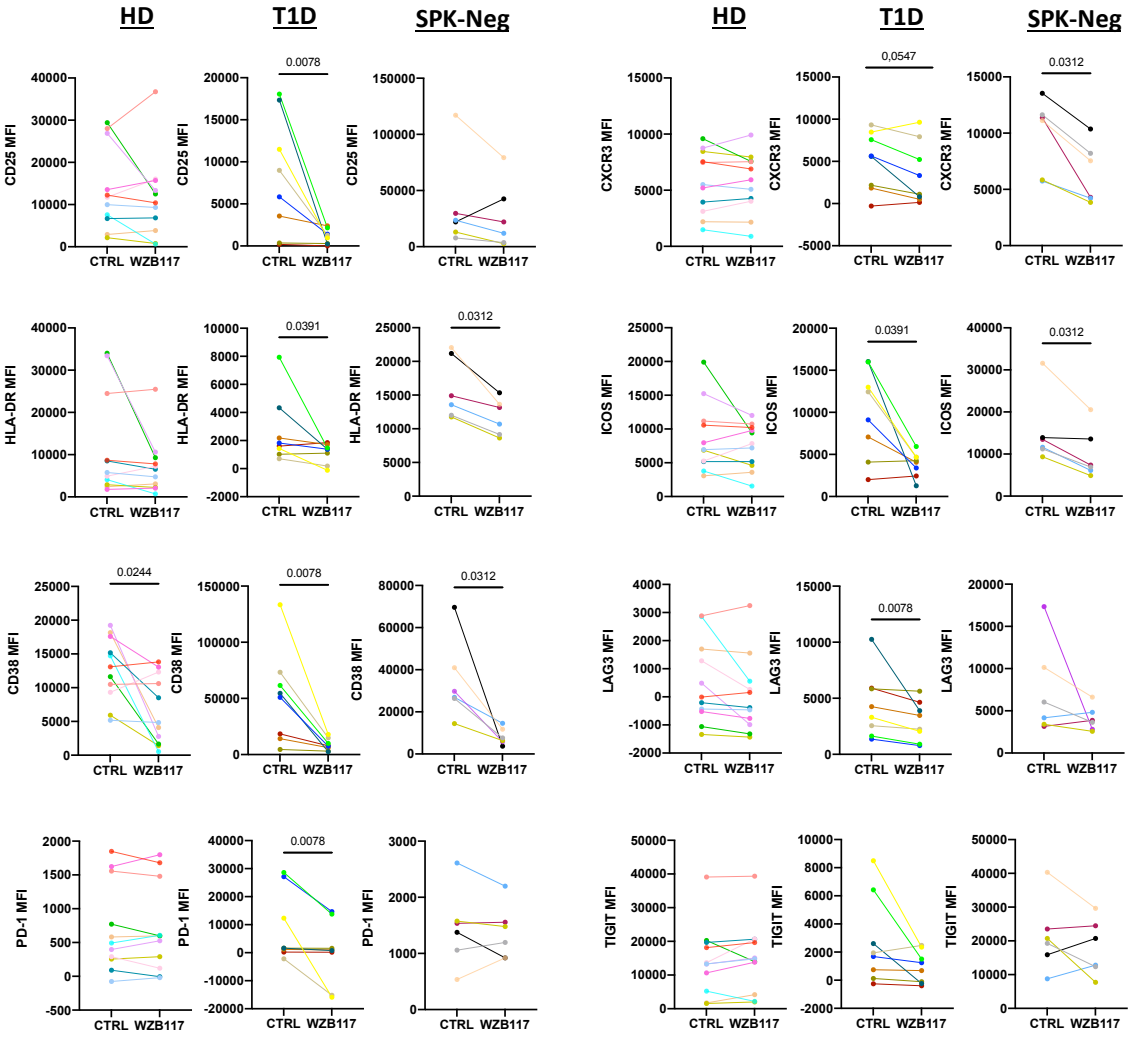


Figure 35. Phenotypic changes associated with GLUT1 inhibition in vitro in CD8$^+$ T cells. *Flow cytometry assessment of key phenotypic markers after 6 days in culture, with/without WZB117, in CD8 T cells. Only selected markers are shown, those showing the most evident changes, from our 37-parameter panel. MFI of CD8-positive gate. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

Among Treg cells, the MFI values of CD25 and CTLA-4 were reduced (**Fig. 36**); however, the expression levels of both molecules in the control cultures, after T cell activation, were higher in Tregs than in the total CD4$^+$ T cell population (for HD CD25 MFI mean 30,062 vs mean 7,291, p=0.0049, respectively; for T1D, CD25 MFI mean 62,051 vs mean 9,474, p=0.0156; for SPK-Neg patients, CD25 MFI mean 64, 971 vs mean 38,767, p=0.06).

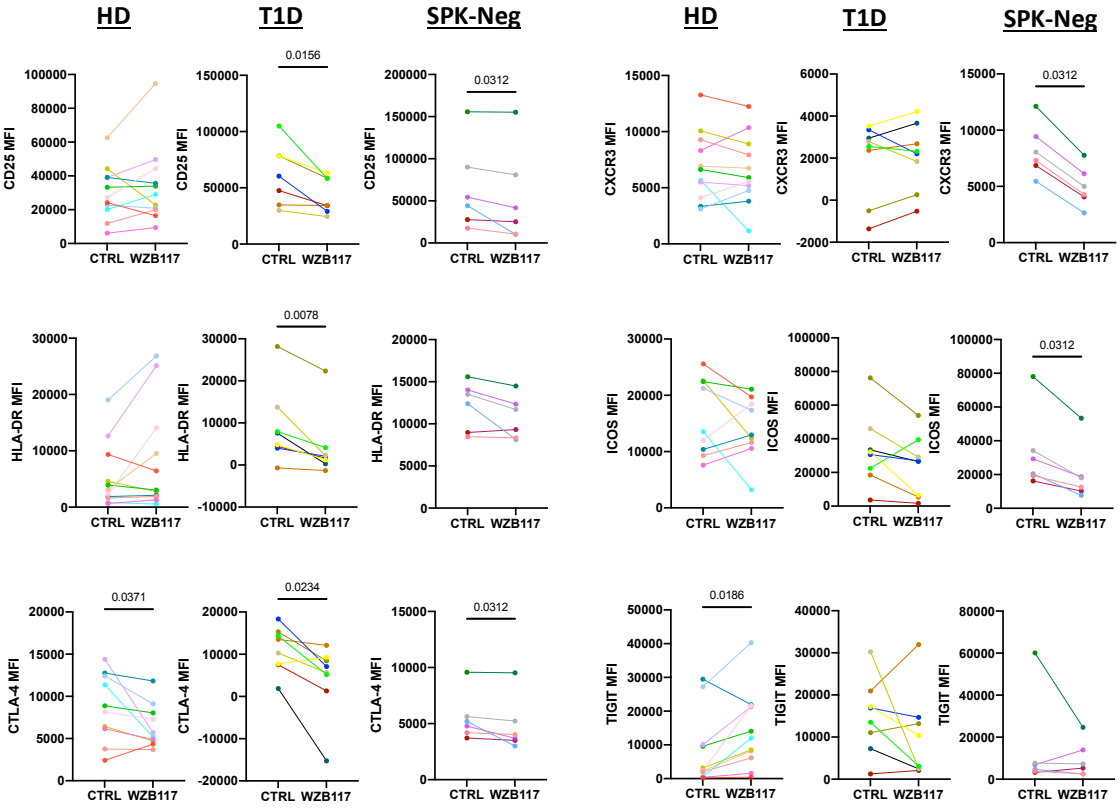


Figure 36. Phenotypic changes associated with GLUT1 inhibition in vitro in Treg cells. *Flow cytometry assessment of key phenotypic markers after 6 days in culture, with/without WZB117, in CD4 Treg cells. Only selected markers are shown, those showing the most evident changes, from our 37-parameter panel. MFI of Tregs-positive gate. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

Similar differences were also observed between Treg cells and total $CD4^+$ T cells upon treatment with WZB117, and importantly the percent reduction in the CD25 MFI was significantly lower in Tregs compared to total $CD4^+$ T cells (mean percent reduction 30% vs 88%, p=0.031 among T1D patients; 17% vs 44%, p=0.031 among SPK-Neg patients; 14% vs 28%, p=0.0674 in HD). Similar trends overall, but less dramatic differences, were noted for CTLA-4, and percent reductions comparing Treg cells and total $CD4^+$ T cells were not statistically different. Data are shown in **Figure 37.**

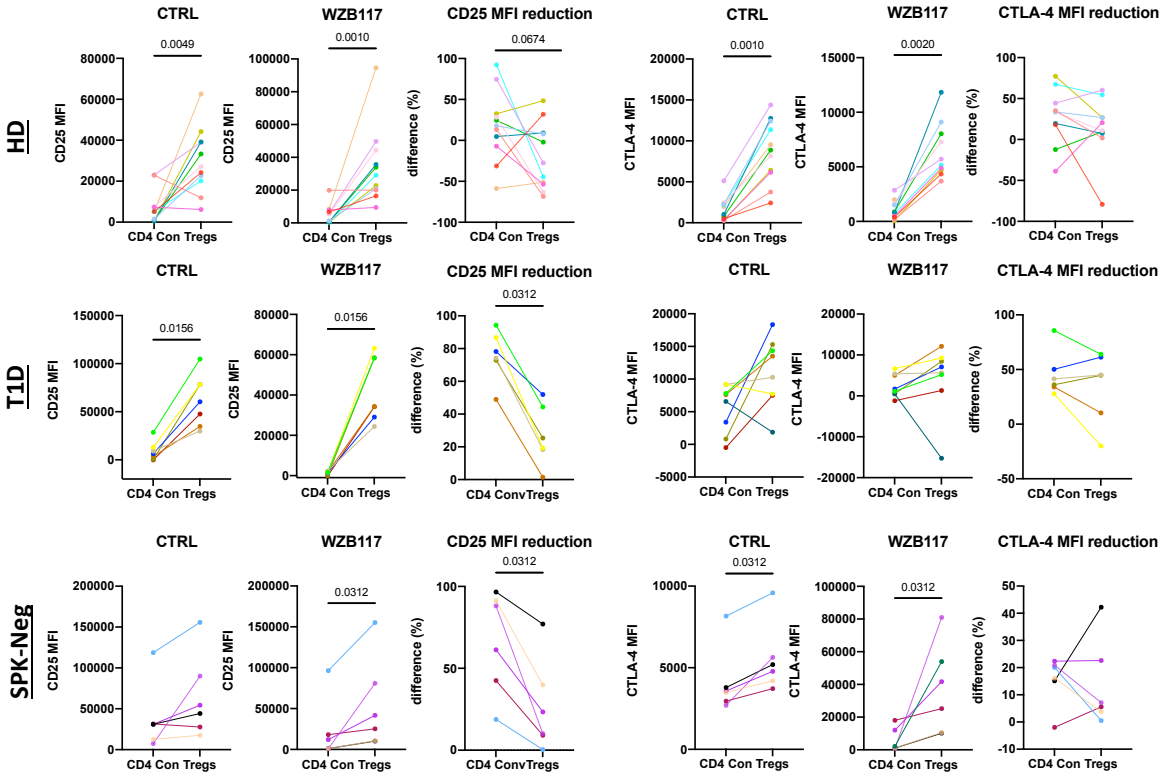


Figure 37. Impact of in vitro GLUT1 inhibition on CD25 and CTLA-4 in total CD4⁺ T cells and CD4⁺ Tregs. *Flow cytometry assessment of CD25 and CTLA-4 expression across CD4⁺ Treg cells and CD4⁺ total (Con) in HD, T1D and SPK-neg subjects, after 6 days in culture, with/without WZB117. MFI of CD4 or Treg-positive gate. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

3.4.4 Impact of in vitro treatment with WZB117 on GLUT1 and GLUT3 expression on CD4⁺, CD8⁺ and Treg cells

Another important goal of my thesis work was to assess the expression levels of GLUT1 and GLUT3. As shown in **Figure 38**, WZB117 treatment significantly reduced GLUT1 MFI levels in $CD8^+$ (2294 Ctrl vs 1727 WZB117, n=8, p=0.0156) and $CD4^+$ (1840 Ctrl vs 1220 WZB117, n=8, p=0.0156) T cells among T1D patients but not in HD ($CD8^+$ T cells: 445 Ctrl vs 478 WZB117, p=0.3203; $CD4^+$ T cells: 142 Ctrl vs 194 WZB117, p=0.2402, n=10) and SPK patients ($CD8^+$ T cells: 1881 Ctrl vs 1653 WZB117, p=0.3125; $CD4^+$ T cells: 2461 Ctrl vs 2261 WZB117, p=0.6875, n=6).

Among SPK-Neg patients, the MFI of GLUT3 trended higher, without reaching statistical significance. When examining $CD4^+$ T cell subsets (**Fig. 39**), we noted significant

reductions or trends in the MFI of both GLUT1 and GLUT3 in CD4^{+} Tcm and Tscm cells from T1D patients. In contrast, among SPK-Neg patients, the MFI levels of GLUT1 and GLUT3 trended higher or were significantly increased after WZB117 treatment, among CD4^{+} Tem, Temra, Tcm and Tscm cells. Divergent changes were also seen between T1D and SPK-Neg patients for some T helper subsets, primarily Th17, Th2 and Th1 cells (**Fig. 40**), and for CD8^{+} T naïve, Tcm and Tscm, in which GLUT3 was upregulated in SPK-Neg patients (**Fig. 41**). Among Treg subsets (**Fig. 42**), we observed significant changes in the MFI levels of GLUT3, only in SPK-Neg patients, which were increased. It is possible that the upregulation of GLUT3 may be compensatory.

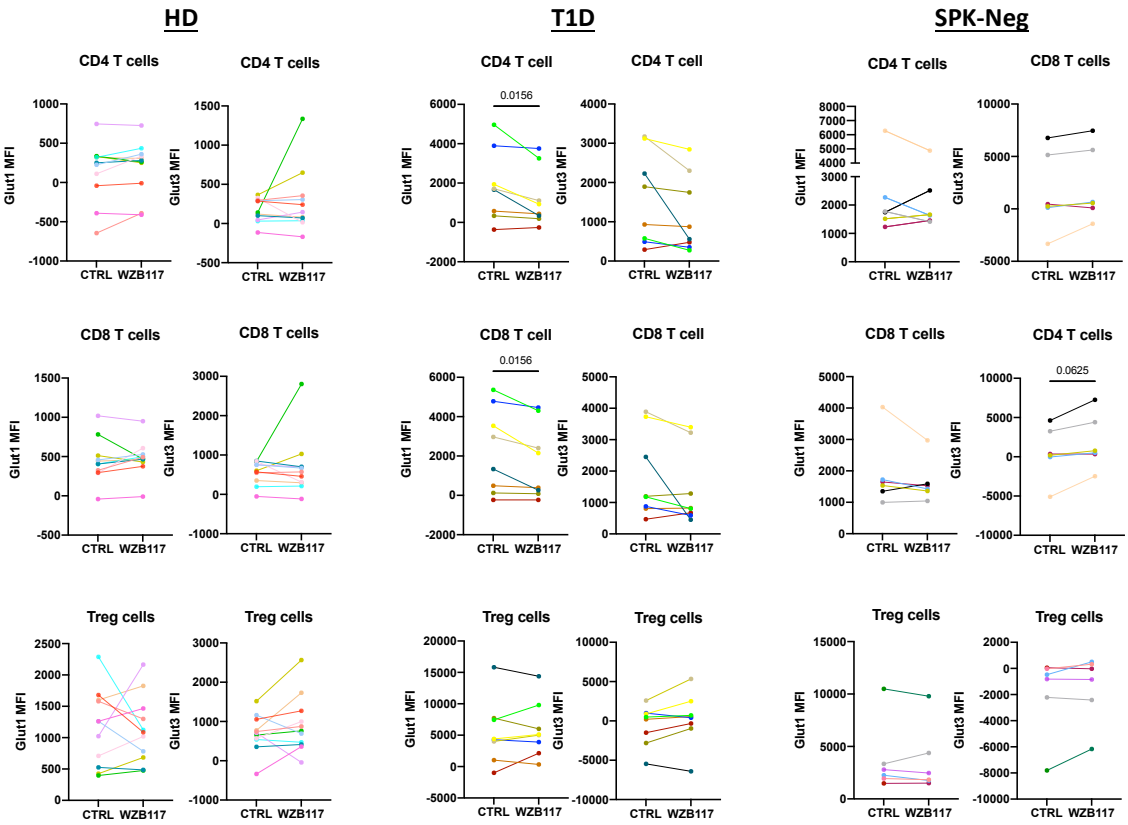


Figure 38. Impact of WZB117 on GLUT1 and GLUT3 expression on CD4^{+}, CD8^{+} and CD4^{+} Treg cells. *Flow cytometry assessment after 6 days in culture, with/without WZB117. Data shown as Mean ± SD. MFI of CD4 or CD8 or Tregs-positive gate. Comparisons were made using a paired Wilcoxon test.*

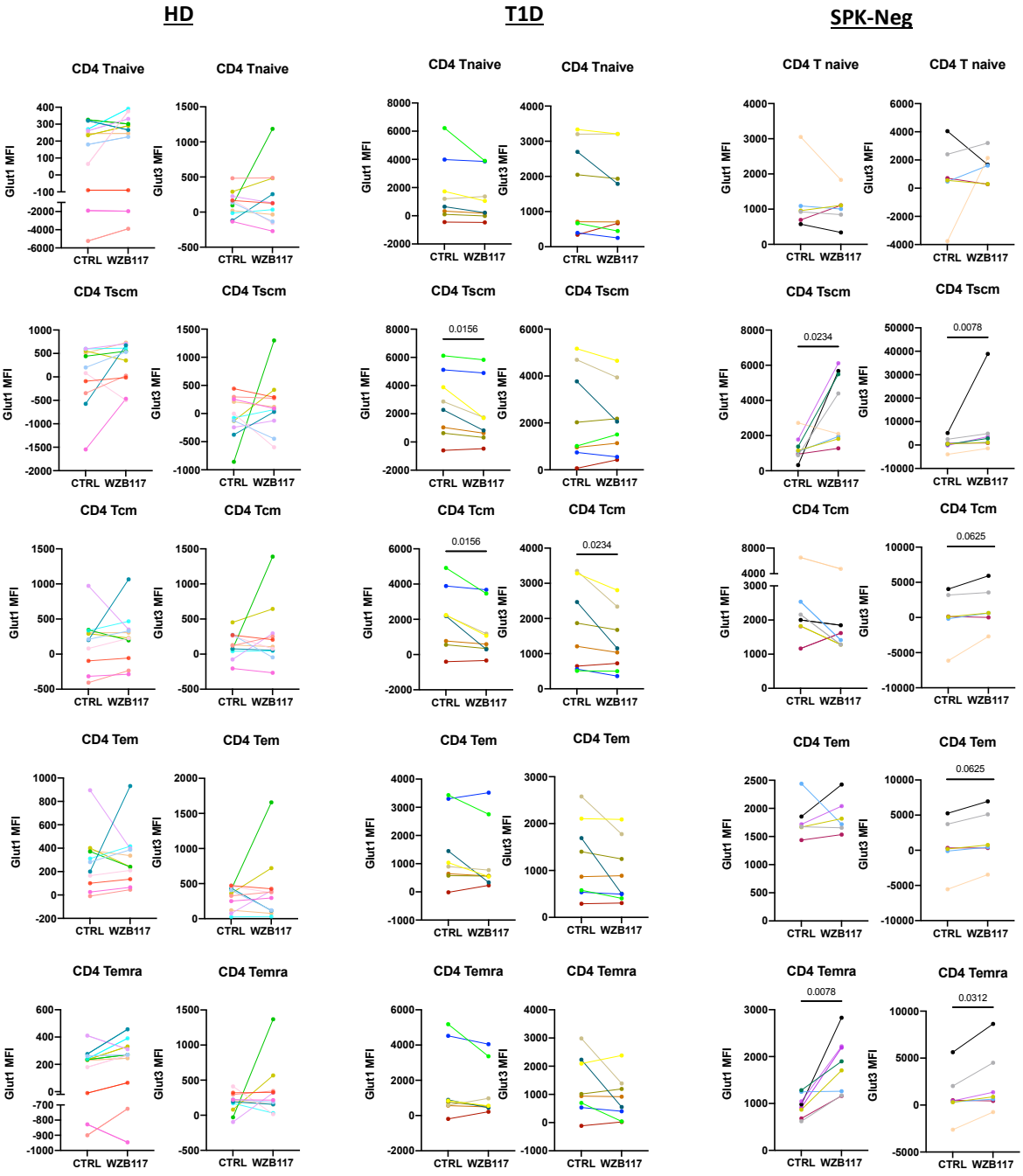


Figure 39. Impact of WZB117 on GLUT1 and GLUT3 expression in CD4$^+$ T cell subsets. *Flow cytometry assessment after 6 days in culture, with/without WZB117. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

