PUBLICATIONS OF
THE UNIVERSITY OF EASTERN FINLAND

Dissertations in Health Sciences

ANNA-MARI SCHRODERUS

T-CELL SIGNATURES DURING THE PROGRESSION OF TYPE 1 DIABETES

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To be presented by permission of the Faculty of Health Sciences, University of Eastern Finland for public examination in CA102 Auditorium, Kuopio on December 13th, 2024, at 12 o'clock noon

Publications of the University of Eastern Finland
Dissertations in Health Sciences
No 870

Institute of Clinical Medicine / School of Medicine
University of Eastern Finland, Kuopio
2024

Printing office
PunaMusta Oy, 2024

Distributor: University of Eastern Finland
Kuopio Campus Library

ISBN: 978-952-61-5422-0 (print/nid.)
ISBN: 978-952-61-5423-7 (PDF)
ISSNL: 1798-5706
ISSN: 1798-5706
ISSN: 1798-5714 (PDF)

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FINLAND

Schroderus, Anna-Mari
T-cell signatures during the progression of type 1 diabetes.
Kuopio: University of Eastern Finland
Publications of the University of Eastern Finland
Dissertations in Health Sciences 870. 2024, 188 p.
ISBN: 978-952-61-5422-0 (print)
ISSNL: 1798-5706
ISSN: 1798-5706
ISBN: 978-952-61-5423-7 (PDF)
ISSN: 1798-5714 (PDF)

ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease where T cells mediate the progressive destruction of insulin-producing β cells in the pancreas. $CD8^+$ T cells and $CD4^+$ B-cell helper T cells, such as follicular (Tfh) and peripheral helper (Tph) T cells, are implicated as major players in T1D disease process but their phenotypes during the disease progression remain elusive. In this thesis, a comprehensive characterization of $CD8^+$ T cells and $CD4^+$ B-cell helper T cells at different stages of T1D development, and also an evaluation of potential T-cell related predictive biomarkers for T1D disease progression were carried out. The single-cell methods, such as multicolor flow cytometry and CITE-seq (cellular indexing of transcriptomes and epitopes) single-cell multiomics approaches were utilized to characterize the T cells. In addition, ultrasensitive plasma cytokine measurements were performed.

$CD8^+$ T-cell and $CD4^+$ B-cell helper T-cell frequencies, functionality and phenotypes, and plasma IL-21 cytokine levels were examined in autoantibody-positive (AAb^+) children at-risk for T1D, children with newly diagnosed T1D, adults with established T1D and age-matched healthy controls. In addition, samples from children before and after seroconversion for islet autoantibodies were analysed to examine whether alterations in $CD8^+$ T cells and $CD4^+$ B-cell helper T-cell frequencies could be detected at the time point reflecting the initiation of the autoimmunity.

Distinct $CD8^+$ T-cell signatures, and alterations in the frequencies of $CD4^+$ B-cell helper T cells were detected in AAb^+ children and children with newly diagnosed T1D. The alterations were confined to these stages of active autoimmunity. In contrast, these alterations were not associated with early autoimmunity i.e. with islet autoantibody seroconversion in the cohort utilized in the study. Adults with established T1D displayed higher plasma levels of interleukin-21 (IL-21), a cytokine poruced by $CD4^+$ B-cell helper T cells, compared to healthy controls. Conversely, this alteration was not detected in children with newly diagnosed T1D or in autoantibody-positive at-risk children.

In conclusion, this thesis presents a comprehensive characterization of $CD8^+$ T-cell and $CD4^+$ B-cell helper T-cell signatures at different stages of T1D development. These findings have a potential to aid in the discovery of additional biomarkers for T1D prediction and could provide new insights for targets or the development of immunotherapy for T1D.

Keywords: Autoimmune Diseases, T-lymphocytes, Diabetes Mellitus, Type 1, Flow Cytometry, Multiomics

Schroderus, Anna-Mari
T-solutyypit tyypin 1 diabeteksen kehittymisen eri vaiheissa
Kuopio: Itä-Suomen yliopisto
Publications of the University of Eastern Finland
Dissertations in Health Sciences 870. 2024, 188 p.
ISBN: 978-952-61-5422-0 (print)
ISSNL: 1798-5706
ISSN: 1798-5706
ISBN: 978-952-61-5423-7 (PDF)
ISSN: 1798-5714 (PDF)

TIIVISTELMÄ

Tyypin 1 diabetes (T1D) on autoimmuunisairaus, jossa haiman insuliinia tuottavat saarekesolut tuhoutuvat T-solujen välittämänä. Tappaja-T-solut (CD8+ T) ja B-soluja auttavat $CD4^+$ auttaja-T-solut, kuten follikulaariset (Tfh) ja perifeeriset (Tph) auttaja-T-solut, ovat keskeisiä T-solutyyppejä, joiden on osoitettu osallistuvan T1D:n syntyprosessiin. Näiden solujen tarkempi fenotyypi kaipaa kuitenkin vielä selvitystä. Tässä väitöskirjassa kuvattiin näiden T-solualatyyppien toiminnallisuutta, fenotyyppiä sekä arvioitiin mahdollisia ennustavia biomarkkereita T1D:n eri vaiheissa hyödyntäen viimeisintä tekniikkaa edustavia yksisolumenetelmiä, kuten monivärivirtaussytometriaa ja yksisolu-multiomiikkaa. Lisäksi väitöskirjassa hyödynnettiin ultrasensitiivista plasman sytokiinimittausmenetelmää.