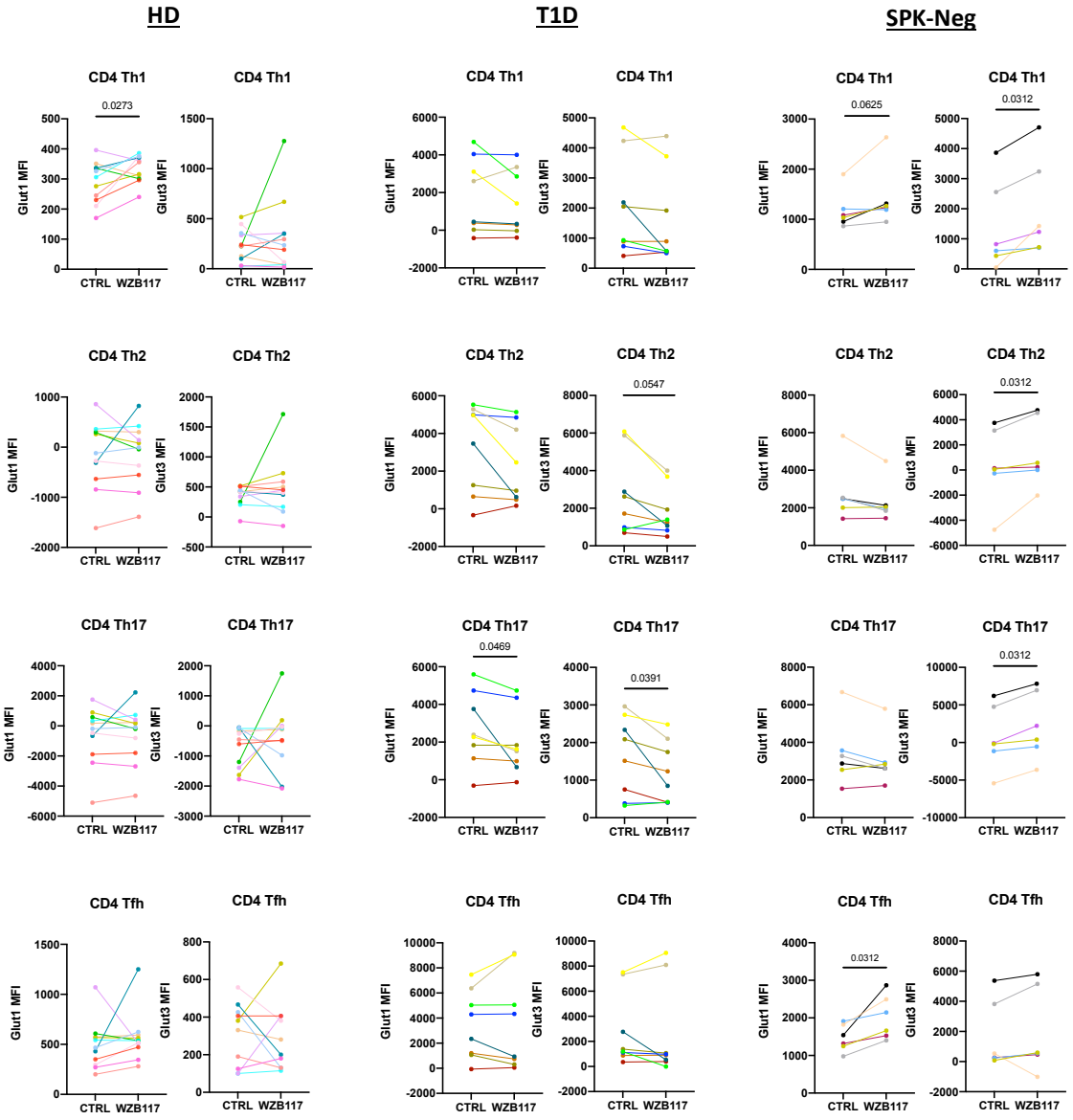


Figure 40. Impact of WZB117 on GLUT1 and GLUT3 expression in CD4⁺ T helper subsets. *Flow cytometry assessment after 6 days in culture, with/without WZB117. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

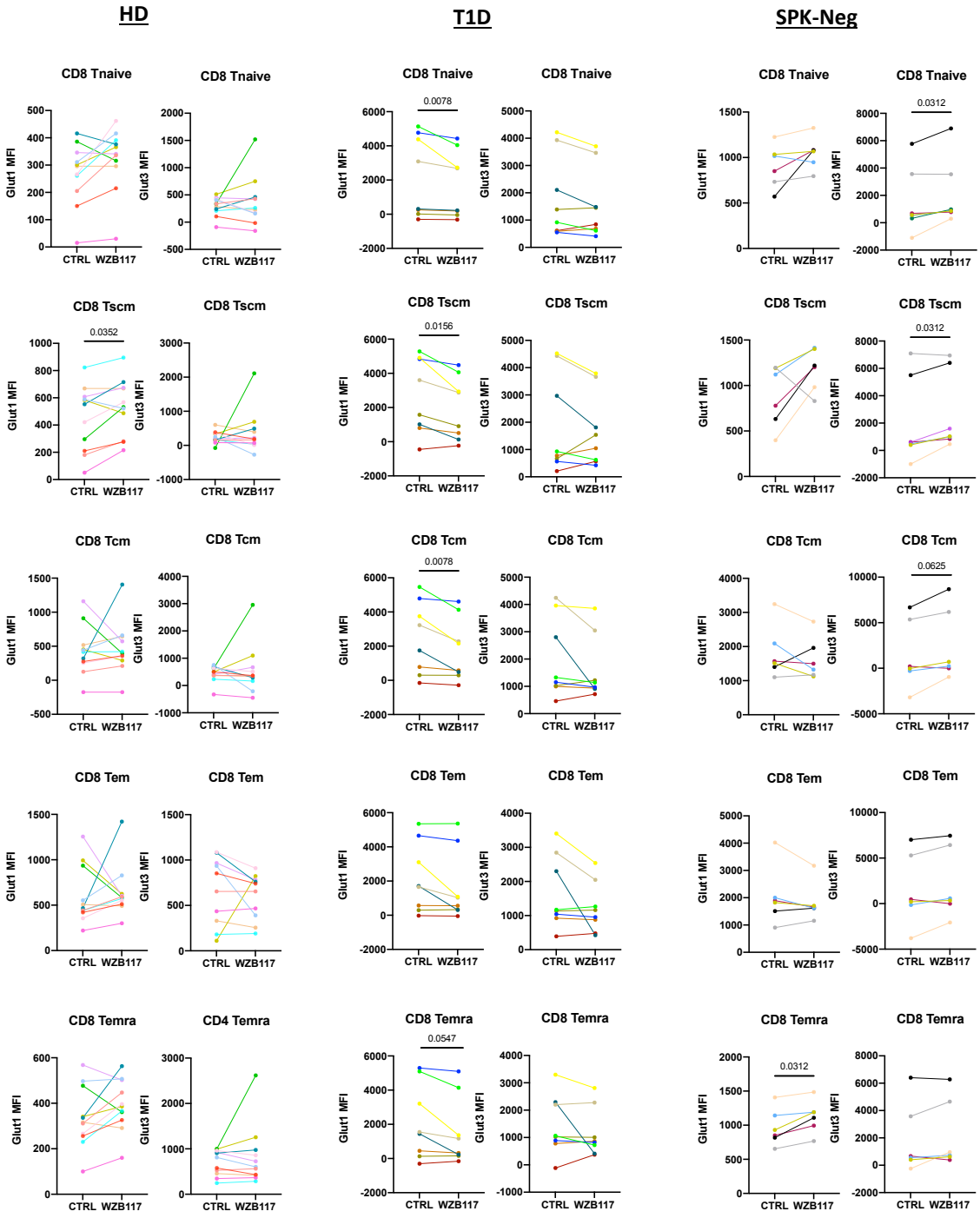


Figure 41. Impact of WZB117 on GLUT1 and GLUT3 expression in CD8$^+$ T cell subsets. *Flow cytometry assessment after 6 days in culture, with/without WZB117. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

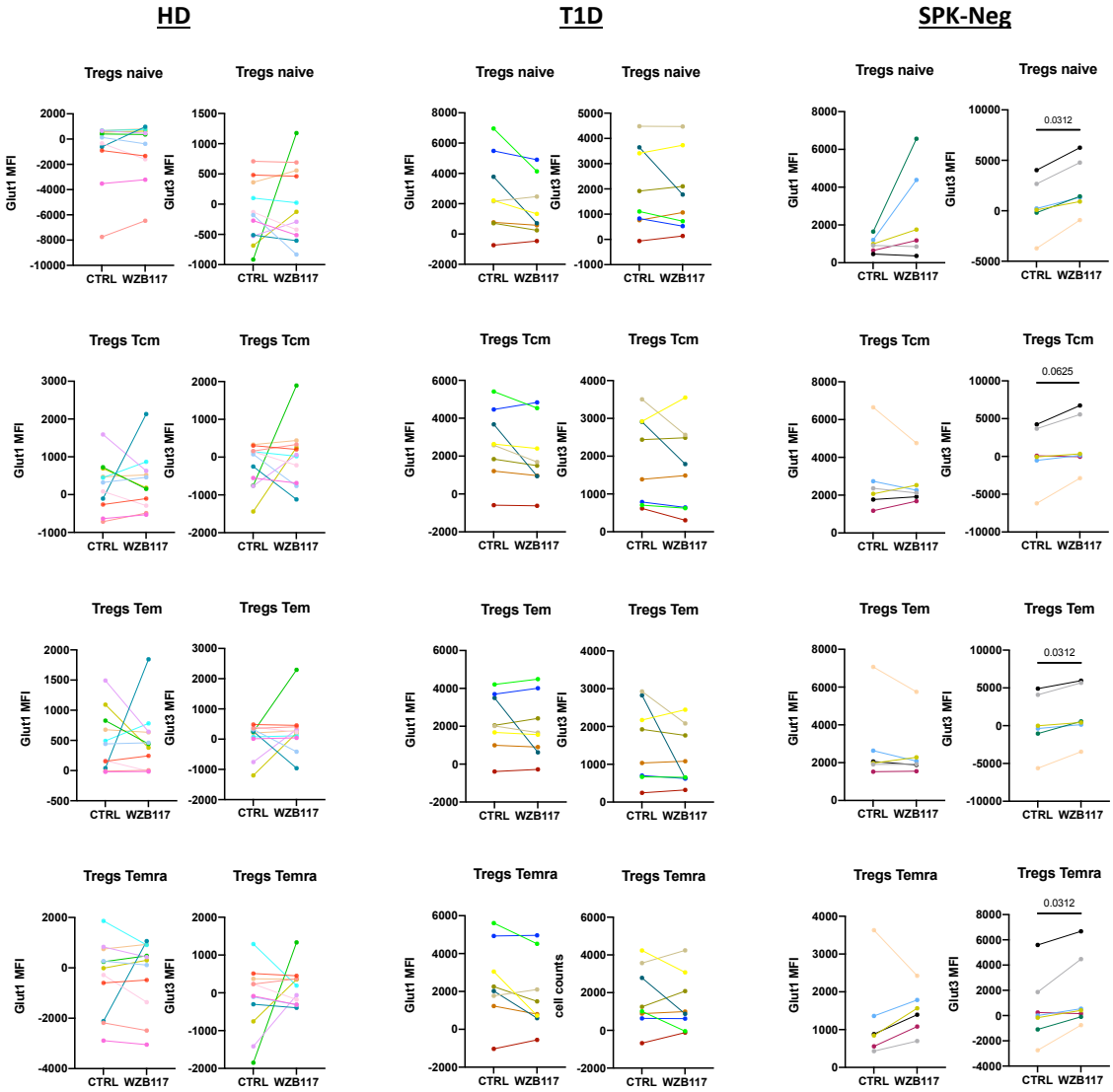


Figure 42. Impact of WZB117 on GLUT1 and GLUT3 expression in CD4$^+$ Treg cell subsets. *Flow cytometry assessment after 6 days in culture, with/without WZB117. Data shown as Mean ± SD. Comparisons were made using a paired Wilcoxon test.*

3.4.5 Evaluation of potential off-target and side effects of WZB117

The widespread expression of the glucose transporter GLUT1 in various human tissues, where it plays a crucial role in basal glucose uptake (insulin-independent), prompts a careful evaluation of potential off-target and side effects of the GLUT1 blockade approach.

To prepare in advance a preclinical model to study the effect of WZB117 in protecting beta cells from immune attack, we performed a preliminary set of experiments aiming at excluding major toxic effects on T cells that exclusively rely on GLUT1 for specific functions. These include beta cells, in which GLUT1 is used as an extracellular glucose sensor to regulate insulin secretion, erythrocytes, where GLUT1 is predominantly used for the uptake of glucose and the oxidized form of vitamin C, and endothelial cells of the blood-brain barrier, where glucose transport across from blood to brain is mediated by GLUT1.

I tested whether WZB117 affected the insulin secretion of pancreatic beta cells. In contrast to islets from NOD mice, human islets predominantly express GLUT1 and, to a much lesser extent GLUT3, instead of GLUT2 [171]. To investigate potential WZB117 off-target effects on pancreatic beta cells we conducted a perifusion assay using the human pancreatic beta cell line EndoC-βH1, widely used as a model of insulin secretion. The Biorep perifusion system allowed dynamic stimulation of insulin secretion from beta cells in the presence of secretory stimuli and drugs.

During the assay, cells were initially stimulated with a low glucose solution (2mM) for 80 minutes, followed by a 20-minute stimulation with a 15mM glucose solution. Subsequently, cells underwent a 20-minute stimulation with 2mM glucose and another 20-minute exposure to 30mM potassium chloride solution (KCl), to depolarize cell membranes facilitating the release of insulin granules. Finally, the last 20 minutes cells were perfused again with a low glucose concentration solution (**Fig. 43**).

In **Figure 43** yellow squares indicate the WZB117 treatment. Results showed that when WZB117 was administered throughout the entire experiment (blue line) a significant reduction in insulin release in response to glucose was observed, compared to the control

condition without WZB117 (black line). When WZB117 was added after the first 80 minutes and maintained until the end of the experiment (red line), a slight decrease in insulin secretion was noted. Notably, the addiction of WZB117 only during the initial 80 minutes (green line) caused a minimal reduction in insulin secretion that showed to be reversible upon the removal of the drug. Importantly, insulin secretion in response to KCl remained preserved, suggesting that the reduction of insulin secretion was not due to toxic effects on beta cells. Similar effects of reduced insulin secretion following GLUT1 blockade were also observed in human pancreatic beta cells treated with the GLUT1 inhibitor STF-31 [172].

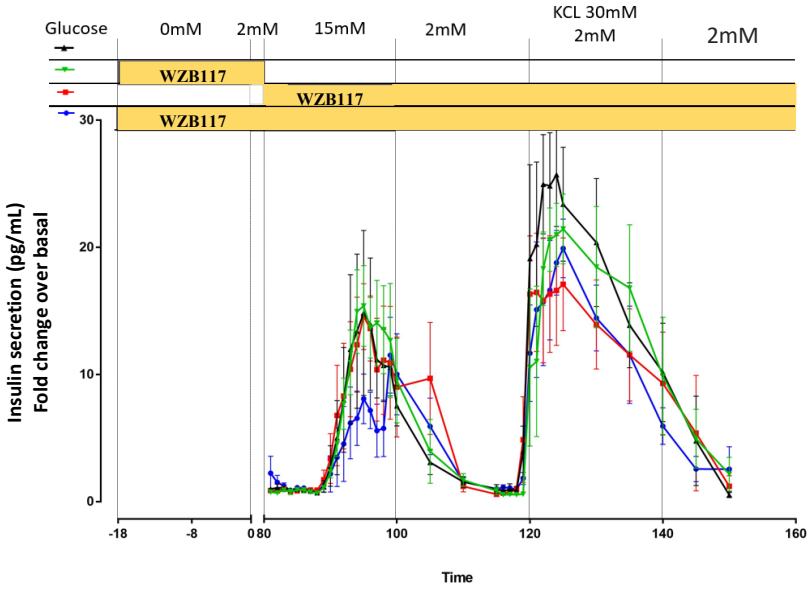


Figure 43. Insulin secretion test of EndoC-βH1 cells. *Cells were perifused with low (2mM) and high (15mM) glucose with or without WZB117 at different time points. Values are expressed as fold change over the basal. Values are presented as mean ± SEM.*

To investigate the direct impact of WZB117 on human red blood cells, an erythrolysis assay was performed. The erythrolysis assay is a quick valuable tool for assessing a molecule's cytotoxicity on erythrocytes. Whole blood samples from HD were diluted in PBS and incubated with 10µM of WZB117 for 30 minutes. As a positive control, whole blood was treated with distilled water, a known inducer of erythrolysis. Results demonstrated that WZB117 did not induce lysis of red blood cells, as evidenced by comparing it to negative control (PBS) (**Fig. 44**). These findings were consistent with previous observations made with the GLUT1 inhibitor STF-31[155].

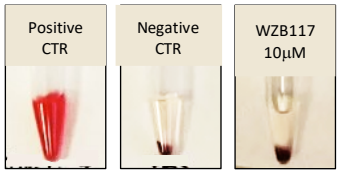


Figure 44. Representative photos of human red blood cells experiment. *Whole blood was treated with distilled water (left), PBS (middle) and WZB117 10µM (right) for 30 minutes.*

Moreover, it was crucial to assess the tolerability of WZB117 administration *in vivo*. Previous experience in my laboratory brought to the set-up of an *in vivo* model to resemble the human setting of islet transplantation. The model consisted of diabetic MHC class I and class II deficient NSG mice re-populated with human HLA-A*0201 PBMCs and transplanted with human HLA-A2*0201 islets. The administration of WZB117 at a dose of 10mg/kg/day was achieved using 1004D ALZET osmotic pumps, which constantly released the drug for 28 consecutive days. The results revealed that the treatment was well tolerated with no observed major toxic effects or mortality, and the growth curve was similar to mice treated with vehicle (**Fig. 45**).

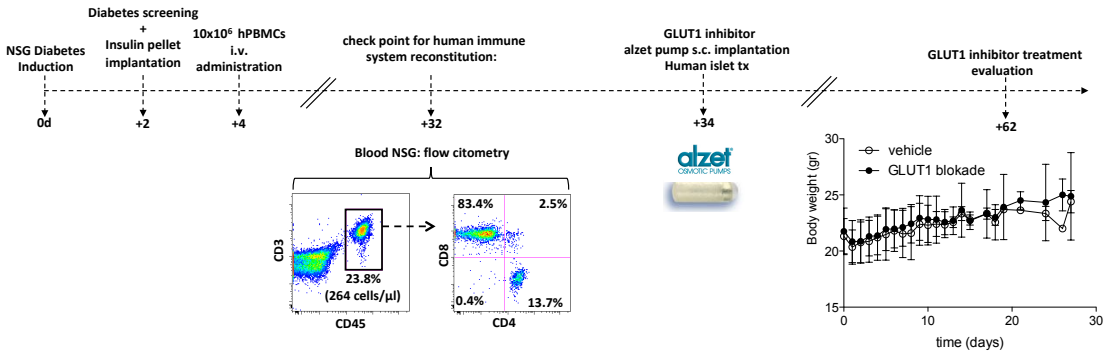


Figure 45. Inhibition of GLUT1 in preclinical model of T1D*. Set-up of NSG preclinical diabetic humanized model for the treatment with WZB117. FACS plots are representative of a staining of CD45RA⁺ CD3⁺ CD4⁺ or CD8⁺ T cells in an NSG mouse 4 weeks after PBMCs infusion. The graph shows the weight (Y axis) over time (X axis) of mice during treatment with vehicle or WZB117.*

Chapter 4

DISCUSSION

DISCUSSION

Controlling the activation of autoreactive T cells in patients with T1D, as well as their reactivation in patients following pancreas transplantation, remains a therapeutic challenge. An ideal target molecule to selectively control the activation of autoreactive T cells should spare other T cells that are not involved in the process. GLUT1 represents the rate-limiting step in cellular glucose uptake, and glycolysis is required for T cell activation, proliferation and function. Starting from this standpoint, targeting GLUT1 during the metabolic switch of activated T cells from the resting state can provide the opportunity to selectively target autoreactive and pathogenic T cell clones.

In the first part of the thesis, I have investigated three different groups of SPK transplant recipients, who are classified as AABs negative, converter and T1D recurrent, to delve into the immune phenotypes associated with recurrence of autoimmunity. In particular, I investigated changes in the size of the major cellular subsets and their subpopulations, as well as differences in the expression of key phenotypic markers, to delineate the metabolic and differentiated status of T cells, within the study cohorts. The study of GLUT1 expression garnered specific attention during this phase of the thesis, aiming to elucidate whether its levels could be associated with recurrence of T1D. I then focused on the detection and characterization of autoreactive T cell populations inclusive of T cells targeting the diabetes-associated autoantigens GAD65, IGRP and (Pro)-insulin in the transplanted patients. This part of the work aimed to identifying patients' characteristics associated with T1DR in pancreas transplantation.

A key finding emerging from our analysis of T cells from SPK patients with T1DR is the increased expression levels of GLUT1 by islet autoreactive T cells, and not by T cells with irrelevant reactivity. This provides a rationale to further investigate GLUT1 as a therapeutic target that meets the above criteria for selectively targeting autoreactive T cells, prevent or inhibit their activation and survival, and promote their exhaustion.

Therefore, I focused the second part of the work on the characterization of the effect of a small molecule that inhibits GLUT1 on T cells, WZB117. WZB117 has been widely studied *in vitro* and *in vivo* over the past decade in the context of tumors, showing

encouraging effects in reducing cancer cell proliferation and tumor growth [160, 173-175]; however, its effects on T cells are largely unknown. I conducted an extensive characterization of cells treated with WZB117, in terms of alterations of cell subsets and phenotypic features among healthy donors, patients with T1D and patients who had undergone pancreas transplantation and have a functional, healthy pancreas (SPK-Neg).

4.1 Analysis of immune subsets and phenotypic features associated with recurrence of T1D in SPK patients

From its initial description in 1984 [102] to the present, the causes that lead to the recurrence of autoimmunity following pancreas or islet transplantation have remained elusive. In an attempt to identify a phenotypic signature and/or changes in the prevalence of naïve/memory subsets of T cells in patients with T1D recurrence, I performed *ex-vivo* analysis on samples from three different groups of patients who have undergone SPK transplantation and are chronically immunosuppressed, along with patients with T1D (mean age 13.6 years $\pm$ SD 2.4) and healthy donors (mean age 50 years $\pm$ SD 9). SPK patients who remained normal glucose tolerant, with a functional, healthy pancreas and no AABs, represented the control group (SPK-Neg; mean age 48.4 years $\pm$ SD 10) for the other SPK groups. SPK patients who showed post-transplant AAB positivity (SPK-Conv; mean age 50.1 years $\pm$ SD 11.4) were considered to have a higher risk of developing T1D recurrence, based on an earlier study of the University of Miami SPK cohort [176]. SPK-T1DR patients experienced hyperglycemia and had to return to insulin treatment, having developed recurrent diabetes; most of them had experience AABs seroconversion during the post-transplant follow-up (mean age 49.2 years $\pm$ SD 8.8).

When examining whole lymphocyte populations, all SPK groups demonstrated higher frequencies of $CD4^+$ Tem cells compared to HD and T1D patients, which may be consistent with expansion of effector memory cells in immunosuppressed subjects. I also observed some differences among SPK groups in terms of frequency of cell subsets or phenotypic markers: $CD8^+$ Tem cells were increased in frequency among T1DR patients compared to HD, T1D, and importantly compared to SPK-neg patients. This is consistent with a greater expansion of effector memory T cells in those SPK patients who developed T1DR.

Moreover, SPK-Neg patients showed a significant higher GLUT3 expression than other SPK groups and compared to T1D and HD groups. This result suggests that in a group of SPK patients with normal graft function and no AABs, their T cells may preferentially transport glucose using GLUT3.

We have demonstrated that T cells in T1D patients exhibit a notably more activated phenotype when compared to HD, highlighted by increased expression of various activation markers. Furthermore, we observed an increase in the $CD4^+$ Temra subpopulation and a reduction in the levels of markers critical for Treg function, including CTLA-4, PD-1, FoxP3, and TIGIT. These findings collectively suggest an enhanced state of immune activation in T1D patients.

SPK-Conv patients had a higher proportion of $CD8^+$ Tscm and a lower proportion of $CD4^+$ and $CD8^+$ Temra compared to T1D patients. A common trend observed across T cell subpopulations was the reduction of Tcm in all T1D and SPK groups compared to HD. The naïve T cells were reduced in SPK groups, while the frequency of Tem was increased. There was also a trend towards increased Tscm, although the statistical significance was no obvious. In interpreting these findings, it is crucial to consider that HD and all SPK groups are age-matched, whereas T1D patients are notably younger, indicating that T cell characteristics may be influenced by the age of the patients, but also by the effects of immunosuppression, and the underlying disease state. The HD group was matched to the SPK groups, and a group of younger control subjects to match the age of the T1D patients was not easily obtainable.

Notably, GLUT1 expression levels were consistently higher in $CD8^+$ and $CD4^+$ Tscm compared to other T cell subsets, implying that targeting GLUT1 may preferentially impact this specific T cell population which was reported to be a major driver of islet autoimmunity in NOD mice [95]. This observation aligns with previous research conducted in our laboratory, which identified elevated GLUT1 expression in $CD8^+$ Tscm cells from both HD and T1D patients [97].

However, due to the limitations of our study, including a relatively small sample size and inconsistencies in certain markers across the groups, further investigations are warranted to provide a more comprehensive understanding of the complex immune alterations in these patient populations.

4.2 Characterization of autoreactive T cells in recurrent autoimmunity

The presence of autoreactive T cells specific for T1D autoantigens in patients who have undergone pancreas or islet transplantation has been extensively described [70,79,80]. Literature data reported the presence of autoreactive T cells both in patients who developed recurrence and in patients who remained normal glucose tolerant, but also showed that frequently autoreactive $CD4^+$ and $CD8^+$ T cells in SPK patients with T1DR have a memory phenotype [80][110]. Interestingly, it has been recently described that these cells have increased expression of CXCR3 both in T1DR and SPK-Conv, compared to SPK-Negative, suggesting the potential ability to migrate to the inflamed pancreas. Also, in T1DR patients, the frequency of the Temra subset was dramatically decreased among autoreactive $CD4^+$ T cells, while naïve and memory subsets were increased [177].

In order to identify phenotypic markers that characterize the function and better define populations of autoreactive T cells, or cell subsets that are likely to play a role in the recurrence of autoimmunity, I examined and characterized autoreactive T cells in SPK patients. I used dextramers to detect autoreactive T cells, a sensitive method that was shown to be effective in the detection of antigen-specific T cell populations [178]. Compared to classical tetramer staining, which is still the most widely used technology to identify autoreactive T cells, dextramers consist of a dextran backbone with multiple pMHC complexes and stain more brightly than tetramers. Moreover, dextramers can detect T cells with a low TCR–pMHC affinity. A remaining limitation of the dextramers-based staining is that the assay requires a larger number of cells.

I provided evidence that autoreactive T cells specific for diabetes-associated autoantigens were present in all groups. The only quantitative difference I noted was an increase in autoreactive $CD8^+$ T cells in SPK-Conv compared to SPK-Neg. The presence of autoreactive T cells in SPK recipients who remained normal glucose tolerant is not *per*

se surprising, given that many groups demonstrated that these cells are detectable even in subjects with no sign of autoimmunity or diabetes [79, 80, 87]. I asked, therefore, whether autoreactive T cells may be qualitatively different between SPK-Neg patients, SPK patients found to be seroconverting for GAD65, IA2 or ZnT8 AABs, and SPK patients with T1D recurrence. A comprehensive flow cytometry analysis revealed that autoreactive $CD4^+$ T cells in T1DR patients showed a more activated and less exhausted phenotype compared to SPK-Neg and SPK-Conv, which is consistent with an active autoimmune response against beta cells. Similar but less profound differences were noted in $CD8^+$ T cells. Of note, there was increased GLUT1 expression (MFI) in the $CD4^+$ and $CD8^+$ T cells of T1DR patients overall.

However, we could discern two distinct subgroups within the T1DR patients: a subgroup exhibiting high levels of GLUT1 expression, concomitant with heightened expression of activation markers and diminished exhaustion markers. Conversely, the other subgroup displays lower GLUT1 expression alongside contrasting marker profiles. Such heterogeneity could depend on several factors. We explored potential differences among the patients in terms of age and year of transplantation, HLA matching, and intervals from transplantation to hyperglycemia or from hyperglycemia to sample collection, but we found no associations. We also examined potential differences in immunosuppression regimens, given the evolving protocols over time. Before 1997, the protocol consisted of induction therapy with OKT3 and maintenance therapy with cyclosporine or FK506, azathioprine or MMF, and steroids. Between 1997 and 2000, most patients received no induction therapy, while after 2000, the current immunosuppressive protocol was introduced, including ATG and anti-CD25 as induction therapy, and FK506, MMF, or rapamycin, along with steroids, as maintenance [107]. In our cohort, except for two patients transplanted before 1997, who are allocated in the two different groups, all other patients received the same immunosuppression regimen. Therefore, different immunosuppression treatments may not explain the observed variability. Furthermore, other parameters such as C-peptide, HbA1c, and HLA-matching were also non-informative. Finally, the time interval between the recurrence of hyperglycemia and the collection of the sample tested showed no clear association. On the other hand, we cannot exclude that the variability in the detection of these autoreactive T cells with an activated

phenotype may reflect disease activity, an hypothesis that would require access to much larger populations and longitudinal samples to explore.

Unsupervised analysis of the flow cytometry data confirmed the existence of these populations of activated autoreactive T cells and provided a deeper dimension to our multi-parameter studies. The unsupervised analyses revealed that autoreactive T cells from T1DR patients exist in distinct, spatially segregated clusters from those of autoreactive T cells in patients who maintained normal glucose tolerance (SPK-Neg and SPK-Conv). Even in the unsupervised analysis, T1DR autoreactive T cells showed a unique phenotypic pattern characterized by high GLUT1 expression, upregulation of activation markers and downregulation of exhaustion markers.

This pattern was not observed in non-autoreactive $CD4^+$ and $CD8^+$ T cell, supporting the hypothesis that GLUT1 expression serves as a discriminating marker for recurrent T1D. This supports the concept that a potential treatment to block GLUT1 may preferentially impact autoreactive, activated T cells with high GLUT1 expression, minimizing off target effects and enhancing the specificity of therapeutic interventions.

Moreover, I assessed the $CD4^+$ and $CD8^+$ T cell differentiation states, based on the distinctive expression of CD62L and CD45RA, to define the distribution of naïve/memory subsets of autoreactive T cells. I found that the frequency of $CD4^+$ and $CD8^+$ Tem cells was significantly increased in T1DR patients compared to SPK-Conv and SPK-Neg (only for $CD8^+$). On the contrary, the frequency of Temra and naïve was strongly decreased, compared to the other groups.

By examining *ex-vivo* T cells of patients receiving pancreas transplantation, we were able to identify autoreactive T cells with a memory phenotype. The phenotypic assessment revealed notable differences, particularly within the $CD4^+$ and $CD8^+$ Tem subsets, where some activation markers were upregulated and some exhaustion markers were downregulated. However, the most striking difference observed was the overexpression of GLUT1.

The modulation of autoimmune memory response represents a challenge, as it has proven to be remarkably resistant to immunosuppressive and immune-modulating treatments [179, 180]. In our quest for a novel avenue to inhibit autoreactive memory clones to hinder autoimmunity recurrence, we propose the targeting of GLUT1, a mechanism also relevant for various disease contexts. Further studies are needed to address whether the level of GLUT1 in autoreactive T cells pre and post-transplant may be a predictive biomarker of autoimmunity recurrence.

4.3 Glucose availability regulates T cell survival and proliferation

I then addressed the issue of the relative contribution of glucose to the survival of T cells in culture. My thesis data were consistent with the common notion that glucose plays a fundamental and non-redundant role in the survival and proliferation of activated T cells. However, the glucose concentration needed for survival was surprisingly low [151]. Indeed, the 1mM glucose concentration, which preserved T cell viability *in vitro* is considered a lethal glucose concentration for the whole organism. Moreover, when cells were cultured in the absence of glucose but in the presence of growth factors (dialyzed FBS), differences in responses between $CD4^+$ and $CD8^+$ T cells were observed. One possible explanation is that T cell activation does not result in uniform metabolic reprogramming: $CD4^+$ T cells have higher metabolic adaptivity in terms of energy-rich substrates compared to effector $CD8^+$ T cells. Upon activation $CD4^+$ T cells may increase both glycolysis and oxidative phosphorylation, contrarily to $CD8^+$ T cells, which are then extremely sensitive to glucose availability [181].

Although T cells showed great adaptation to very low glucose concentrations in terms of viability, T cell proliferation was strongly affected. Using CFSE dilution assay I studied the proliferation of $CD4^+$ and $CD8^+$ T cells in response to activation (anti-CD3/CD28 beads) in healthy donors. The results revealed that the proliferative response of T cells cultured in low glucose concentration (1mM), was halved compared to the cells cultured in the normal one (5.5mM). These findings are in line with previous literature [151, 182].

Since earlier work in our laboratory demonstrated the capacity of the GLUT1 inhibitor WZB117 to reduce glucose uptake in T cells [97], I investigated its effect on cell survival and proliferation. To the best of our knowledge, so far, no other groups have published data with respect to the effects of WZB117 on lymphocytes. Thus, our work provides new insights into the mechanisms and intervention targets in the field of immunometabolism. The results of my *in vitro* analysis highlighted that 6 days of WZB117 treatment, at a concentration of 10μM, resulted in an increase in late apoptotic cells by 8% and a significant decrease in proliferative response by 20% in $CD4^+$ and $CD8^+$ T cells from healthy donors. These findings align with literature reporting that WZB117

can induce cell death specifically in cancer cells, which exhibit higher metabolic activity than healthy, quiescent cells [160, 163, 174].

One important observation was the duration of the GLUT1 inhibitory effect. In contrast to the cells cultured for the first 6 days in low glucose, which completely recovered proliferation, cells cultured for the first 6 days with WZB117 did not show any recovery but a further reduction in T cell proliferation upon extended culture. This experiment demonstrated that the state of hypo-responsiveness induced by WZB117 on T cells lasted beyond the treatment and was not modified by re-stimulation. Thus, the effect of WZB117 on T cells is not solely related to restriction in glucose availability but other mechanisms can be potentially involved. A long-term therapeutic effect has been noted in cancer cells, manifesting as the incapacity of treated cells to form colonies for a period extending up to 9 days following 24-hour treatment with WZB117 [162]. This persistence of the WZB117 inducing hypo-responsiveness could be considered a real advantage of a therapeutic approach that involves GLUT1 blockade. Further characterization of this effect is needed to examine potential additive or synergistic mechanisms induced by WZB117 that may explain the persistence of the effect. From the standpoint of controlling T cell activation, a long-lasting, if not permanent impact of GLUT1 blockade on T cells is a promising result, as it would allow to impart a persistent inhibition of T cell proliferation with a time-limited administration of the drug.

Finally, since we hypothesized a translation of this GLUT1 blockade as part of an induction therapy in patients with T1D undergoing pancreas or islet transplantation, we aimed to test the combinatorial effect of WZB117 with drugs used in standard immunosuppression for organ transplant. We found that WZB117 had an additive effect with rapamycin (Sirolimus) and FK506 (Tacrolimus) in inhibiting T cell proliferation.
Similar effects have been observed on cancer cells, when WZB117 was used in combination with anticancer agents, such as apatinib [163], imatinib [161] and MK-2206 [162]. This result, if confirmed, could potentially allow for a reduction of the target plasma concentration of immunosuppressive drugs when used in combination with WZB117.

In our study we have to consider the potential for acquired resistance to GLUT1 blockade in T cells, which could overcome GLUT1 inhibition. One possibility is the upregulation of metabolic pathways that rely on alternative sources of energy, such as fatty acids and amino acids. Existing literature suggests that transient glucose deprivation in T cells *in vitro* resulted in adaptive responses likely driven by mitochondrial compensation [183]. While compensatory mechanisms in T cells remain less explored, analogous processes have been extensively studied in cancer cells. Inhibition of glycolysis in cancer cells has shown promise in reducing cell survival and proliferation, enhancing chemosensitivity, and triggering metabolic adaptations such as a shift to oxidative phosphorylation [184].

In the context of glucose deprivation, studies involving monocytes activated with LPS revealed a compensatory increase in oxidative phosphorylation via fatty acid oxidation, along with the activation of autophagy to provide nutrients by degrading cellular components [185]. Similarly, stem cell-like tumor subpopulations with high glycolytic activity exhibited upregulated autophagy in response to glycolysis inhibition [186]. Studies on macrophages from GLUT1-deficient mice have indicated compensatory increases in glutamine and fatty acid consumption in the tricarboxylic acid (TCA) cycle [187]. Studies on $CD8^+$ T cells from GLUT2-deficient mice showed increased glutamine uptake and a switch towards glutamine metabolism [150]. However, it is crucial to note that existing evidence suggests that increased oxidative phosphorylation alone may not entirely compensate for glucose deprivation, because AMP increases and NADH, used as cofactor for several enzymes, decreases [185].

Moreover, despite the higher efficiency of oxidative phosphorylation in producing ATP compared to glycolysis, the extremely high rate of glycolysis results in higher ATP production. Glycolysis produces an amount approximately 13 times greater of ATP per unit of time than oxidative phosphorylation [188]. In our future experiments, we aim to delve deeper into the metabolic signatures of T cells using Seahorse XF technology, providing a more comprehensive understanding of their adaptive responses after GLUT1 inhibition.

4.4 The *in vitro* effect of GLUT1 inhibitor WZB117 on T cells

Pancreas or islet transplantation is considered an effective strategy for T1D reversal, overcoming the complications associated with the direct injection of insulin. However, many groups have reported recurrence in autoimmunity, in addition to the risk of allogenic graft rejection, either in islet [105, 189, 190] or pancreas [98, 107, 108, 191] transplantation. One possible explanation is that the chronic immunosuppression, required to prevent rejection, may in the long term favor the emergence of autoreactive T cells via homeostatic proliferation, driven by IL-7. IL-7 is largely ignored by immunosuppressive drugs, which target the IL-2 pathway [109, 179], and is implicated in the generation of long-lived memory T cells that persist in the patient for decades [97]. Despite being an active area of research, the precise mechanisms for the reactivation of autoreactive memory T cell clones in transplanted patients remain unknown. Within the cohort of SPK patients experiencing T1D recurrence I studied in this thesis work, the time interval between the transplantation and the reoccurrence of hyperglycemia shows considerable variability, ranging from 4 to 17 years, with an average of 8.5 years. This is consistent with the gradual emergence and activation of autoreactive T cells that may be expanding from the memory pool because of homeostatic proliferation.

GLUT1 is known to be critical for T cell activation and proliferation, therefore I wanted to investigate the effect of WZB117 on T cells obtained from patients with T1D and transplanted patients. Specifically, I studied samples from patients with SPK transplants, who became and remained normal glucose tolerant after transplantation.

Importantly, and in further support of my initial studies in samples from healthy subjects, my *in vitro* data confirmed that GLUT1 blockade inhibited T cell activation and proliferation, and revealed that the inhibition of GLUT1 caused greater effects on cells from T1D and SPK patients, compared to healthy subjects. T cell proliferation was reduced in all the three groups but this was much more pronounced in T1D and SPK patients, for both $CD4^+$ and $CD8^+$ T cells. Other key changes that consistently occurred in T1D and SPK patients were reduction in the size of naïve, Tcm and Tscm subsets, accompanied by a downregulation of several activation markers. While only T1D patients showed decreased GLUT1 expression following WZB117 treatment, especially in Tscm

and Tcm subsets, the expression of GLUT3 trended higher in SPK patients with functional grafts. One hypothesis to explain the observed GLUT1 reduction following WZB117 treatment only in T1D T cells, which is supported by recent evidence [192, 193], is that glucose uptake in T cells is significantly increased in T1D patients, compared to HD, and this was mainly reflected as increased glycolysis or increased expression of GLUT1.

Of note, GLUT3, together with GLUT1, is a hypoxia-responsive glucose transporter [194]. Its role has been thoroughly characterized in the brain but little is known about GLUT3 in T cells.

Some authors reported that GLUT3-dependent glucose uptake supports the differentiation and effector functions of pathogenic Th17 cells, and its overexpression leads to a more severe outcome of autoimmune disease in mouse model of EAE [195]. Other authors claimed that GLUT3 plays a role in suppressive activities and glucose metabolism of Treg cells in tumors, contributing to their homeostasis [196]. However, the authors suggest that the increased glucose consumption caused by GLUT3 can lead to Treg cell senescence and suppression of effector T cells, which hinder the immune response against tumors. By blocking GLUT3, the suppressive effects of Tregs on effector T cells are reversed and the induction of senescence in effector T cells is prevented, enhancing the immune response against tumors.

In my experiments, increases of GLUT3 levels were only noted among SPK patients. As the pharmaceutical industry has directed substantial resources towards the development of GLUT1 inhibitors, particularly in the context of tumor therapeutics, the repertoire of molecules that selectively target GLUT1 is considerably broader than those targeting GLUT3. In a perspective of a future application, we focused our studies on GLUT1 because several inhibitors are available and other more potent are likely to be developed in the near future. However, also GLUT3 need to be considered as a potential target. Specifically, the distinct association of GLUT3 with SPK patients negative for autoantibodies highlights the importance of conducting a comprehensive characterization of the impact of GLUT3 inhibition, advancing our understanding of potential therapeutic avenues in this context.