$CD8^+$ T-solujen ja B-soluja auttavien $CD4^+$ T-solujen määrää, toiminnallisuutta ja fenotyyppiä sekä plasman interleukiini-21 (IL-21) tasoja tutkittiin näytteistä, jotka oli kerätty autovasta-ainepositiivisista sairastumisriskissä olevista lapsista (AAb^+), lapsista, joilla oli juuri diagnosoitu T1D, aikuis-T1D-potilaista sekä ikävakioiduista terveissä kontrolleista. Lisäksi T-soluja tutkittiin myös lapsilta ennen ja jälkeen autovasta-ainepositiivisuuden kerätyistä näyteistä, toisin sanoen autoimmuniteetin käynnistymisajankohdan molemmin puolin.

Tutkimuksissa havaittiin, että $CD8^+$ T-solujen fenotyyppi erosi AAb^+ lapsilla ja juuri T1D:hen sairastuneilla lapsilla terveisiin kontrolleihin verrattuna. Lisäksi B-soluja auttavien $CD4^+$ T-solujen määrä oli kasvanut AAb^+ lapsilla ja T1D:hen sairastuneilla lapsilla verrattuna terveisiin kontrolleihin. Samanlaisia muutoksia ei havaittu aikuis-T1D-potilailla tai lapsilla pitkittäisissä näytteissä, jotka kerättiin ennen ja juuri autovasta-aineiden kehittymisen jälkeen. Sen sijaan aikuis-T1D-potilailla havaittiin suurempi pitoisuus muun muassa B-soluja auttavien $CD4^+$ T-solujen tuottamaa IL-21 sytokiinia plasmanäytteissä terveisiin kontrolleihin verrattuna. Vastaavaa ei kuitenkaan havaittu AAb^+ lasten tai T1D-lapsipotilaiden näytteissä.

Yhteenvetona, tässä väitöskirjaprojektissa osoitettiin muutoksia eri T-solupopulaatioissa T1D:n tautiprosessin eri vaiheissa. Nämä löydökset voivat auttaa uusien T1D:n riskiä ennustavien biomarkkereiden kehityksessä ja luoda uutta tietoa T1D:n immunoterapioiden kehittämistä varten.

Avainsanat: autoimmuunisairaudet, T-imusolut, nuoruustyypin diabetes

ACKNOWLEDGEMENTS

Looking way, way back, I think it is now very clear that at this moment I'm in the path where I'm supposed to be. "I can feed the caterpillar and I can whisper through the chrysalis, but what hatches, follows its own nature and is beyond me."[1]

I would like to express my gratitude to my supervisors Professor Tuure Kinnunen and Professor Emeritus Jorma Ilonen for making my PhD journey possible. Tuure, I think I cannot thank you enough for the past years. Everything I try to write in words feels too little, but here we go. You have taught me the best of arts, T-cell immunology. Thank you for the mentoring, believing in me and pushing me to always go a bit further. It has been much appreciated. Thank you for all the conversations over the years, about work and life. And thank you for all the opportunities: with the flow cytometers & single-cell technologies, teaching and traveling, to mention a few. It is safe to say that I won in the supervisor lottery. I hope you know that I'm in your debt forever.

I would like to thank my previous colleague & dear friend Emmi for the friendship over the years. You were my lab work supervisor during my master's thesis, and you naturally had a big impact on the fact that I wanted to continue to do the PhD in the lab. You were kind and understanding. I hope I have been able to provide such kindness and understanding also to my students and fellow co-workers. I would like to show gratitude also my former colleague & friend, Céline. You taught me one of the most valuable lessons during my PhD studies: you inspired me to do better. I was inspired by your courage and expertise, and still am.