Recently GLUT2, the well-established glucose transporter in liver, pancreas and kidney [197, 198], has been shown to be expressed in T cells [150]. Unlike $CD4^+$ T cells, which predominantly rely on GLUT1, $CD8^+$ T cells demonstrate a dependence on both GLUT1 and GLUT2 for their metabolic and functional processes. An elegant study from Fu et al [150], showed that the absence or inhibition of GLUT2 in $CD8^+$ T cells resulted in diminished glucose uptake and impaired glycolysis, impacting the proliferation, differentiation, and overall function of $CD8^+$ T cells. This may affect their capacity of infiltrating tumors or mounting robust immune responses to infections. Moreover, GLUT2 expression is dynamically regulated in response to environmental factors such as glucose availability, oxygen levels, and extracellular acidification. In glucose-deprived inflammatory environments GLUT2 is downregulated and a concomitant upregulation of GLUT1 may serve to prevent the loss of $CD8^+$ T cell functions. Given the recently recognized significance of GLUT2 in T cells, evaluating its potential changes in my experimental setting emerges as a future development for the project.

4.5 Exploring the potential off-target and side effects of GLUT1 inhibition

Given the broad expression of GLUT1 across various tissues (**Fig. 42**) and its role in physiological processes, a crucial phase in the investigation of a potential molecule for translation into a clinical setting is the identification of potential off-target and side effects. This glucose transporter, more than others, is highly expressed during early embryo development in the brain, epithelial cells of the mammary gland, placenta and fetal tissue, but also in transformed cells, skeletal muscle, and myocardium during the suckling phase. Subsequently, its expression decreases in most tissues except for the brain, where it remains consistent [199]. While the expression of GLUT1 may be redundant in some tissues, due to the presence of other GLUTs, certain organs rely on GLUT1 for specific functions. Notable examples include endothelial cells of the blood-brain barrier, where GLUT1 facilitates glucose uptake from the blood to the brain; human, but not mouse, pancreatic beta cells, which utilize GLUT1 for glucose sensing and subsequent insulin secretion; erythrocytes, which only express GLUT1 and use it for glucose and dehydroascorbic acid (DHA) uptake.

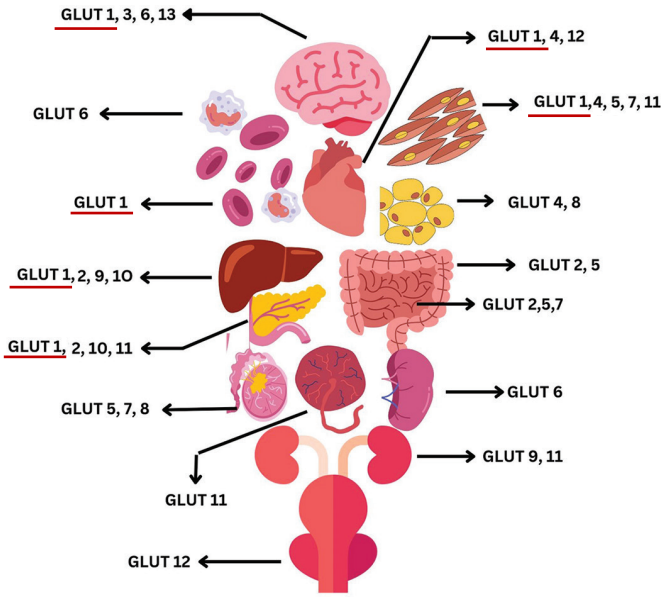


Figure 42. Expression of GLUTs in different organs and cell types*. Modified by SSQ Jafri, SI Ali Shah and SHA Jaffari [199]*

Inhibiting GLUT1 via STF-31, BAY-876 and WZB117 is likely to cause systemic effects. While potential side effects on non-immune cells, such as erythrocytes and insulin-secreting cells, have been considered, *in vivo* experiments in mice have not revealed such

effects, likely due to species-specific differences in glucose transporter usage. In humans, beta cells and erythrocytes use GLUT1 for glucose uptake, while in rodents, this function is provided by GLUT2 for beta cells [200] and GLUT4 for erythrocytes [201], respectively. A recent finding has challenged current understanding of the importance of GLUT1 in human erythroid cells. Using CRISPR gene editing Freire and colleagues [202] generated erythroid cells completely lacking GLUT1 expression and found that the absence of GLUT1 did not impair erythroblast proliferation and terminal differentiation. However, erythroid cells lacking GLUT1 showed increased osmotic fragility, that may be indicative of altered membrane properties and potential disruptions in cellular homeostasis.

The neurological impact of GLUT1 inhibition is likely to be significant, especially in neurons and glia cells, where GLUT1 is essential for both glucose and DHA uptake. Reduced GLUT1 expression at the blood-brain barrier has been linked to Alzheimer's disease vasculo-neuronal dysfunction and degeneration [203].

Experimental evidence from rats at 5 days post-natal suggests that WZB117 has an impact on glial cell branching, disrupting the Vitamin C recycling process between neurons and astrocytes. Notably, WZB117 affected DHA uptake in astrocytes but not in neurons, which also express GLUT3 [204]. This observed effect at an early developmental stage emphasizes the significance of GLUT1 during this phase, suggesting that reliance on GLUT1 may diminish over time.

While STF-31, BAY-876 and WZB117 seem to be well tolerated in *in vivo* models, further research is needed to understand the full range of systemic effects and potential side effects of these inhibitors.

Initial studies conducted in our lab involving mice treated with WZB117 for 28 days have shown no evident side effects, with normal growth curves observed. However, given its central role in the brain, the impact of GLUT1 inhibition on the central nervous system has emerged as a critical concern. In mice, the germline deletion of GLUT1 led to embryonic lethality, while mice with a heterozygous deletion survived but had central

nervous system alterations, such as seizures, increased glucose levels in cerebrospinal fluid and impaired motor activity [205]. The impact of systemic administration of WZB117 in adult mice at a concentration of 5, 10 or 20 mg/kg/day has been investigated [160, 175, 206, 207]. The treatment has demonstrated to be well tolerated with no adverse events over a 10-week period of daily administration.

Also in humans, a GLUT1 deficiency syndrome (GLUT1-DS) has been described as having relevant neurological defects and disorders. GLUT1-DS patients are characterized by *de novo* or inherited mutations of the *Slc2a1* gene that affect assembly, three-dimensional folding, trafficking to the cell membrane, or GLUT1 protein activation [208]. Measurement of *in vitro* glucose uptake in GLUT1-DS patients' erythrocytes showed a reduction in glucose transport ranging from 30% to 70% compared to healthy subjects, which correlates with clinical neurological symptoms such as epilepsy, ataxia, and movement disorders. Of note, these patients show no signs of anemia, this might be explained by recent findings on erythroid cells lacking GLUT1 [202]. Neurological symptoms tend to alleviate over time and into adulthood, and some patients can live regular and fulfilling lives [209]. The ketogenic diet, which compels the brain to utilize ketone bodies instead of glucose as energy source, represents the standard treatment and is highly efficient in improving neurological outcomes [210].

Given these premises, to avoid neurological side effects, pharmacological GLUT1 blockade to control T cell activation should be restricted to adolescent or adult patients with T1D and for a limited period. A phase I clinical trial on adult patients with tumors showed that using 2-DG for several weeks caused only mild and reversible neurological side effects, mainly dizziness [211]. An alternative could be combining WZB117 treatment with a ketogenic diet. Low-carb or ketogenic diet regimens have also been evaluated in patients with T1D, showing benefits in the improvements of glycaemic variability, but limited long-term tolerability and patient compliance [212].

Preliminary *in vitro* data produced in our laboratory, using WZB117, have excluded erythrolysis and have shown a slight reduction in glucose-stimulated insulin secretion on human pancreatic beta cell line EndoC-βH1 when cells were treated with WZB117.

While impaired insulin secretion might be considered a detrimental side effect in a clinical setting where beta cell replacement aims to restore insulin production, on the other hand insulin secretion itself can induce stress in beta cells [213]. In fact, immediately after islet transplantation, approximately 25% of islets are lost [214], primarily due to cellular stress and inflammatory responses resulting from isolation, *in vitro* culture, and transfusion procedures. Therefore, a reduction in glucose sensitivity and insulin secretion could exert a positive effect on beta cell survival in the early post-transplantation period.

Moreover, the impact of GLUT1 inhibitors on reducing cytokine production and glycolysis could have consequences on overall T cell functionality, potentially affecting their ability to respond to infections. Specifically, the treatment with WZB117 may impact pathogen-specific memory T cell populations, leading to a reduced memory T cell reactivation during the recall infection. However, it is interesting to note that patients with GLUT1-DS do not typically have a history of severe infections.

Our strategy of GLUT1 blockade right after transplant as induction therapy relies on the fact that in this specific time window only autoreactive T cells are activated and overexpress GLUT1 upon antigen re-exposure. This would increase the selectivity of the treatment for autoreactive T cells in this specific phase and, as the effect lasts beyond the treatment, GLUT1 blockade will be discontinued after few weeks to preserve the immune competence against pathogens.

4.6 Limitations of the study

The *ex-vivo* flow cytometry analysis conducted in the first part of the thesis allowed for a comprehensive comparison of different patient cohorts, including HD, T1D patients, and three distinct groups of SPK patients (those that do not seroconvert, those that seroconvert without any clinical signs of T1DR, and those that experienced T1DR). This analysis provided valuable insights into the phenotypic profiles of individuals across these different groups.

However, it is crucial to consider and discuss certain limitations that might influence the interpretation of the results. Notably, the age difference among the cohorts, particularly the markedly younger age of T1D patients compared to the other groups, poses a potential caveat for direct comparisons. Age-related differences could contribute to differences observed across the groups, impacting the interpretation of the data and making it difficult to draw linear conclusions.

Additionally, all SPK patient groups are under chronic immunosuppression, a factor that distinguishes them from both HD and T1D patients. This immunosuppressive treatment may significantly influence the immune profiles and could contribute to the observed disparities, potentially masking or confounding some of the underlying similarities between T1D and T1DR patients. Moreover, the transplantation itself could elicit similar effects.

Given these considerations, it is important to interpret the findings with caution and recognize that the observed differences between T1D and T1DR patients may be influenced not only by the nature of the diseases but also by age-related variations and the effects of immunosuppressive treatments in SPK patients. However, it is important to recognize that the main goal of our study was to examine T cell phenotypes in SPK patients, comparing groups of patients with/without T1DR, and at different risk for T1DR. From that point of view, the study design and the subjects were well matched, and access to these well characterized SPK cohort is a unique strength of our study.

FUTURE PLANS

The overall aim of my thesis work was to characterize the GLUT1 dynamic of autoreactive T cells in T1D patients who had undergone pancreas transplantation, and to test pharmacological GLUT1 blockade as a potential strategy to control activation of autoreactive T cells in T1D patients and prevent autoimmunity recurrence in transplanted patients. To better characterize genes and pathways that are associated with the recurrence of T1D in SPK recipients, RNAseq studies will be conducted on human T cell populations that have been sorted. The objective is to identify potential risk markers and predictors of recurrence.

In order to investigate how GLUT1 inhibition by WZB117 would affect energy metabolism, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) will be measured with or without WZB117, using Seahorse XF technology. Moreover, in view of a future translation of a GLUT1 blockade approach, fundamental aspects need to be determined. RNAseq approach will also be used to study cells treated with WZB117, in order to reveal whether the persistent effects of WZB117 can be explained by changes at the transcriptome level.

Another important issue is to investigate the safety and efficacy of GLUT1 inhibition *in vivo*. In future studies, we plan to use two mouse models: (i) NOD mice and (ii) a preclinical model of beta cell replacement.

(i) We will test the ability of WZB117 to prevent or reverse diabetes in NOD mice, soon after diagnosis, when mice have residual beta cell mass. These set of experiments will be conducted by our collaborator Dr. Allison Bayer, at the University of Florida. NOD mice will be followed up for up to 70 days. We hope that the intervention with WZB117 at this early time point can reverse diabetes. This model was used to generate preclinical data on anti-CD3 mAbs [215, 216], ATG [217], alefacept [218], rituximab [219], and abatacept [220]. These molecules afforded significant yet partial preservation of insulin secretion following diagnosis, however, their therapeutic benefit waned over time. A recent study [221] demonstrated that a Treg cells-based immunomodulatory regimen when combined with anti-CD3 mAb successfully reversed autoimmune diabetes in NOD mice

without compromising the remainder of the immune system and without the need for chronic immunosuppression. Thus, besides testing WZB117 as a single agent, we will also determine whether improved therapeutic outcomes can be achieved when WZB117 is combined with anti-CD3 treatment.

(ii) We will test the WZB117 effect in a preclinical model of beta cell replacement. Human induced pluripotent stem (iPS) cell- derived beta cells will be transplanted in diabetic, class I and class II deficient NSG mice, reconstituted with human PBMCs from patients with T1D. The main advantage of this model is the lack of GvHD following the adoptive transfer of human PBMCs, allowing a long-term follow-up of the graft function. Both iPS-derived beta cells and PBMCs express HLA-A*0201 to recapitulate the co-existence of autoimmunity and allo-immunity. Moreover, HLA-A*0201 matching will allow us to study the presence, activation and expansion of autoreactive T cells using the MHC dextramers we previously used in this thesis. We expect this model to provide insight into the *in vivo* capacity of a GLUT1 blockade strategy to prevent or delay graft rejection by selectively targeting activated T cells that upregulate GLUT1 upon recognition of autoantigens and alloantigens. Moreover, we will include assessments that will inform about the safety of GLUT1 inhibition. The model will also provide insights into transplantation with iPS-derived beta cells. Little is known about their natural capacity to expand and activate and be targeted by auto- and alloreactive T cells. We will also test *in vivo* the hypothesis that WZB117 exhibits an additive effect with rapamycin and FK506, as observed *in vitro* in T cell proliferation. This will be performed by comparing the experimental outcomes resulting from WZB117 treatment alone with those from WZB117 in combination with standard immunosuppression.

If the safety of WZB117 *in vivo* will be confirmed, in future investigations we will also consider combining WZB117 with Dimethyl Fumarate (DMF) treatment. DMF is a fumaric acid ester derivative of the Krebs cycle intermediate fumarate which was approved by the FDA in 2013 (Tecfidera) for the treatment of relapsing-remitting multiple sclerosis (RRMS). Unpublished data from the Diabetes Research Institute in

Miami (Dr. Bayer's laboratory), showed that 12-week DMF treatment can prevent diabetes development and reverse diabetes in NOD mice. Given the known mechanism of action of DMF, combination with WZB117 could be synergistic and further benefit therapeutic outcomes [222].

Chapter 5

MATERIALS AND METHODS

5.1 Subjects

5.1.1 Healthy donors

For the experiments performed in Milan (Italy), PBMCs were obtained from venous blood samples provided by the Immunohematology and Transfusion Medicine Service of San Raffaele Hospital, Milan, Italy. For the experiments performed in Miami (US), PBMCs were obtained from venous blood samples provided by the Continental Services Group, Inc., blood bank, Miami, Florida, US. The age range of the subjects was 29 - 63 years, with a mean age of 47.8 $\pm$ SD 10.8 years. The control subjects were selected to match the age range of the SPK recipients. Healthy donors' characteristics are described in **Table 6**.

Subjects ID	*Age*	*Sex*
1345	57	M
1346	37	M
1349	63	M
1352	53	M
1353	29	M
1355	57	M
1360	51	M
1363	46	M
1364	42	M
1365	47	M

***Table 6**. Demographic features of healthy donors.*

5.1.2 Patients with T1D

Fourteen patients with clinical T1D were recruited for the study. The age range of the patients was 8-19 years, with a mean age of 14.1 ± SD 2.6 years. The patients had a mean T1D duration of 4.3 years ± SD 2.8 years. T1D patients' characteristics are described in **Table 7**.

Subjects ID	*Age*	*Sex*	*T1D duration (years)*
1342	14	M	3
343	9	M	0.6
372	15	F	7
1373	16	F	8
1374	17	F	8
1388	15	M	4
1389	11	M	2
1401	11	F	6
1402	14	M	4
EN0005LA	14	M	0,4
EN0006JB	14	M	0,6
EN0008NF	19	M	0,2
EN0013MP	13	M	1
EN0014DC	16	M	0,9

***Table** 7. Demographic features of patients with T1D.*

5.1.3 SPK recipients

We studied T1D patients with ESRD who received SPK transplants at the University of Miami Miller School of Medicine. Before transplantation, all patients had no detectable C-peptide response to a Sustacal/Boost test (Soci et e des Produits Nestle S.A., Vevey, Switzerland). The pancreas transplants were bladder-drained, with systemic venous effluent; in this setting, urine amylase levels are used to monitor pancreatic exocrine graft function and rejection. A stable range of urine amylase levels is observed for well-functioning pancreas grafts, while reduced amylase levels are observed in the event of pancreas rejection. SPK patients voluntarily participated in a study to evaluate islet autoimmunity, which was approved by the University of Miami Institutional Review Board (Protocol #20053039).

Participation was voluntary and subjects could withdraw at any time. For some patients, outcome data and archived serum samples were evaluated under waiver of consent. Islet autoimmunity was assessed by measuring levels of AABs associated with T1D, in serum samples obtained before transplantation and on follow-up. Specifically, autoantibodies to the autoantigens GAD65 (GADA), IA-2 (IA-2A), and ZnT8 (ZnT8A) were measured using radioimmunoassays (RIA), as previously described [107].

We examined four groups of SPK patients who were previously classified based on the monitoring of islet autoimmunity and clinical course post-transplantation, specifically: those who developed T1D recurrence on follow-up, or T1DR; those who remained normal glucose tolerant (NGT), divided into four sub-groups by longitudinal assessment of autoantibodies: negative (NGT-AAB-neg), sporadic (NGT-AAB-spor), persistent (NGT-AAB-pers), and converter (NGT-AAB-conv). As previously reported [107], SPK patients who are autoantibody-negative, sporadic, or have autoantibodies persisting from before transplantation have very low risk of developing T1DR, while most future cases of T1DR are expected in those who experienced autoantibody conversion on follow-up. Because of the low risk of T1D recurrence, the NGT-AAB-pers, NGT-AAB-spor and NGT-AAB-neg groups were combined into a single control group.

I. SPK recipients NGT-AAB-Neg

Patients were categorized as NGT if their HbA1c level was within the normal range of 4.5–6.1% and they were not receiving insulin therapy or oral antidiabetic medications. This group includes patients who had no evidence of autoantibodies either before transplantation or during subsequent follow-up (Neg), patients who sporadically exhibited low levels and inconsistent positivity for various autoantibodies (Spor) and patients who displayed pre-transplant autoantibody positivity and remained positive without acquiring additional autoantibodies (Pers). The characteristics of these 17 NGT-AAB-neg SPK recipients are summarized in **Table 8**.

Subjects ID	*Age*	*Age at TX*	*Sex*	*T1D Duration*	*HLA-A1*	*HLA-A2*	*HLA-DR1*	*HLA-DR2*
A-3632	46	36	F	34.6	24	2	4	17
A-3639	40	30	F	20.3	2	3	1	9
A-3695	56	48	M	46.2	24	30	4	17
A-3702	46	40	M	30.6	2	24	17	17
A-3710	50	48	M	43.2	2	3	17	
A-3716	56	51	M		1	3	4	17
A-3772	59	51	M	30.3	3	29	1	4
A-3816	58	47	M	35.5	23	2	17	
A-3724	38	36	M		30	33	4	17
A-3641	53	51	F	28.1	11	29	4	8
A-3683	46	40	F	36.8	24	29	4	17
A-3657	61	48	M	37.3	2	26	4	7
A-3670	35	32	F	18.2	2	3	4	7
A-3721	62	45	M	27.8	2	24	4	15
A-3729			F		11	26	4	4
A-3662	51	43	F	19.8	2	24	4	9
A-3747	32	29	M		3	31	3	4

Table 8. *Demographic features of SPK recipients* NGT-AAB-neg. *TX. transplantation*

II. SPK recipients NGT-AAB-conv

This group includes SPK patients who remained NGT on post-transplantation follow-up and showed positivity of autoantibodies only post-transplant (conversion) or acquired additional autoantibodies in the longitudinal assessment on follow-up. The characteristics of these 10 SPK recipients NGT-AAB-Conv are summarized in **Table 9**.

Subjects ID	*Age*	*Age at TX*	*Sex*	T1D Duration	*HLA-A1*	*HLA-A2*	*HLA-DR1*	*HLA-DR2*
A-3643	49	41	M	37.5	2	3	4	17
A-3652	75	60	F	49.5	2	26	4	17
A-3661	59	58	M	35.7	2	11	1	13
A-3665	48	43	F	23.4	2	11	17	
A-3723	44	31	M	18.9	2	29	1	13
A-3745	42	39	M		2	24	4	11
A-3699	38	33	F	26.4	2	3	4	
A-3704	44	40	F	32	1	26	4	17
A-3714	49	46	M	21.2	3	68	4	7
A-3654	64	41	F	27.7	1	3	3	4

***Table 9**. Demographic features of SPK recipients* NGT-AAB-Conv

III. SPK recipients with T1D recurrence

This group includes patients who developed hyperglycemia and clinical symptoms of T1D, several years after post-transplant normalization of glucose metabolism, requiring reinstitution of insulin therapy. These patients also exhibited progressive elevation of HbA1c levels and severe reduction of c-peptide levels was noted typically in the absence of laboratory and clinical evidence of rejection (stable urine amylase and serum creatinine levels). This group showed a higher prevalence of post-transplant positivity for all autoantibodies, and 8/9 were converters.

Figure 43 describes the features of T1DR patients. This patient in particular developed autoantibodies conversion for GAD65 and IA-2 about 2.5 years after transplantation (**Fig. 43A**). Biopsies were obtained at several time points within 6 months and 1 year after the recurrence of hyperglycemia. The presence of peri-insulitis affecting islets is supported by the detection of infiltrating T-cells (CD3, CD4, CD8) and B-cells (CD20) (**Fig. 43B**). indicated the function of the residual beta cells The patient had residual pancreatic cells function, as demonstrated by detectable C-peptide levels (**Fig. 43C**), which declined 2 years after the recurrence, when circulating GAD-autoreactive $CD4^+$ T-cells increased (**Fig. 43C,D**). The characteristics of these 9 SPK recipients with T1DR are summarized in **Table 10**.

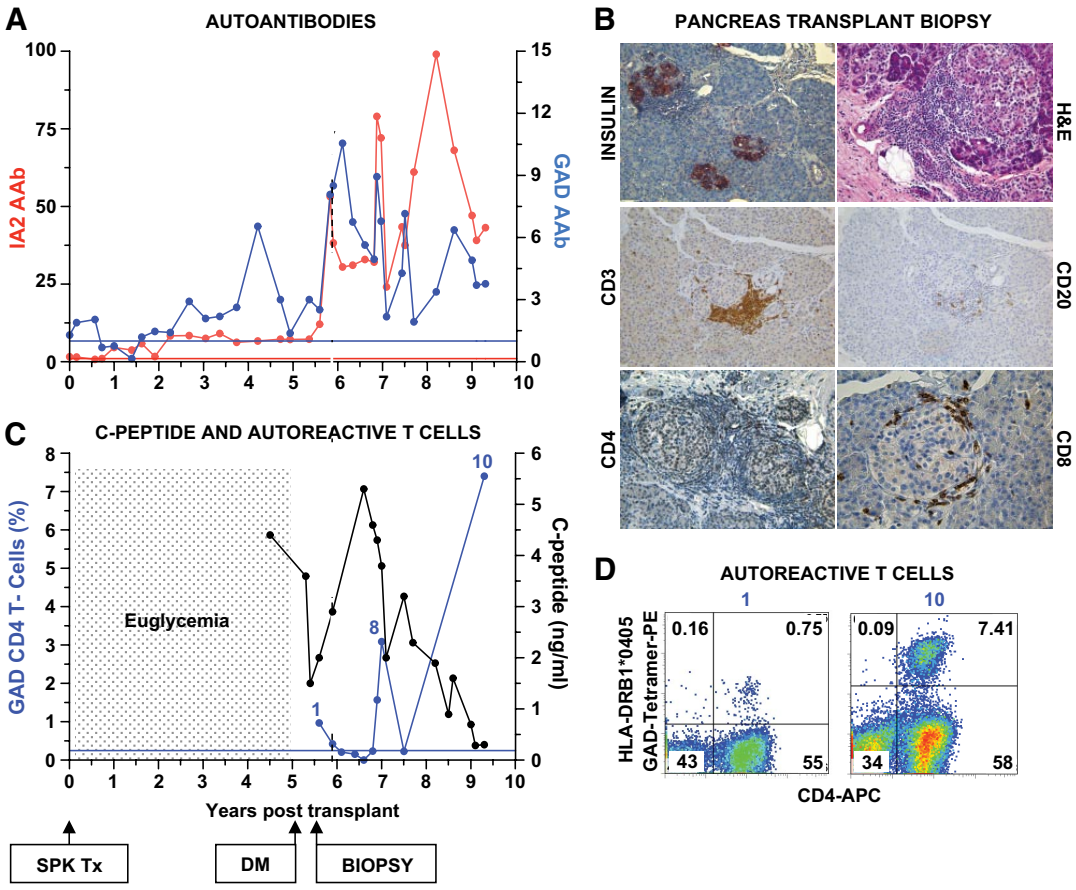


Figure 43. Recurrence of diabetes in an SPK transplant patient in the absence of organ rejection. *The patient was a 41-year-old male who developed T1D at the age of 7. He received an SPK transplant when he was 32 years old, which successfully reversed his diabetes. However, 5 years post-transplantation, the patient experienced a return to insulin dependence, despite normal functioning of the kidney and exocrine pancreas allografts.* ***(A)*** *Autoantibody levels before the transplant and during the follow-up are expressed as the ratio of the patient's index level over the upper limit of the normal index (cut-off). For all autoantibodies, a value greater*

than 1 indicates a positive result. ***(B)*** *A pancreas transplant biopsy, obtained approximately 6 months after the recurrence of hyperglycemia, is shown. Insulitis is marked by the presence of CD3, CD4 and CD8 for T cells and CD20 for B cells infiltrating the islets, while insulin staining indicates the loss of beta cells.* ***(C)*** *The serum C-peptide levels have decreased from the time when hyperglycemia recurred.* ***(D)*** *Flow cytometry plots demonstrating GAD-autoreactive $CD4^+$ T cells.[98]*

Subjects ID	*Age*	*Age at TX*	*Sex*	*T1D Duration*	*HLA-A1*	*HLA-A2*	*HLA-DR1*	*HLA-DR2*	*TX-T1DR interval*
A-3626	63	43	M	14.4	2	2	3	4	17
A-3635	49	38	M	27	2	3	4	17	12
A-3678	51	39	M	26.2	2	26	7	17	8
A-3681	41	35	M	24.1	1	29	4	17	4
A-3698	52	40	F	26.6	2	29	4	17	4
A-3720	47	41	M	31.9	32	33	4	17	7
A-3628	43	29	M	27.4	29	31	4	17	8
A-3717	39	29	F	13.5	24	26	4	13	9
A-3604	64	47	M	39.3	2	29	3	4	8

Table 10*. Demographic features of SPK recipients who experienced T1D recurrence.*

5.2 Methods

5.2.1 Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from sodium-heparinized venous blood samples, using Ficoll-Paque (Fisher Scientific) density gradient centrifugation (400*g* for 30 minutes, with no brake at 18-20°C) and washed twice in PBS (300*g* x 10 minutes, with brake, at room temperature). Pellet was resuspended in 10mL of ACK solution (NH_4Cl 0.15M, $KHCO_3$ 10mM, EDTA 0.1mM) and incubated for 10 minutes at room temperature, sterile PBS was added to stop the reaction, followed by a 300*g* centrifugation for 10 minutes.

5.2.2 In vitro T cell culture and treatments

PBMCs were cultured at $2x10^5$ cells/well in 96 well plates in 5.5mM glucose medium: RPMI 1640 containing 50% RPMI 1640 without glucose, (Gibco, Thermofisher scientific) supplemented with 6.5% FBS, 100U/mL penicillin and 100U/mL streptomycin. Cells were stimulated with Dynabeads™ Human T-Activator CD3/CD28 (Gibco) for 3 or 6 days. WZB117 (Merck) has been used at the concentration of 10µM in all the experiments.

5.2.3 T cell proliferation assay

In vitro proliferation was evaluated using CellTrace proliferation kit (CFSE or Violet, Thermofisher Scientific). PBMCs were resuspended in PBS and incubated with 0.5µM of CellTrace for 10 minutes at 37°C. The reaction was stopped by adding RPMI 1640 containing 5% of FBS. Stained PBMCs were then washed with PBS to remove unreacted CellTrace, resuspended in RPMI medium, as described above, and seeded at $2x10^5$ cells/well in 96 well plates for 6 days.

Proliferating cells were identified as the percentage of cells diluting the fluorescent intensity of CellTrace in flow cytometry analysis.

5.2.4 Assessment of cell apoptosis/necrosis

Annexin V/propidium iodide (PI) staining was performed using the Dead Cells apoptosis kit according to the manufacturer‘s instructions (Invitrogen). PBMCs were washed twice with cold PBS and resuspended at a concentration of $1x10^6$ cells/ml in 1X

binding buffer. 100µL of cell suspension was stained using 5µL of FITC-conjugated annexin V and 5µL PI for 15 minutes in the dark before analysis. The samples were acquired on a Becton Dickinson FACS CANTO II cytometer and analyzed using FlowJo software v10.9.0. All events were intentionally included in this gating strategy, excluding only debris, to ascertain the frequency of dead cells under different conditions. By adopting this inclusive approach to gating, we ensured a comprehensive evaluation that considers the entire cell population.

5.2.5 Erythrolysis assay

Whole blood was collected from healthy subject. 20µL of blood was resuspended in 180µL of PBS and treated with WZB117 10µM for 30 minutes or distilled water. Samples were then centrifuged at 10.000 rpm for 5 minutes and the release of hemoglobin into the surrounding medium was evaluated.

5.2.6 EndoC-βH1 cell line

EndoC-βH1 cells were derived from human fetal pancreatic buds transduced with a lentiviral vector expressing SV40LT under the insulin promoter control, grafted into SCID mice in order to develop mature pancreatic tissue [223]. EndoC-βH1 cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 5.5mM glucose, 2% BSA fraction V, 50µM 2-mercaptoethanol, 10mM nicotinamide, 5.5µg/ml transferrin, 6.7ng/ml sodium selenite, penicillin (100U/ml)/streptomycin (100U/mL). Cells were passaged when confluence was observed.

5.2.7 Dynamic insulin secretion test of EndoC-βH1 cell line

The insulin secretion was performed using the BioRep® Perifusion V2.0.0 system. Krebs buffer (pH 7-7.4), containing 25mM HEPES, 115mM NaCl, 24mM $NaHCO_3$, 5mM KCl, 1mM $MgCl_2$, 2.5mM $CaCl_2$, and 0.1% BSA was used for pre-priming and priming the system before perifusion. The whole system was maintained at a constant temperature of 37°C. The mechanical arm, which holds the plate, was maintained at a temperature of 4°C. Briefly, EndoC-βH1 cells were initially perfused with a low glucose insulin-free perifusion buffer (2mM glucose) for 80 minutes to allow islet equilibration and removal of culture medium. Next, cells were stimulated with insulin-free perifusion buffer at 15mM glucose for 20 minutes, followed by 20 minutes with 2mM glucose. Then

30mM KCl was added for 20 minutes and finally, cells were perfused with low glucose for the last 20 minutes. WZB117 at a concentration of 10μM was administered at different time points. The eluate was collected in 96 well plates and the insulin secretion was measured by ELISA test (Mercodia).

5.2.8 Cell staining for multiparameter flow cytometry analysis

For FACS analysis, PBMCs were stained with fluorochrome-conjugated monoclonal antibodies. After 6 days in culture cells were harvested, resuspended in PBS supplemented with 5% FBS and placed in FACS tubes. After antibodies for external staining were added, cells were incubated on ice for 20 minutes in the dark and then washed twice in 2mL of PBS to remove excess of antibody. Zombie UV fixable viability dye (Biolegend) was added to exclude dead cells from the analysis. After 20 minutes of incubation on ice in the dark, the reaction was brought to a stop by adding cold PBS supplemented with 5% of FBS and centrifuging at 300*g* x 10 minutes. Cells were then resuspended and washed in PBS. For intracellular detection of markers, cells were fixed and permeabilized using eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Invitrogen), according to the manufacturer's instructions. Briefly, cells were incubated for 30 minutes in the dark with 1mL of fixation/permeabilization solution and then washed with 2mL of 1X Permeabilization Buffer at 300*g* for 5 minutes. Cells were stained with monoclonal antibodies for intracellular detection, incubated for 20 minutes on ice, protected from the light, and washed twice with 2mL of cold 1X Permeabilization Buffer. Cellular debris and multi-nucleate events were gated out using forward (FSC) and side scatter (SSC). Samples were acquired on a Cytek Aurora and analyzed using FlowJo software v10.9.0.

Of note, CTLA-4 staining was conducted after permeabilization, as part of the intracellular stains. We used the PE Mouse Anti-Human CD152 from BD Biosciences (https://www.bdbiosciences.com/en-ca/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-mouse-anti-human-cd152.557301), which is designed for flow cytometry intracellular staining. CTLA-4 is expressed primarily as an intracellular antigen with transport to the cell surface under tight regulation of several molecules including Trim, PLD and TIRC7. CTLA-4

undergoes rapid internalization once expressed at the cell surface [224]. In our experiments, cells were either non-stimulated (for *ex-vivo* experiments) or analyzed after 6 days of in culture stimulation (for *in vitro* experiments). Since CTLA-4 surface expression is maintained up to 48 hours after activation [225], surface expression might not have been detectable in our experimental conditions.

5.2.9 MHC class I and II dextramers staining

For dextramers staining, thawed PBMCs were washed twice and resuspended at $2x10^7$ cells/mL in PBS supplemented with 5% of FBS. The cell suspension was then incubated for 90 minutes at room temperature in the dark with a pool of dextramers, containing 7 MHC Dextramer reagents (10µL each) (Immudex), plus 2.8µL of mouse serum and 8.4µL of 10X PBS, according to the manufacturer's instruction. The pool solution was previously centrifuged at 10.000*g* for 1 minute, to avoid potential precipitate.

The following HLA-A*0201 dextramers were used for $CD8^+$ T cells identification:

- PE-conjugated HLA-A*0201 $GAD65_{114-123}$ VMNILLQYVV
- PE-conjugated HLA-A*0201 $Insulin_{10-19}$ HLVEALYLV
- PE-conjugated HLA-A*0201 $IGRP_{265-273}$ VLFGLGFAI
- APC-conjugated HLA-A*0201 CMV IE-$1_{316-324}$ VLEETSVML

The following HLA-DR*0401 dextramers were used for $CD4^+$ T cells identification:

- PE-conjugated DRB1*0401 $GAD65_{555-567}$ NFIRMVISNPAAT
- PE-conjugated DRB1*0401 $Proinsulin_{73-90}$ GAGSLQPLALEGSLQKRG
- APC-conjugated DRB1*0401 Flu $HA_{306-318}$ PKYVKQNTLKLAT

The following dextramers were used as negative control:

- PE-conjugated DRB1*0401 PVSKMRMATPLLMQA
- PE-conjugated HLA-A*0201ALIAPVHAV

Antibodies for external staining were immediately added to the dextramers-stained cell suspension and incubated for 20 minutes at room temperature. Cells were then washed in PBS supplemented with 5% of FBS. The protocol followed as described in the paragraph "**Cell staining for multiparameter flow cytometry analysis**". Cells were resuspended in 500µL of 1X Permeabilization Buffer and immediately analyzed on a Cytek Aurora.

We followed the MHC Dextramer® Staining Protocol provided by Immudex, the manufacturer of the dextramers, which includes a fixation step after dextramer staining. Fixation was also required for the intracellular stains in our protocol. Previous studies support the compatibility of MHC dextramer staining with fixation [226, 227].

The gating strategy was as follows: the lymphocytes were identified by the forward (FSC) and side scatter (SSC) profile, doublets were discarded and living cells were distinguished from dead by plotting SSC vs ZombieUV. $CD3^+$ T cells were identified by the SSC and CD3 channel, $CD4^+$ and $CD8^+$ T cells were gated on $CD3^+$ positive T cells. Then, autoreactive T cells were considered as events in the gate positive for pool of dextramers in PE.

5.2.10 Statistical analysis

Flow cytometry data were analyzed using FlowJo software v10.9.0 and calculated as mean fluorescence intensity (MFI) of the total $CD4^+$, $CD8^+$ or Treg cells, and subpopulations as percentage of positive cells.

Data for the protein expression levels among the dextramers positive T cells in SPK subgroups were compared by calculating the Log2 of the fold change for each protein and represented as heatmaps.

Statistical analyses were performed using GraphPad Prism software version 9.0. Data were presented as mean and SD, tested for normal distribution and analyzed with the paired Student's t-test (parametric data) or Wilcoxon rank test (nonparametric data). One-way ANOVA, followed by post hoc Turkey test, or nonparametric unpaired Mann-Whitney test, was used for comparison between groups of patients with T1D, HD and SPK recipients.

Dimensionality reduction was performed with the UMAP FlowJo plugin v4.0.4. Dextramer-positive or negative populations from each individual were down-sampled for comparability (FlowJo Downsample plugin v3.3), barcoded and concatenated. FlowSOM algorithm v3.0.18 [169] was applied for unsupervised clustering, using default combinations of parameter values.

Outliers were excluded using the robust regression and outlier detection method (ROUT) on GraphPad Prism software. For all analyses, a two-tailed $p<0.05$ was considered significant.

5.3 Materials

5.3.1 Antibodies and reagents for flow cytometry

5.3.1.1. Antibodies

Specificity	Clone	Producer
CD3	SK7	Biolegend
CD4	SK3	Biolegend
CD95	DX2	Biolegend
CD127	A019D5	Biolegend
CD194	L291H4	Biolegend
CD195	J418F1	Biolegend
CD196	G034E3	Biolegend
CD197	G043H7	Biolegend
LAG-3	11C3C65	Biolegend
CD27	O323	Biolegend
PD-1	EH12.2H7	Biolegend
CD38	S17017A	Biolegend
CD45RA	HI100	Biolegend
CD62L	DREG-56	Biolegend
HLA-DR	L243	Biolegend
HELIOS	22F6	Biolegend
FoxP3	206D	Biolegend
CD122	5H4	BD Bioscience
CD183	1C6/CXCR3	BD Bioscience
CD25	M-A251	BD Bioscience
CD278	DX29	BD Bioscience
CD28	CD28.2	BD Bioscience
CD69	FN50	BD Bioscience
CD73	AD2	BD Bioscience
TIGIT	741182	BD Bioscience
CD272	J168-540	BD Bioscience
CD39	TU66	BD Bioscience
CD152	BNI3	BD Bioscience
KI-67	B56	BD Bioscience
CD98	590559	R&D System
GLUT3	202017	R&D System
CD8	RPA-T8	eBioscience
GLUT1	Polyclonal	Novus Bio

5.3.1.2 Other reagents for staining

5.3.1.2.1 Fluorescent dyes for cell staining

Reagent	**Producer**
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Thermofisher scientific
CellTrace Violet	Thermofisher scientific
eBioscience™ Foxp3 / Transcription Factor Fixation/Permeabilization Concentrate and Diluent	Invitrogen
Dead Cell Apoptosis Kits with Annexin V	Invitrogen
Zombie UV fixable viability dye	Biolegend

5.3.1.2.2 MHC multimers

The following MHC dextramers were purchased from Immudex (Copenhagen, Denmark):

Reagent	**Fluorochrome**
HLA-A*0201 GAD65$_{114-122}$ (VMNILLQYV)	PE
HLA-A*0201 Insulin$_{10-19}$ (HLVEALYLV)	PE
HLA-A*0201 IGRP$_{265-273}$ (VLFGLGFAI)	PE
HLA-A*0201 CMV IE-1$_{316-324}$ (VLEETSVML)	APC
HLA-A*0201ALIAPVHAV	PE
DRB1*0401 GAD65$_{555-567}$ (NFIRMVISNPAAT)	PE
DRB1*0401 Proinsulin$_{73-90}$ (GAGSLQPLALEGSLQKRG)	PE
DRB1*0401 Flu HA$_{306-318}$ (PKYVKQNTLKLAT)	APC
DRB1*0401 PVSKMRMATPLLMQA	PE

5.3.2 Cell culture reagents and buffers

5.3.2.1 Cell culture medium

Reagent	Producer
RPMI 1640	Gibco
RPMI 1640 without glucose	Gibco
Penicillin-Streptomycin (5,000 U/mL)	Gibco
Fetal Bovine Serum (FBS)	Gibco
Dialyzed FBS	Gibco
Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation	Gibco

5.3.2.2 Drugs

Reagent	Producer
WZB117	Merck
Rapamycin	Merck
FK506	Merck
Mycophenolate mofetil (MMF)	Roche Laboratories

5.3.3 Other reagents

Reagent	Producer
Cytiva Ficoll-Paque™ PLUS	Fisher Scientific
Ammonium Chloride (NH_4Cl)	Merck
Potassium bicarbonate ($KHCO_3$)	Merck
Ethylenediaminetetraacetic acid disodium salt solution (EDTA)	Merck
Phosphate Buffered Saline (PBS)	Gibco

REFERENCES

1. Babaya N, Nakayama M, Eisenbarth GS: **The stages of type 1A diabetes**. *Ann N Y Acad Sci* 2005, **1051**:194-204.
2. Kent SC, Chen Y, Bregoli L, Clemmings SM, Kenyon NS, Ricordi C, Hering BJ, Hafler DA: **Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope**. *Nature* 2005, **435**(7039):224-228.
3. Coppieters KT, Dotta F, Amirian N, Campbell PD, Kay TW, Atkinson MA, Roep BO, von Herrath MG: **Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients**. *J Exp Med* 2012, **209**(1):51-60.
4. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, Colagiuri S, Guariguata L, Motala AA, Ogurtsova K *et al*: **Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition**. *Diabetes Res Clin Pract* 2019, **157**:107843.
5. Kamrath C, Rosenbauer J, Eckert AJ, Siedler K, Bartelt H, Klose D, Sindichakis M, Herrlinger S, Lahn V, Holl RW: **Incidence of Type 1 Diabetes in Children and Adolescents During the COVID-19 Pandemic in Germany: Results From the DPV Registry**. *Diabetes Care* 2022, **45**(8):1762-1771.
6. Gottesman BL, Yu J, Tanaka C, Longhurst CA, Kim JJ: **Incidence of New-Onset Type 1 Diabetes Among US Children During the COVID-19 Global Pandemic**. *JAMA Pediatr* 2022, **176**(4):414-415.
7. Rahmati M, Keshvari M, Mirnasuri S, Yon DK, Lee SW, Il Shin J, Smith L: **The global impact of COVID-19 pandemic on the incidence of pediatric new-onset type 1 diabetes and ketoacidosis: A systematic review and meta-analysis**. *J Med Virol* 2022, **94**(11):5112-5127.
8. Marca V, Gianchecchi E, Fierabracci A: **Type 1 Diabetes and Its Multi-Factorial Pathogenesis: The Putative Role of NK Cells**. *Int J Mol Sci* 2018, **19**(3).
9. Gomez-Munoz L, Perna-Barrull D, Villalba A, Rodriguez-Fernandez S, Ampudia RM, Teniente-Serra A, Vazquez F, Murillo M, Perez J, Corripio R *et al*: **NK Cell Subsets Changes in Partial Remission and Early Stages of Pediatric Type 1 Diabetes**. *Front Immunol* 2020, **11**:611522.
10. Diana J, Simoni Y, Furio L, Beaudoin L, Agerberth B, Barrat F, Lehuen A: **Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes**. *Nat Med* 2013, **19**(1):65-73.
11. Smith MJ, Simmons KM, Cambier JC: **B cells in type 1 diabetes mellitus and diabetic kidney disease**. *Nat Rev Nephrol* 2017, **13**(11):712-720.
12. Ilonen J, Lempainen J, Veijola R: **The heterogeneous pathogenesis of type 1 diabetes mellitus**. *Nat Rev Endocrinol* 2019, **15**(11):635-650.
13. Bottazzo GF, Florin-Christensen A, Doniach D: **Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies**. *Lancet* 1974, **2**(7892):1279-1283.
14. Wenzlau JM, Hutton JC: **Novel diabetes autoantibodies and prediction of type 1 diabetes**. *Curr Diab Rep* 2013, **13**(5):608-615.
15. Fabris M, Zago S, Liguori M, Trevisan MT, Zanatta M, Comici A, Zanette G, Carlin E, Curcio F, Tonutti E: **Anti-zinc transporter protein 8 autoantibodies**

significantly improve the diagnostic approach to type 1 diabetes: an Italian multicentre study on paediatric patients. *Auto Immun Highlights* 2015, **6**(1-2):17-22.
16. Kawasaki E: **Anti-Islet Autoantibodies in Type 1 Diabetes**. *Int J Mol Sci* 2023, **24**(12).
17. Gonzalez-Duque S, Azoury ME, Colli ML, Afonso G, Turatsinze JV, Nigi L, Lalanne AI, Sebastiani G, Carre A, Pinto S *et al*: **Conventional and Neo-antigenic Peptides Presented by beta Cells Are Targeted by Circulating Naive CD8+ T Cells in Type 1 Diabetic and Healthy Donors**. *Cell Metab* 2018, **28**(6):946-960 e946.
18. Sidney J, Vela JL, Friedrich D, Kolla R, von Herrath M, Wesley JD, Sette A: **Low HLA binding of diabetes-associated CD8+ T-cell epitopes is increased by post translational modifications**. *BMC Immunol* 2018, **19**(1):12.
19. Rodriguez-Calvo T, Johnson JD, Overbergh L, Dunne JL: **Neoepitopes in Type 1 Diabetes: Etiological Insights, Biomarkers and Therapeutic Targets**. *Front Immunol* 2021, **12**:667989.
20. Baker RL, Jamison BL, Wiles TA, Lindsay RS, Barbour G, Bradley B, Delong T, Friedman RS, Nakayama M, Haskins K: **CD4 T Cells Reactive to Hybrid Insulin Peptides Are Indicators of Disease Activity in the NOD Mouse**. *Diabetes* 2018, **67**(9):1836-1846.
21. Wiles TA, Delong T: **HIPs and HIP-reactive T cells**. *Clin Exp Immunol* 2019, **198**(3):306-313.
22. Mitchell AM, Alkanani AA, McDaniel KA, Pyle L, Waugh K, Steck AK, Nakayama M, Yu L, Gottlieb PA, Rewers MJ *et al*: **T-cell responses to hybrid insulin peptides prior to type 1 diabetes development**. *Proc Natl Acad Sci U S A* 2021, **118**(6).
23. Kracht MJ, van Lummel M, Nikolic T, Joosten AM, Laban S, van der Slik AR, van Veelen PA, Carlotti F, de Koning EJ, Hoeben RC *et al*: **Autoimmunity against a defective ribosomal insulin gene product in type 1 diabetes**. *Nat Med* 2017, **23**(4):501-507.
24. Atkinson MA, Eisenbarth GS, Michels AW: **Type 1 diabetes**. *Lancet* 2014, **383**(9911):69-82.
25. Insel RA, Dunne JL, Atkinson MA, Chiang JL, Dabelea D, Gottlieb PA, Greenbaum CJ, Herold KC, Krischer JP, Lernmark A *et al*: **Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association**. *Diabetes Care* 2015, **38**(10):1964-1974.
26. Andersson C, Kolmodin M, Ivarsson SA, Carlsson A, Forsander G, Lindblad B, Ludvigsson J, Kockum I, Marcus C, Samuelsson U *et al*: **Islet cell antibodies (ICA) identify autoimmunity in children with new onset diabetes mellitus negative for other islet cell antibodies**. *Pediatr Diabetes* 2014, **15**(5):336-344.
27. Knip M, Simell O: **Environmental triggers of type 1 diabetes**. *Cold Spring Harb Perspect Med* 2012, **2**(7):a007690.
28. Warncke K, Weiss A, Achenbach P, von dem Berge T, Berner R, Casteels K, Groele L, Hatzikotoulas K, Hommel A, Kordonouri O *et al*: **Elevations in blood glucose before and after the appearance of islet autoantibodies in children**. *J Clin Invest* 2022, **132**(20).

29. American Diabetes A: **2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018**. *Diabetes Care* 2018, **41**(Suppl 1):S13-S27.
30. Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, Winkler C, Ilonen J, Veijola R, Knip M *et al*: **Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children**. *JAMA* 2013, **309**(23):2473-2479.
31. Evans-Molina C, Oram RA: **Teplizumab approval for type 1 diabetes in the USA**. *The Lancet Diabetes & Endocrinology* 2023, **11**(2):76-77.
32. Herold KC, Bundy BN, Long SA, Bluestone JA, DiMeglio LA, Dufort MJ, Gitelman SE, Gottlieb PA, Krischer JP, Linsley PS *et al*: **An Anti-CD3 Antibody, Teplizumab, in Relatives at Risk for Type 1 Diabetes**. *N Engl J Med* 2019, **381**(7):603-613.
33. Thakkar S, Chopra A, Nagendra L, Kalra S, Bhattacharya S: **Teplizumab in Type 1 Diabetes Mellitus: An Updated Review**. *touchREV Endocrinol* 2023, **19**(2):22-30.
34. Keymeulen B, van Maurik A, Inman D, Oliveira J, McLaughlin R, Gittelman RM, Roep BO, Gillard P, Hilbrands R, Gorus F *et al*: **A randomised, single-blind, placebo-controlled, dose-finding safety and tolerability study of the anti-CD3 monoclonal antibody otelixizumab in new-onset type 1 diabetes**. *Diabetologia* 2021, **64**(2):313-324.
35. Hagopian W, Ferry RJ, Jr., Sherry N, Carlin D, Bonvini E, Johnson S, Stein KE, Koenig S, Daifotis AG, Herold KC *et al*: **Teplizumab preserves C-peptide in recent-onset type 1 diabetes: two-year results from the randomized, placebo-controlled Protege trial**. *Diabetes* 2013, **62**(11):3901-3908.
36. Long SA, Thorpe J, Herold KC, Ehlers M, Sanda S, Lim N, Linsley PS, Nepom GT, Harris KM: **Remodeling T cell compartments during anti-CD3 immunotherapy of type 1 diabetes**. *Cell Immunol* 2017, **319**:3-9.
37. Todd JA, Walker NM, Cooper JD, Smyth DJ, Downes K, Plagnol V, Bailey R, Nejentsev S, Field SF, Payne F *et al*: **Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes**. *Nat Genet* 2007, **39**(7):857-864.
38. Valdes AM, Wapelhorst B, Concannon P, Erlich HA, Thomson G, Noble JA: **Extended DR3-D6S273-HLA-B haplotypes are associated with increased susceptibility to type 1 diabetes in US Caucasians**. *Tissue Antigens* 2005, **65**(1):115-119.
39. Pugliese A, Boulware D, Yu L, Babu S, Steck AK, Becker D, Rodriguez H, DiMeglio L, Evans-Molina C, Harrison LC *et al*: **HLA-DRB1*15:01-DQA1*01:02-DQB1*06:02 Haplotype Protects Autoantibody-Positive Relatives From Type 1 Diabetes Throughout the Stages of Disease Progression**. *Diabetes* 2016, **65**(4):1109-1119.
40. Mameli C, Triolo TM, Chiarelli F, Rewers M, Zuccotti G, Simmons KM: **Lessons and gaps in the prediction and prevention of type 1 diabetes**. *Pharmacol Res* 2023, **193**:106792.
41. Zhang L, Nakayama M, Eisenbarth GS: **Insulin as an autoantigen in NOD/human diabetes**. *Curr Opin Immunol* 2008, **20**(1):111-118.
42. Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM, Smith AN, Di Genova G *et al*: **Association of the T-cell**

regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 2003, **423**(6939):506-511.
43. Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K, Rostamkhani M, MacMurray J, Meloni GF, Lucarelli P, Pellecchia M *et al*: **A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes**. *Nat Genet* 2004, **36**(4):337-338.
44. Vella A, Cooper JD, Lowe CE, Walker N, Nutland S, Widmer B, Jones R, Ring SM, McArdle W, Pembrey ME *et al*: **Localization of a type 1 diabetes locus in the IL2RA/CD25 region by use of tag single-nucleotide polymorphisms**. *Am J Hum Genet* 2005, **76**(5):773-779.
45. Cho JH, Feldman M: **Heterogeneity of autoimmune diseases: pathophysiologic insights from genetics and implications for new therapies**. *Nat Med* 2015, **21**(7):730-738.
46. Oram RA, Patel K, Hill A, Shields B, McDonald TJ, Jones A, Hattersley AT, Weedon MN: **A Type 1 Diabetes Genetic Risk Score Can Aid Discrimination Between Type 1 and Type 2 Diabetes in Young Adults**. *Diabetes Care* 2016, **39**(3):337-344.
47. Ferrat LA, Vehik K, Sharp SA, Lernmark A, Rewers MJ, She JX, Ziegler AG, Toppari J, Akolkar B, Krischer JP *et al*: **A combined risk score enhances prediction of type 1 diabetes among susceptible children**. *Nat Med* 2020, **26**(8):1247-1255.
48. Sharp SA, Rich SS, Wood AR, Jones SE, Beaumont RN, Harrison JW, Schneider DA, Locke JM, Tyrrell J, Weedon MN *et al*: **Development and Standardization of an Improved Type 1 Diabetes Genetic Risk Score for Use in Newborn Screening and Incident Diagnosis**. *Diabetes Care* 2019, **42**(2):200-207.
49. Oram RA, Sharp SA, Pihoker C, Ferrat L, Imperatore G, Williams A, Redondo MJ, Wagenknecht L, Dolan LM, Lawrence JM *et al*: **Utility of Diabetes Type-Specific Genetic Risk Scores for the Classification of Diabetes Type Among Multiethnic Youth**. *Diabetes Care* 2022, **45**(5):1124-1131.
50. Xia Y, Xie Z, Huang G, Zhou Z: **Incidence and trend of type 1 diabetes and the underlying environmental determinants**. *Diabetes Metab Res Rev* 2019, **35**(1):e3075.
51. Soderstrom U, Aman J, Hjern A: **Being born in Sweden increases the risk for type 1 diabetes - a study of migration of children to Sweden as a natural experiment**. *Acta Paediatr* 2012, **101**(1):73-77.
52. Nistico L, Iafusco D, Galderisi A, Fagnani C, Cotichini R, Toccaceli V, Stazi MA, Study Group on Diabetes of the Italian Society of Pediatric E, Diabetology: **Emerging effects of early environmental factors over genetic background for type 1 diabetes susceptibility: evidence from a Nationwide Italian Twin Study**. *J Clin Endocrinol Metab* 2012, **97**(8):E1483-1491.
53. Metcalfe KA, Hitman GA, Rowe RE, Hawa M, Huang X, Stewart T, Leslie RD: **Concordance for type 1 diabetes in identical twins is affected by insulin genotype**. *Diabetes Care* 2001, **24**(5):838-842.
54. Chehadeh W, Kerr-Conte J, Pattou F, Alm G, Lefebvre J, Wattre P, Hober D: **Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells**. *J Virol* 2000, **74**(21):10153-10164.

55. Nekoua MP, Yessoufou A, Alidjinou EK, Badia-Boungou F, Moutairou K, Sane F, Hober D: **Salivary anti-coxsackievirus-B4 neutralizing activity and pattern of immune parameters in patients with type 1 diabetes: a pilot study**. *Acta Diabetol* 2018, **55**(8):827-834.
56. Lloyd RE, Tamhankar M, Lernmark A: **Enteroviruses and Type 1 Diabetes: Multiple Mechanisms and Factors?** *Annu Rev Med* 2022, **73**:483-499.
57. Nekoua MP, Mercier A, Alhazmi A, Sane F, Alidjinou EK, Hober D: **Fighting Enteroviral Infections to Prevent Type 1 Diabetes**. *Microorganisms* 2022, **10**(4).
58. Krogvold L, Mynarek IM, Ponzi E, Mork FB, Hessel TW, Roald T, Lindblom N, Westman J, Barker P, Hyoty H *et al*: **Pleconaril and ribavirin in new-onset type 1 diabetes: a phase 2 randomized trial**. *Nat Med* 2023.
59. Wilkin TJ: **The accelerator hypothesis: a unifying explanation for type-1 and type-2 diabetes**. *Nestle Nutr Workshop Ser Clin Perform Programme* 2006, **11**:139-153.
60. Harder T, Roepke K, Diller N, Stechling Y, Dudenhausen JW, Plagemann A: **Birth weight, early weight gain, and subsequent risk of type 1 diabetes: systematic review and meta-analysis**. *Am J Epidemiol* 2009, **169**(12):1428-1436.
61. Thomas RM, Jobin C: **Microbiota in pancreatic health and disease: the next frontier in microbiome research**. *Nat Rev Gastroenterol Hepatol* 2020, **17**(1):53-64.
62. Verduci E, Mameli C, Amatruda M, Petitti A, Vizzuso S, El Assadi F, Zuccotti G, Alabduljabbar S, Terranegra A: **Early Nutrition and Risk of Type 1 Diabetes: The Role of Gut Microbiota**. *Front Nutr* 2020, **7**:612377.
63. Lo Conte M, Antonini Cencicchio M, Ulaszewska M, Nobili A, Cosorich I, Ferrarese R, Massimino L, Andolfo A, Ungaro F, Mancini N *et al*: **A diet enriched in omega-3 PUFA and inulin prevents type 1 diabetes by restoring gut barrier integrity and immune homeostasis in NOD mice**. *Front Immunol* 2022, **13**:1089987.
64. Girdhar K, Huang Q, Chow IT, Vatanen T, Brady C, Raisingani A, Autissier P, Atkinson MA, Kwok WW, Kahn CR *et al*: **A gut microbial peptide and molecular mimicry in the pathogenesis of type 1 diabetes**. *Proc Natl Acad Sci U S A* 2022, **119**(31):e2120028119.
65. Boddu SK, Aurangabadkar G, Kuchay MS: **New onset diabetes, type 1 diabetes and COVID-19**. *Diabetes Metab Syndr* 2020, **14**(6):2211-2217.
66. Holman N, Knighton P, Kar P, O'Keefe J, Curley M, Weaver A, Barron E, Bakhai C, Khunti K, Wareham NJ *et al*: **Risk factors for COVID-19-related mortality in people with type 1 and type 2 diabetes in England: a population-based cohort study**. *Lancet Diabetes Endocrinol* 2020, **8**(10):823-833.
67. Qeadan F, Tingey B, Egbert J, Pezzolesi MG, Burge MR, Peterson KA, Honda T: **The associations between COVID-19 diagnosis, type 1 diabetes, and the risk of diabetic ketoacidosis: A nationwide cohort from the US using the Cerner Real-World Data**. *PLoS One* 2022, **17**(4):e0266809.
68. Nentwich MM, Ulbig MW: **Diabetic retinopathy - ocular complications of diabetes mellitus**. *World J Diabetes* 2015, **6**(3):489-499.
69. Hovind P, Tarnow L, Rossing P, Jensen BR, Graae M, Torp I, Binder C, Parving HH: **Predictors for the development of microalbuminuria and**

macroalbuminuria in patients with type 1 diabetes: inception cohort study. *BMJ* 2004, **328**(7448):1105.
70. Lim A: **Diabetic nephropathy - complications and treatment**. *Int J Nephrol Renovasc Dis* 2014, **7**:361-381.
71. Groop PH, Thomas MC, Moran JL, Waden J, Thorn LM, Makinen VP, Rosengard-Barlund M, Saraheimo M, Hietala K, Heikkila O *et al*: **The presence and severity of chronic kidney disease predicts all-cause mortality in type 1 diabetes**. *Diabetes* 2009, **58**(7):1651-1658.
72. Sandholm N, Van Zuydam N, Ahlqvist E, Juliusdottir T, Deshmukh HA, Rayner NW, Di Camillo B, Forsblom C, Fadista J, Ziemek D *et al*: **The Genetic Landscape of Renal Complications in Type 1 Diabetes**. *J Am Soc Nephrol* 2017, **28**(2):557-574.
73. Harjutsalo V, Thomas MC, Forsblom C, Groop PH, FinnDiane Study G: **Risk of coronary artery disease and stroke according to sex and presence of diabetic nephropathy in type 1 diabetes**. *Diabetes Obes Metab* 2018, **20**(12):2759-2767.
74. Rawshani A, Sattar N, Franzen S, Rawshani A, Hattersley AT, Svensson AM, Eliasson B, Gudbjornsdottir S: **Excess mortality and cardiovascular disease in young adults with type 1 diabetes in relation to age at onset: a nationwide, register-based cohort study**. *Lancet* 2018, **392**(10146):477-486.
75. Bodman MA, Varacallo M: **Peripheral Diabetic Neuropathy**. In: *StatPearls.* Treasure Island (FL) ineligible companies. Disclosure: Matthew Varacallo declares no relevant financial relationships with ineligible companies.; 2023.
76. Lizzo JM, Goyal A, Gupta V: **Adult Diabetic Ketoacidosis**. In: *StatPearls.* Treasure Island (FL) ineligible companies. Disclosure: Amandeep Goyal declares no relevant financial relationships with ineligible companies. Disclosure: Vikas Gupta declares no relevant financial relationships with ineligible companies.; 2023.
77. Graveling AJ, Frier BM: **The risks of nocturnal hypoglycaemia in insulin-treated diabetes**. *Diabetes Res Clin Pract* 2017, **133**:30-39.
78. Amiel SA: **The consequences of hypoglycaemia**. *Diabetologia* 2021, **64**(5):963-970.
79. Boehncke WH, Brembilla NC: **Autoreactive T-Lymphocytes in Inflammatory Skin Diseases**. *Front Immunol* 2019, **10**:1198.
80. Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW: **Autoreactive T cells in healthy individuals**. *J Immunol* 2004, **172**(10):5967-5972.
81. Heninger AK, Monti P, Wilhelm C, Schwaiger P, Kuehn D, Ziegler AG, Bonifacio E: **Activation of islet autoreactive naive T cells in infants is influenced by homeostatic mechanisms and antigen-presenting capacity**. *Diabetes* 2013, **62**(6):2059-2066.
82. Culina S, Lalanne AI, Afonso G, Cerosaletti K, Pinto S, Sebastiani G, Kuranda K, Nigi L, Eugster A, Osterbye T *et al*: **Islet-reactive CD8(+) T cell frequencies in the pancreas, but not in blood, distinguish type 1 diabetic patients from healthy donors**. *Sci Immunol* 2018, **3**(20).
83. Linsley PS, Barahmand-Pour-Whitman F, Balmas E, DeBerg HA, Flynn KJ, Hu AK, Rosasco MG, Chen J, O'Rourke C, Serti E *et al*: **Autoreactive T cell receptors with shared germline-like alpha chains in type 1 diabetes**. *JCI Insight* 2021, **6**(22).

84. Kaddis JS, Pugliese A, Atkinson MA: **A run on the biobank: what have we learned about type 1 diabetes from the nPOD tissue repository?** *Curr Opin Endocrinol Diabetes Obes* 2015, **22**(4):290-295.
85. Morgan NG, Richardson SJ: **Fifty years of pancreatic islet pathology in human type 1 diabetes: insights gained and progress made**. *Diabetologia* 2018, **61**(12):2499-2506.
86. Seay HR, Yusko E, Rothweiler SJ, Zhang L, Posgai AL, Campbell-Thompson M, Vignali M, Emerson RO, Kaddis JS, Ko D *et al*: **Tissue distribution and clonal diversity of the T and B cell repertoire in type 1 diabetes**. *JCI Insight* 2016, **1**(20):e88242.
87. Monti P, Scirpoli M, Rigamonti A, Mayr A, Jaeger A, Bonfanti R, Chiumello G, Ziegler AG, Bonifacio E: **Evidence for in vivo primed and expanded autoreactive T cells as a specific feature of patients with type 1 diabetes**. *J Immunol* 2007, **179**(9):5785-5792.
88. Endl J, Rosinger S, Schwarz B, Friedrich SO, Rothe G, Karges W, Schlosser M, Eiermann T, Schendel DJ, Boehm BO: **Coexpression of CD25 and OX40 (CD134) receptors delineates autoreactive T-cells in type 1 diabetes**. *Diabetes* 2006, **55**(1):50-60.
89. Velthuis JH, Unger WW, Abreu JR, Duinkerken G, Franken K, Peakman M, Bakker AH, Reker-Hadrup S, Keymeulen B, Drijfhout JW *et al*: **Simultaneous detection of circulating autoreactive CD8+ T-cells specific for different islet cell-associated epitopes using combinatorial MHC multimers**. *Diabetes* 2010, **59**(7):1721-1730.
90. Kronenberg D, Knight RR, Estorninho M, Ellis RJ, Kester MG, de Ru A, Eichmann M, Huang GC, Powrie J, Dayan CM *et al*: **Circulating preproinsulin signal peptide-specific CD8 T cells restricted by the susceptibility molecule HLA-A24 are expanded at onset of type 1 diabetes and kill beta-cells**. *Diabetes* 2012, **61**(7):1752-1759.
91. Yeo L, Pujol-Autonell I, Baptista R, Eichmann M, Kronenberg-Versteeg D, Heck S, Dolton G, Sewell AK, Harkonen T, Mikk ML *et al*: **Circulating beta cell-specific CD8(+) T cells restricted by high-risk HLA class I molecules show antigen experience in children with and at risk of type 1 diabetes**. *Clin Exp Immunol* 2020, **199**(3):263-277.
92. Skowera A, Ladell K, McLaren JE, Dolton G, Matthews KK, Gostick E, Kronenberg-Versteeg D, Eichmann M, Knight RR, Heck S *et al*: **beta-cell-specific CD8 T cell phenotype in type 1 diabetes reflects chronic autoantigen exposure**. *Diabetes* 2015, **64**(3):916-925.
93. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C *et al*: **A human memory T cell subset with stem cell-like properties**. *Nat Med* 2011, **17**(10):1290-1297.
94. Monti P, Heninger AK, Bonifacio E: **Differentiation, expansion, and homeostasis of autoreactive T cells in type 1 diabetes mellitus**. *Curr Diab Rep* 2009, **9**(2):113-118.
95. Gearty SV, Dundar F, Zumbo P, Espinosa-Carrasco G, Shakiba M, Sanchez-Rivera FJ, Socci ND, Trivedi P, Lowe SW, Lauer P *et al*: **An autoimmune stem-like CD8 T cell population drives type 1 diabetes**. *Nature* 2022, **602**(7895):156-161.

96. Vrisekoop N, den Braber I, de Boer AB, Ruiter AF, Ackermans MT, van der Crabben SN, Schrijver EH, Spierenburg G, Sauerwein HP, Hazenberg MD *et al*: **Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool**. *Proc Natl Acad Sci U S A* 2008, **105**(16):6115-6120.
97. Vignali D, Cantarelli E, Bordignon C, Canu A, Citro A, Annoni A, Piemonti L, Monti P: **Detection and Characterization of CD8(+) Autoreactive Memory Stem T Cells in Patients With Type 1 Diabetes**. *Diabetes* 2018, **67**(5):936-945.
98. Vendrame F, Pileggi A, Laughlin E, Allende G, Martin-Pagola A, Molano RD, Diamantopoulos S, Standifer N, Geubtner K, Falk BA *et al*: **Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation, despite immunosuppression, is associated with autoantibodies and pathogenic autoreactive CD4 T-cells**. *Diabetes* 2010, **59**(4):947-957.
99. Laughlin E, Burke G, Pugliese A, Falk B, Nepom G: **Recurrence of autoreactive antigen-specific CD4+ T cells in autoimmune diabetes after pancreas transplantation**. *Clin Immunol* 2008, **128**(1):23-30.
100. Dholakia S, Oskrochi Y, Easton G, Papalois V: **Advances in pancreas transplantation**. *J R Soc Med* 2016, **109**(4):141-146.
101. Lombardo C, Perrone VG, Amorese G, Vistoli F, Baronti W, Marchetti P, Boggi U: **Update on Pancreatic Transplantation in the Management of Diabetes**. In: *Endotext.* Edited by Feingold KR, Anawalt B, Blackman MR, Boyce A, Chrousos G, Corpas E, de Herder WW, Dhatariya K, Dungan K, Hofland J *et al*. South Dartmouth (MA); 2000.
102. Sutherland DE, Sibley R, Xu XZ, Michael A, Srikanta AM, Taub F, Najarian J, Goetz FC: **Twin-to-twin pancreas transplantation: reversal and reenactment of the pathogenesis of type I diabetes**. *Trans Assoc Am Physicians* 1984, **97**:80-87.
103. Petruzzo P, Andreelli F, McGregor B, Lefrancois N, Dawahra M, Feitosa LC, Dubernard JM, Thivolet C, Martin X: **Evidence of recurrent type I diabetes following HLA-mismatched pancreas transplantation**. *Diabetes Metab* 2000, **26**(3):215-218.
104. Sibley RK, Sutherland DE: **Pancreas transplantation. An immunohistologic and histopathologic examination of 100 grafts**. *Am J Pathol* 1987, **128**(1):151-170.
105. Tyden G, Reinholt FP, Sundkvist G, Bolinder J: **Recurrence of autoimmune diabetes mellitus in recipients of cadaveric pancreatic grafts**. *N Engl J Med* 1996, **335**(12):860-863.
106. Burke GW, 3rd, Vendrame F, Virdi SK, Ciancio G, Chen L, Ruiz P, Messinger S, Reijonen HK, Pugliese A: **Lessons From Pancreas Transplantation in Type 1 Diabetes: Recurrence of Islet Autoimmunity**. *Curr Diab Rep* 2015, **15**(12):121.
107. Vendrame F, Hopfner YY, Diamantopoulos S, Virdi SK, Allende G, Snowhite IV, Reijonen HK, Chen L, Ruiz P, Ciancio G *et al*: **Risk Factors for Type 1 Diabetes Recurrence in Immunosuppressed Recipients of Simultaneous Pancreas-Kidney Transplants**. *Am J Transplant* 2016, **16**(1):235-245.
108. Argente-Pla M, Martinez-Millana A, Del Olmo-Garcia MI, Espi-Reig J, Perez-Rojas J, Traver-Salcedo V, Merino-Torres JF: **Autoimmune Diabetes Recurrence After Pancreas Transplantation: Diagnosis, Management, and Literature Review**. *Ann Transplant* 2019, **24**:608-616.

109. Monti P, Scirpoli M, Maffi P, Ghidoli N, De Taddeo F, Bertuzzi F, Piemonti L, Falcone M, Secchi A, Bonifacio E: **Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells**. *J Clin Invest* 2008, **118**(5):1806-1814.
110. Burke GW, Ciancio G, Morsi M, Figueiro J, Chen L, Vendrame F, Pugliese A: **Type 1 Diabetes Recurrence After Simultaneous Pancreas-KidneyTransplantation**. *Current Transplantation Reports* 2018, **5**(4):295-303.
111. Murata K, Tsukahara T, Emori M, Shibayama Y, Mizushima E, Matsumiya H, Yamashita K, Kaya M, Hirohashi Y, Kanaseki T *et al*: **Identification of a novel human memory T-cell population with the characteristics of stem-like chemo-resistance**. *Oncoimmunology* 2016, **5**(6):e1165376.
112. Coppieters KT, Amirian N, Pagni PP, Baca Jones C, Wiberg A, Lasch S, Hintermann E, Christen U, von Herrath MG: **Functional redundancy of CXCR3/CXCL10 signaling in the recruitment of diabetogenic cytotoxic T lymphocytes to pancreatic islets in a virally induced autoimmune diabetes model**. *Diabetes* 2013, **62**(7):2492-2499.
113. Satin LS, Soleimanpour SA, Walker EM: **New Aspects of Diabetes Research and Therapeutic Development**. *Pharmacol Rev* 2021, **73**(3):1001-1015.
114. Hogrebe NJ, Ishahak M, Millman JR: **Developments in stem cell-derived islet replacement therapy for treating type 1 diabetes**. *Cell Stem Cell* 2023, **30**(5):530-548.
115. Gruessner AC, Gruessner RW: **Pancreas transplant outcomes for United States and non United States cases as reported to the United Network for Organ Sharing and the International Pancreas Transplant Registry as of December 2011**. *Clin Transpl* 2012:23-40.
116. Marchetti P, Occhipinti M, Rondinini L, Mariotti R, Amorese G, Barsotti M, Vistoli F, Boggi U: **Metabolic and cardiovascular effects of beta cell replacement in type 1 diabetes**. *Intern Emerg Med* 2013, **8 Suppl 1**:S55-56.
117. Mineo D, Pileggi A, Alejandro R, Ricordi C: **Point: steady progress and current challenges in clinical islet transplantation**. *Diabetes Care* 2009, **32**(8):1563-1569.
118. Kochar IS, Jain R: **Pancreas transplant in type 1 diabetes mellitus: the emerging role of islet cell transplant**. *Ann Pediatr Endocrinol Metab* 2021, **26**(2):86-91.
119. Nagendra L, Fernandez CJ, Pappachan JM: **Simultaneous pancreas-kidney transplantation for end-stage renal failure in type 1 diabetes mellitus: Current perspectives**. *World J Transplant* 2023, **13**(5):208-220.
120. Sutherland DER: **Pancreas and Islet Transplant Population**. In: *Transplantation of the Pancreas*. Edited by Gruessner RWG, Sutherland DER. New York, NY: Springer New York; 2004: 91-102.
121. Kootstra G, Daemen JH, Oomen AP: **Categories of non-heart-beating donors**. *Transplant Proc* 1995, **27**(5):2893-2894.
122. El-Hennawy H, Stratta RJ, Smith F: **Exocrine drainage in vascularized pancreas transplantation in the new millennium**. *World J Transplant* 2016, **6**(2):255-271.
123. De Roover A, Coimbra C, Detry O, Van Kemseke C, Squifflet JP, Honore P, Meurisse M: **Pancreas graft drainage in recipient duodenum: preliminary experience**. *Transplantation* 2007, **84**(6):795-797.

124. Shokouh-Amiri H, Zakhary JM, Zibari GB: **A novel technique of portal-endocrine and gastric-exocrine drainage in pancreatic transplantation**. *J Am Coll Surg* 2011, **212**(4):730-738; discussion 738-739.
125. Adler JT, Zaborek N, Redfield RR, 3rd, Kaufman DB, Odorico JS, Sollinger HW: **Enteric conversion after bladder-drained pancreas transplantation is not associated with worse allograft survival**. *Am J Transplant* 2019, **19**(9):2543-2549.
126. Corry RJ, Chakrabarti P, Shapiro R, Jordan ML, Scantlebury VP, Vivas CA: **Comparison of enteric versus bladder drainage in pancreas transplantation**. *Transplant Proc* 2001, **33**(1-2):1647-1651.
127. Lablanche S, Borot S, Wojtusciszyn A, Skaare K, Penfornis A, Malvezzi P, Badet L, Thivolet C, Morelon E, Buron F *et al*: **Ten-year outcomes of islet transplantation in patients with type 1 diabetes: Data from the Swiss-French GRAGIL network**. *Am J Transplant* 2021, **21**(11):3725-3733.
128. Bertuzzi F, Ricordi C: **Beta-cell replacement in immunosuppressed recipients: old and new clinical indications**. *Acta Diabetol* 2007, **44**(4):171-176.
129. McCoy RG, Van Houten HK, Ziegenfuss JY, Shah ND, Wermers RA, Smith SA: **Increased mortality of patients with diabetes reporting severe hypoglycemia**. *Diabetes Care* 2012, **35**(9):1897-1901.
130. Venturini M, Angeli E, Maffi P, Fiorina P, Bertuzzi F, Salvioni M, De Cobelli F, Socci C, Aldrighetti L, Losio C *et al*: **Technique, complications, and therapeutic efficacy of percutaneous transplantation of human pancreatic islet cells in type 1 diabetes: the role of US**. *Radiology* 2005, **234**(2):617-624.
131. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW: **Automated method for isolation of human pancreatic islets**. *Diabetes* 1988, **37**(4):413-420.
132. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: **Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen**. *N Engl J Med* 2000, **343**(4):230-238.
133. Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, Rabinovitch A, Elliott JF, Bigam D, Kneteman NM *et al*: **Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol**. *Diabetes* 2001, **50**(4):710-719.
134. Fishman JA: **Infection in Organ Transplantation**. *Am J Transplant* 2017, **17**(4):856-879.
135. Beilhack GF, Scheffold YC, Weissman IL, Taylor C, Jerabek L, Burge MJ, Masek MA, Shizuru JA: **Purified allogeneic hematopoietic stem cell transplantation blocks diabetes pathogenesis in NOD mice**. *Diabetes* 2003, **52**(1):59-68.
136. Nikolic B, Onoe T, Takeuchi Y, Khalpey Z, Primo V, Leykin I, Smith RN, Sykes M: **Distinct requirements for achievement of allotolerance versus reversal of autoimmunity via nonmyeloablative mixed chimerism induction in NOD mice**. *Transplantation* 2010, **89**(1):23-32.
137. Persaud SP, Ritchey JK, Kim S, Lim S, Ruminski PG, Cooper ML, Rettig MP, Choi J, DiPersio JF: **Antibody-drug conjugates plus Janus kinase inhibitors enable MHC-mismatched allogeneic hematopoietic stem cell transplantation**. *J Clin Invest* 2021, **131**(24).
138. Wang M, Racine J, Zhang M, Wu T, Deng R, Johnston H, Shen C, Siswanto K, Zeng D: **MHC-mismatched chimerism is required for induction of**

transplantation tolerance in autoimmune nonobese diabetic recipients. *J Immunol* 2014, **193**(4):2005-2015.
139. Bellin MD, Dunn TB: **Transplant strategies for type 1 diabetes: whole pancreas, islet and porcine beta cell therapies**. *Diabetologia* 2020, **63**(10):2049-2056.
140. Butler PC, Gale EA: **Reversing type 1 diabetes with stem cell-derived islets: a step closer to the dream?** *J Clin Invest* 2022, **132**(3).
141. Roelen DL, Huurman VA, Hilbrands R, Gillard P, Duinkerken G, van der Meer-Prins PW, Versteeg-van der Voort Maarschalk MF, Mathieu C, Keymeulen B, Pipeleers DG *et al*: **Relevance of cytotoxic alloreactivity under different immunosuppressive regimens in clinical islet cell transplantation**. *Clin Exp Immunol* 2009, **156**(1):141-148.
142. Garyu JW, Uduman M, Stewart A, Rui J, Deng S, Shenson J, Staron MM, Kaech SM, Kleinstein SH, Herold KC: **Characterization of Diabetogenic CD8+ T Cells: IMMUNE THERAPY WITH METABOLIC BLOCKADE**. *J Biol Chem* 2016, **291**(21):11230-11240.
143. Martins CP, New LA, O'Connor EC, Previte DM, Cargill KR, Tse IL, Sims-Lucas S, Piganelli JD: **Glycolysis Inhibition Induces Functional and Metabolic Exhaustion of CD4(+) T Cells in Type 1 Diabetes**. *Front Immunol* 2021, **12**:669456.
144. Sun Y, Tian T, Gao J, Liu X, Hou H, Cao R, Li B, Quan M, Guo L: **Metformin ameliorates the development of experimental autoimmune encephalomyelitis by regulating T helper 17 and regulatory T cells in mice**. *J Neuroimmunol* 2016, **292**:58-67.
145. Di Dedda C, Vignali D, Piemonti L, Monti P: **Pharmacological Targeting of GLUT1 to Control Autoreactive T Cell Responses**. *Int J Mol Sci* 2019, **20**(19).
146. Macintyre AN, Gerriets VA, Nichols AG, Michalek RD, Rudolph MC, Deoliveira D, Anderson SM, Abel ED, Chen BJ, Hale LP *et al*: **The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function**. *Cell Metab* 2014, **20**(1):61-72.
147. Maher F, Davies-Hill TM, Simpson IA: **Substrate specificity and kinetic parameters of GLUT3 in rat cerebellar granule neurons**. *Biochem J* 1996, **315 (Pt 3)**(Pt 3):827-831.
148. Barger SW: **Glucose transport in the regulation of T-cell activation: the journey may be as important as the destination**. *Immunometabolism (Cobham)* 2022, **4**(3):e00003.
149. Hochrein SM, Wu H, Eckstein M, Arrigoni L, Herman JS, Schumacher F, Gerecke C, Rosenfeldt M, Grün D, Kleuser B *et al*: **The glucose transporter GLUT3 controls T helper 17 cell responses through glycolytic-epigenetic reprogramming**. *Cell Metabolism* 2022, **34**(4):516-532.e511.
150. Fu H, Vuononvirta J, Fanti S, Bonacina F, D'Amati A, Wang G, Poobalasingam T, Fankhaenel M, Lucchesi D, Coleby R *et al*: **The glucose transporter 2 regulates CD8(+) T cell function via environment sensing**. *Nat Metab* 2023, **5**(11):1969-1985.
151. Jacobs SR, Herman CE, Maciver NJ, Wofford JA, Wieman HL, Hammen JJ, Rathmell JC: **Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways**. *J Immunol* 2008, **180**(7):4476-4486.

152. Wofford JA, Wieman HL, Jacobs SR, Zhao Y, Rathmell JC: **IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T-cell survival**. *Blood* 2008, **111**(4):2101-2111.
153. Palmer MJ, Mahajan VS, Trajman LC, Irvine DJ, Lauffenburger DA, Chen J: **Interleukin-7 receptor signaling network: an integrated systems perspective**. *Cell Mol Immunol* 2008, **5**(2):79-89.
154. Wang J, Ye C, Chen C, Xiong H, Xie B, Zhou J, Chen Y, Zheng S, Wang L: **Glucose transporter GLUT1 expression and clinical outcome in solid tumors: a systematic review and meta-analysis**. *Oncotarget* 2017, **8**(10):16875-16886.
155. Chan DA, Sutphin PD, Nguyen P, Turcotte S, Lai EW, Banh A, Reynolds GE, Chi JT, Wu J, Solow-Cordero DE *et al*: **Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality**. *Sci Transl Med* 2011, **3**(94):94ra70.
156. Kraus D, Reckenbeil J, Veit N, Kuerpig S, Meisenheimer M, Beier I, Stark H, Winter J, Probstmeier R: **Targeting glucose transport and the NAD pathway in tumor cells with STF-31: a re-evaluation**. *Cell Oncol (Dordr)* 2018, **41**(5):485-494.
157. Kavanagh Williamson M, Coombes N, Juszczak F, Athanasopoulos M, Khan MB, Eykyn TR, Srenathan U, Taams LS, Dias Zeidler J, Da Poian AT *et al*: **Upregulation of Glucose Uptake and Hexokinase Activity of Primary Human CD4+ T Cells in Response to Infection with HIV-1**. *Viruses* 2018, **10**(3).
158. Wang L, Pavlou S, Du X, Bhuckory M, Xu H, Chen M: **Glucose transporter 1 critically controls microglial activation through facilitating glycolysis**. *Mol Neurodegener* 2019, **14**(1):2.
159. Ojelabi OA, Lloyd KP, Simon AH, De Zutter JK, Carruthers A: **WZB117 (2-Fluoro-6-(m-hydroxybenzoyloxy) Phenyl m-Hydroxybenzoate) Inhibits GLUT1-mediated Sugar Transport by Binding Reversibly at the Exofacial Sugar Binding Site**. *J Biol Chem* 2016, **291**(52):26762-26772.
160. Liu Y, Cao Y, Zhang W, Bergmeier S, Qian Y, Akbar H, Colvin R, Ding J, Tong L, Wu S *et al*: **A small-molecule inhibitor of glucose transporter 1 downregulates glycolysis, induces cell-cycle arrest, and inhibits cancer cell growth in vitro and in vivo**. *Mol Cancer Ther* 2012, **11**(8):1672-1682.
161. Shima T, Taniguchi K, Tokumaru Y, Inomata Y, Arima J, Lee SW, Takabe K, Yoshida K, Uchiyama K: **Glucose transporter-1 inhibition overcomes imatinib resistance in gastrointestinal stromal tumor cells**. *Oncol Rep* 2022, **47**(1).
162. Li YL, Weng HC, Hsu JL, Lin SW, Guh JH, Hsu LC: **The Combination of MK-2206 and WZB117 Exerts a Synergistic Cytotoxic Effect Against Breast Cancer Cells**. *Front Pharmacol* 2019, **10**:1311.
163. Zhang RS, Li ZK, Liu J, Deng YT, Jiang Y: **WZB117 enhanced the anti-tumor effect of apatinib against melanoma via blocking STAT3/PKM2 axis**. *Front Pharmacol* 2022, **13**:976117.
164. Ma Y, Wang W, Idowu MO, Oh U, Wang XY, Temkin SM, Fang X: **Ovarian Cancer Relies on Glucose Transporter 1 to Fuel Glycolysis and Growth: Anti-Tumor Activity of BAY-876**. *Cancers (Basel)* 2018, **11**(1).
165. Siebeneicher H, Cleve A, Rehwinkel H, Neuhaus R, Heisler I, Muller T, Bauser M, Buchmann B: **Identification and Optimization of the First Highly Selective GLUT1 Inhibitor BAY-876**. *ChemMedChem* 2016, **11**(20):2261-2271.

166. Chen Z, Vaeth M, Eckstein M, Delgobo M, Ramos G, Frantz S, Hofmann U, Gladow N: **Characterization of the effect of the GLUT-1 inhibitor BAY-876 on T cells and macrophages**. *Eur J Pharmacol* 2023, **945**:175552.
167. Yang H, Zhang MZ, Sun HW, Chai YT, Li X, Jiang Q, Hou J: **A Novel Microcrystalline BAY-876 Formulation Achieves Long-Acting Antitumor Activity Against Aerobic Glycolysis and Proliferation of Hepatocellular Carcinoma**. *Front Oncol* 2021, **11**:783194.
168. Chen X, Zhao Y, He C, Gao G, Li J, Qiu L, Wang X, Gao Y, Qi Y, Sun K *et al*: **Identification of a novel GLUT1 inhibitor with in vitro and in vivo anti-tumor activity**. *Int J Biol Macromol* 2022, **216**:768-778.
169. Van Gassen S, Callebaut B, Van Helden MJ, Lambrecht BN, Demeester P, Dhaene T, Saeys Y: **FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data**. *Cytometry Part A* 2015, **87**(7):636-645.
170. Chakrabarti R, Jung CY, Lee TP, Liu H, Mookerjee BK: **Changes in glucose transport and transporter isoforms during the activation of human peripheral blood lymphocytes by phytohemagglutinin**. *J Immunol* 1994, **152**(6):2660-2668.
171. Coppieters KT, Wiberg A, Amirian N, Kay TW, von Herrath MG: **Persistent glucose transporter expression on pancreatic beta cells from longstanding type 1 diabetic individuals**. *Diabetes Metab Res Rev* 2011, **27**(8):746-754.
172. Pingitore A, Ruz-Maldonado I, Liu B, Huang GC, Choudhary P, Persaud SJ: **Dynamic Profiling of Insulin Secretion and ATP Generation in Isolated Human and Mouse Islets Reveals Differential Glucose Sensitivity**. *Cell Physiol Biochem* 2017, **44**(4):1352-1359.
173. Ooi AT, Gomperts BN: **Molecular Pathways: Targeting Cellular Energy Metabolism in Cancer via Inhibition of SLC2A1 and LDHA**. *Clin Cancer Res* 2015, **21**(11):2440-2444.
174. Peng Y, Xing SN, Tang HY, Wang CD, Yi FP, Liu GL, Wu XM: **Influence of glucose transporter 1 activity inhibition on neuroblastoma in vitro**. *Gene* 2019, **689**:11-17.
175. Shibuya K, Okada M, Suzuki S, Seino M, Seino S, Takeda H, Kitanaka C: **Targeting the facilitative glucose transporter GLUT1 inhibits the self-renewal and tumor-initiating capacity of cancer stem cells**. *Oncotarget* 2015, **6**(2):651-661.
176. Vendrame F, Hopfner YY, Diamantopoulos S, Virdi SK, Allende G, Snowhite IV, Reijonen HK, Chen L, Ruiz P, Ciancio G *et al*: **Risk Factors for Type 1 Diabetes Recurrence in Immunosuppressed Recipients of Simultaneous Pancreas–Kidney Transplants**. *American Journal of Transplantation* 2016, **16**(1):235-245.
177. Reijonen W, Falk **Association of autoreactive memory CD4+ T cells expressing the chemokine receptor CXCR3 in the peripheral blood of pancreas-kidney transplant recipients with type 1 diabetes recurrence**. In. Presented at the immunology of diabetes society meeting, san Francisco, January 2017; 2017.
178. Batard P, Peterson DA, Devevre E, Guillaume P, Cerottini JC, Rimoldi D, Speiser DE, Winther L, Romero P: **Dextramers: new generation of fluorescent MHC class I/peptide multimers for visualization of antigen-specific CD8+ T cells**. *J Immunol Methods* 2006, **310**(1-2):136-148.

179. Monti P, Brigatti C, Heninger AK, Scirpoli M, Bonifacio E: **Disengaging the IL-2 receptor with daclizumab enhances IL-7-mediated proliferation of CD4(+) and CD8(+) T cells**. *Am J Transplant* 2009, **9**(12):2727-2735.
180. Sherry N, Hagopian W, Ludvigsson J, Jain SM, Wahlen J, Ferry RJ, Jr., Bode B, Aronoff S, Holland C, Carlin D *et al*: **Teplizumab for treatment of type 1 diabetes (Protege study): 1-year results from a randomised, placebo-controlled trial**. *Lancet* 2011, **378**(9790):487-497.
181. Kouidhi S, Noman MZ, Kieda C, Elgaaied AB, Chouaib S: **Intrinsic and Tumor Microenvironment-Induced Metabolism Adaptations of T Cells and Impact on Their Differentiation and Function**. *Front Immunol* 2016, **7**:114.
182. Maciver NJ, Jacobs SR, Wieman HL, Wofford JA, Coloff JL, Rathmell JC: **Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival**. *J Leukoc Biol* 2008, **84**(4):949-957.
183. Rangel Rivera GO, Knochelmann HM, Dwyer CJ, Smith AS, Wyatt MM, Rivera-Reyes AM, Thaxton JE, Paulos CM: **Fundamentals of T Cell Metabolism and Strategies to Enhance Cancer Immunotherapy**. *Front Immunol* 2021, **12**:645242.
184. Nile DL, Rae C, Walker DJ, Waddington JC, Vincent I, Burgess K, Gaze MN, Mairs RJ, Chalmers AJ: **Inhibition of glycolysis and mitochondrial respiration promotes radiosensitisation of neuroblastoma and glioma cells**. *Cancer Metab* 2021, **9**(1):24.
185. Raulien N, Friedrich K, Strobel S, Rubner S, Baumann S, von Bergen M, Korner A, Krueger M, Rossol M, Wagner U: **Fatty Acid Oxidation Compensates for Lipopolysaccharide-Induced Warburg Effect in Glucose-Deprived Monocytes**. *Front Immunol* 2017, **8**:609.
186. Martell E, Kuzmychova H, Senthil H, Kaul E, Chokshi CR, Venugopal C, Anderson CM, Singh SK, Sharif T: **Compensatory cross-talk between autophagy and glycolysis regulates senescence and stemness in heterogeneous glioblastoma tumor subpopulations**. *Acta Neuropathol Commun* 2023, **11**(1):110.
187. Freemerman AJ, Zhao L, Pingili AK, Teng B, Cozzo AJ, Fuller AM, Johnson AR, Milner JJ, Lim MF, Galanko JA *et al*: **Myeloid Slc2a1-Deficient Murine Model Revealed Macrophage Activation and Metabolic Phenotype Are Fueled by GLUT1**. *J Immunol* 2019, **202**(4):1265-1286.
188. Zheng J: **Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review)**. *Oncol Lett* 2012, **4**(6):1151-1157.
189. Balamurugan AN, Bottino R, Giannoukakis N, Smetanka C: **Prospective and challenges of islet transplantation for the therapy of autoimmune diabetes**. *Pancreas* 2006, **32**(3):231-243.
190. Gamble A, Pepper AR, Bruni A, Shapiro AMJ: **The journey of islet cell transplantation and future development**. *Islets* 2018, **10**(2):80-94.
191. Assalino M, Genevay M, Morel P, Demuylder-Mischler S, Toso C, Berney T: **Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation in the absence of GAD and IA-2 autoantibodies**. *Am J Transplant* 2012, **12**(2):492-495.

192. Tang R, Zhong T, Fan L, Xie Y, Li J, Li X: **Enhanced T Cell Glucose Uptake Is Associated With Progression of Beta-Cell Function in Type 1 Diabetes**. *Front Immunol* 2022, **13**:897047.
193. Kong BS, Min SH, Lee C, Cho YM: **Mitochondrial-encoded MOTS-c prevents pancreatic islet destruction in autoimmune diabetes**. *Cell Rep* 2021, **36**(4):109447.
194. Chang Y-L, Chang S-D, Chao A-S, Sieber M, Tsai C-L, Cheng P-J: **Effect of Hypoxia on Glucose Transporter 1 and 3 Gene Expression in Placental Mesenchymal Stem Cells Derived from Growth-Restricted Fetuses**. *Genes* 2022, **13**(5):752.
195. Hochrein SM, Wu H, Eckstein M, Arrigoni L, Herman JS, Schumacher F, Gerecke C, Rosenfeldt M, Grun D, Kleuser B *et al*: **The glucose transporter GLUT3 controls T helper 17 cell responses through glycolytic-epigenetic reprogramming**. *Cell Metab* 2022, **34**(4):516-532 e511.
196. Li L, Liu X, Sanders KL, Edwards JL, Ye J, Si F, Gao A, Huang L, Hsueh EC, Ford DA *et al*: **TLR8-Mediated Metabolic Control of Human Treg Function: A Mechanistic Target for Cancer Immunotherapy**. *Cell Metab* 2019, **29**(1):103-123 e105.
197. Bae JS, Kim TH, Kim MY, Park JM, Ahn YH: **Transcriptional regulation of glucose sensors in pancreatic beta-cells and liver: an update**. *Sensors (Basel)* 2010, **10**(5):5031-5053.
198. Ghezzi C, Loo DDF, Wright EM: **Physiology of renal glucose handling via SGLT1, SGLT2 and GLUT2**. *Diabetologia* 2018, **61**(10):2087-2097.
199. Jafri SSQ, Ali Shah SI, Jaffari SHA: **Molecular Structure, Biochemical Functions, Genetics, and Emerging Clinical Relevance of Glucose Transporters**. *Global Journal of Medical, Pharmaceutical, and Biomedical Update*, **18**:23.
200. Berger C, Zdzieblo D: **Glucose transporters in pancreatic islets**. *Pflugers Arch* 2020, **472**(9):1249-1272.
201. Montel-Hagen A, Blanc L, Boyer-Clavel M, Jacquet C, Vidal M, Sitbon M, Taylor N: **The Glut1 and Glut4 glucose transporters are differentially expressed during perinatal and postnatal erythropoiesis**. *Blood* 2008, **112**(12):4729-4738.
202. Freire C, King N, Dzieciatkowska M, Stephenson D, Moura P, Dobbe JGG, Streekstra G, D'Alessandro A, Toye A, Satchwell T: **Complete absence of GLUT1 does not impair human terminal erythroid differentiation**. *bioRxiv* 2024:2024.2001.2010.574621.
203. Winkler EA, Nishida Y, Sagare AP, Rege SV, Bell RD, Perlmutter D, Sengillo JD, Hillman S, Kong P, Nelson AR *et al*: **GLUT1 reductions exacerbate Alzheimer's disease vasculo-neuronal dysfunction and degeneration**. *Nature Neuroscience* 2015, **18**(4):521-530.
204. Salazar K, Espinoza F, Cerda-Gallardo G, Ferrada L, Magdalena R, Ramírez E, Ulloa V, Saldivia N, Troncoso N, Oviedo MJ *et al*: **SVCT2 Overexpression and Ascorbic Acid Uptake Increase Cortical Neuron Differentiation, Which Is Dependent on Vitamin C Recycling between Neurons and Astrocytes**. *Antioxidants* 2021, **10**(9):1413.

205. Wang D, Pascual JM, Yang H, Engelstad K, Mao X, Cheng J, Yoo J, Noebels JL, De Vivo DC: **A mouse model for Glut-1 haploinsufficiency**. *Hum Mol Genet* 2006, **15**(7):1169-1179.
206. Suzuki S, Okada M, Takeda H, Kuramoto K, Sanomachi T, Togashi K, Seino S, Yamamoto M, Yoshioka T, Kitanaka C: **Involvement of GLUT1-mediated glucose transport and metabolism in gefitinib resistance of non-small-cell lung cancer cells**. *Oncotarget* 2018, **9**(66):32667-32679.
207. Wang Y, Li Q, Liu F, Jin S, Zhang Y, Zhang T, Zhu Y, Zhou Y: **Transcriptional activation of glucose transporter 1 in orthodontic tooth movement-associated mechanical response**. *Int J Oral Sci* 2018, **10**(3):27.
208. Wang D, Pascual JM, De Vivo D: **Glucose Transporter Type 1 Deficiency Syndrome**. In: *GeneReviews((R)).* Edited by Adam MP, Mirzaa GM, Pagon RA, Wallace SE, Bean LJH, Gripp KW, Amemiya A. Seattle (WA); 1993.
209. Olivotto S, Duse A, Bova SM, Leonardi V, Biganzoli E, Milanese A, Cereda C, Bertoli S, Previtali R, Veggiotti P: **Glut1 deficiency syndrome throughout life: clinical phenotypes, intelligence, life achievements and quality of life in familial cases**. *Orphanet J Rare Dis* 2022, **17**(1):365.
210. Varesio C, Pasca L, Parravicini S, Zanaboni MP, Ballante E, Masnada S, Ferraris C, Bertoli S, Tagliabue A, Veggiotti P *et al*: **Quality of Life in Chronic Ketogenic Diet Treatment: The GLUT1DS Population Perspective**. *Nutrients* 2019, **11**(7).
211. Raez LE, Papadopoulos K, Ricart AD, Chiorean EG, Dipaola RS, Stein MN, Rocha Lima CM, Schlesselman JJ, Tolba K, Langmuir VK *et al*: **A phase I dose-escalation trial of 2-deoxy-D-glucose alone or combined with docetaxel in patients with advanced solid tumors**. *Cancer Chemother Pharmacol* 2013, **71**(2):523-530.
212. Bolla AM, Caretto A, Laurenzi A, Scavini M, Piemonti L: **Low-Carb and Ketogenic Diets in Type 1 and Type 2 Diabetes**. *Nutrients* 2019, **11**(5).
213. Szabat M, Page MM, Panzhinskiy E, Skovso S, Mojibian M, Fernandez-Tajes J, Bruin JE, Bround MJ, Lee JT, Xu EE *et al*: **Reduced Insulin Production Relieves Endoplasmic Reticulum Stress and Induces beta Cell Proliferation**. *Cell Metab* 2016, **23**(1):179-193.
214. Eriksson O, Eich T, Sundin A, Tibell A, Tufveson G, Andersson H, Felldin M, Foss A, Kyllonen L, Langstrom B *et al*: **Positron emission tomography in clinical islet transplantation**. *Am J Transplant* 2009, **9**(12):2816-2824.
215. Hale G, Rebello P, Al Bakir I, Bolam E, Wiczling P, Jusko WJ, Vandemeulebroucke E, Keymeulen B, Mathieu C, Ziegler AG *et al*: **Pharmacokinetics and antibody responses to the CD3 antibody otelixizumab used in the treatment of type 1 diabetes**. *J Clin Pharmacol* 2010, **50**(11):1238-1248.
216. Herold KC, Hagopian W, Auger JA, Poumian-Ruiz E, Taylor L, Donaldson D, Gitelman SE, Harlan DM, Xu D, Zivin RA *et al*: **Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus**. *N Engl J Med* 2002, **346**(22):1692-1698.
217. Haller MJ, Gitelman SE, Gottlieb PA, Michels AW, Rosenthal SM, Shuster JJ, Zou B, Brusko TM, Hulme MA, Wasserfall CH *et al*: **Anti-thymocyte globulin/G-CSF treatment preserves beta cell function in patients with established type 1 diabetes**. *J Clin Invest* 2015, **125**(1):448-455.

218. Rigby MR, DiMeglio LA, Rendell MS, Felner EI, Dostou JM, Gitelman SE, Patel CM, Griffin KJ, Tsalikian E, Gottlieb PA *et al*: **Targeting of memory T cells with alefacept in new-onset type 1 diabetes (T1DAL study): 12 month results of a randomised, double-blind, placebo-controlled phase 2 trial**. *Lancet Diabetes Endocrinol* 2013, **1**(4):284-294.
219. Pescovitz MD, Greenbaum CJ, Bundy B, Becker DJ, Gitelman SE, Goland R, Gottlieb PA, Marks JB, Moran A, Raskin P *et al*: **B-lymphocyte depletion with rituximab and beta-cell function: two-year results**. *Diabetes Care* 2014, **37**(2):453-459.
220. Orban T, Bundy B, Becker DJ, Dimeglio LA, Gitelman SE, Goland R, Gottlieb PA, Greenbaum CJ, Marks JB, Monzavi R *et al*: **Costimulation modulation with abatacept in patients with recent-onset type 1 diabetes: follow-up 1 year after cessation of treatment**. *Diabetes Care* 2014, **37**(4):1069-1075.
221. Cabello-Kindelan C, Mackey S, Sands A, Rodriguez J, Vazquez C, Pugliese A, Bayer AL: **Immunomodulation Followed by Antigen-Specific T(reg) Infusion Controls Islet Autoimmunity**. *Diabetes* 2020, **69**(2):215-227.
222. Vendrame F BA: **Use of Dimethyl Fumarate in Type 1 Diabetes**. In.: Diabetes Research Institute (Miami); 2021.
223. Tsonkova VG, Sand FW, Wolf XA, Grunnet LG, Kirstine Ringgaard A, Ingvorsen C, Winkel L, Kalisz M, Dalgaard K, Bruun C *et al*: **The EndoC-betaH1 cell line is a valid model of human beta cells and applicable for screenings to identify novel drug target candidates**. *Mol Metab* 2018, **8**:144-157.
224. Valk E, Rudd CE, Schneider H: **CTLA-4 trafficking and surface expression**. *Trends Immunol* 2008, **29**(6):272-279.
225. Jago CB, Yates J, Camara NO, Lechler RI, Lombardi G: **Differential expression of CTLA-4 among T cell subsets**. *Clin Exp Immunol* 2004, **136**(3):463-471.
226. Dolton G, Lissina A, Skowera A, Ladell K, Tungatt K, Jones E, Kronenberg-Versteeg D, Akpovwa H, Pentier JM, Holland CJ *et al*: **Comparison of peptide-major histocompatibility complex tetramers and dextramers for the identification of antigen-specific T cells**. *Clin Exp Immunol* 2014, **177**(1):47-63.
227. Maguire O, Chen GL, Hahn TE, Brix L, McCarthy PL, Wallace PK, Minderman H: **Quantifying MHC dextramer-induced NFAT activation in antigen-specific T cells as a functional response parameter**. *Methods* 2017, **112**:75-83.