I would like to express my gratitude to whole personnel of the Department of Clinical Microbiology. My deepest thanks to the colleagues who have helped me over the years and/or participated in my PhD projects: Professor Emeritus Jukka Pelkonen, Ilse, Marja, Laurène, Bella, Lucas, Mirja and Hanna. Viola and Heikki, I want to thank you both for the support especially during my late PhD journey, you both combine expertise and kindness in an inspirational way. Andrea Hanel, a collaborator and a

1 NBC's *Hannibal*

co-author in one of my projects: I would like to specifically thank you for showing me and introducing me to the “world of code”. My journey in this path is in the beginning but I will always remember your kind help. I would like to extend my thank you’s for all the other collaborators and co-authors as well: Merja Heinäniemi, Daniella Stevens, Kirsi Näntö-Salonen, Reeta Rintamäki, Jussi Pihlajamäki, Josh Poorbaugh, Samatha McElyea, Stephanie Beasley, Ling Zhang and Robert J. Benschop. In addition, I would like to express my deepest gratitude to the DIPP study, where we’ve received the precious samples that I’ve worked with throughout the PhD journey: thank you Johanna Lempainen, Jorma Toppari, Riitta Veijola, Mikael Knip. I would like to thank also Anne Suominen (University of Turku/DIPP study) and Matti Laitinen (UEF) for the technical assistance.

Docent Pia Rantakari and Docent Arno Hänninen are thanked for their valuable work as pre-examiners. I also like to express my gratitude to Docent Eliisa Kekälainen for accepting the role of an opponent in my PhD defence. I’m looking forward to our discussion, let’s make it memorable!

Toni, thank you for the love & partnership in life, and supporting me over this process, you are my ultimate partner in crime. Elisa, we’ve known each other “forever”, thank you for your support and your insight on what I would call a human condition. I hope we can continue relish in our “otherness”. Sohvi and Janne, we’ve had so much fun over the years, our little Kuhmo mafia. All of us have been married with the PhD journey and it has been a delight to see you both shine in your chosen fields. Anna, thank you for the friendship and support, I’m so happy that I found you from that one microscopy room years ago. Äiti and Hanna, I’m so grateful for your love and support. You are my home and I constantly remember where I’ve come from.

This research was conducted at the Institute of Clinical Medicine, Faculty of Health Sciences, University of Eastern Finland in Tuure Kinnunen’s research group. The financial support from the Academy of Finland, the Sigrid Jusélius Foundation and the Finnish Diabetes Research Foundation for conducting this research are thanked.

This journey would not have been possible to conquer alone. I feel privileged and grateful.

Kuopio, November 2024

Anna-Mari Schroderus

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I **Schroderus** AM, Pitkänen V, Ekman I, Stevens D, Rytkönen-Nissinen M, Rintamäki R, Pihlajamäki J, Knip M, Veijola R, Toppari J, Ilonen J, Lempainen J, Kinnunen T. Temporal Alterations in CD8+ T Cells During the Progression from Stage 1 to Stage 3 Type 1 Diabetes. Diabetes 2024; db240159.

II **Schroderus** AM, Hanel A, Vandamme C, Pitkänen V, Rytkönen-Nissinen M, Heinäniemi M, Knip M, Veijola R, Toppari J, Ilonen J, Lempainen J, Kinnunen T. The frequency but not the phenotype of circulating peripheral T helper cells is increased at later stages of progression to type 1 diabetes. *Submitted*.

III **Schroderus** AM, Poorbaugh J, McElyea S, Beasley S, Zhang L, Näntö-Salonen K, Rintamäki R, Pihlajamäki J, Knip M, Veijola R, Toppari J, Ilonen J, Benschop RJ and Kinnunen T. Evaluation of plasma IL-21 as a potential biomarker for type 1 diabetes. Frontiers in Immunology: 14: 1157265, 2023.

CONTENTS

ABBREVIATIONS

AAb	Autoantibody
AAb+	Autoantibody positivity
ADCC	Antibody- dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
BCL6	B-cell lymphoma 6
CCA	Canonical correlation analysis
CCR	Chemokine receptor
CD	Cluster of differentiation
CeD	Coeliac disease
TCM	Central memory
CMV	Cytomegalovirus
cTfh	circulating T follicular helper cell
cTph	circulating T peripheral helper cell
CTL	cytolytic
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL	Chemokine (C-X-C motif) ligandAbbreviation
CXCR	CXC Chemokine Receptor
CX3CR1	CX3C motif chemokine receptor 1
DC	Dendritic cell
DZ	Dark zone
EOMES	Eomesodermin
FDA	U. S. Food and Drug Administration
FOXP3	Forkhead box P3
GADA	glutamic acid decarboxylase antibodies
GC	Germinal center
GM-CSF	Granulocyte-monocyte colony stimulating factor
GZM	granzyme

HbA1c	Glycated hemoglobin
HLA	Human leukocyte antigen
IAA	Insulin autoantibody
IA-2A	islet antigen 2 antibodies
ICOS	inducible costimulator
IFG	Impaired fasting glucose
IFN	Interferon
IGT	Impaired glucose tolerance
IL	Interleukin
ILC	Innate lymphoid cell
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	Killer-cell immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte-activation gene 3
LLOQ	Lower limit of quantification
LOD	Lower limit of detection
LZ	Light zone
MAIT	Mucosal associated invariant T
MR1	Major histocompatibility complex class I-related gene protein
MS	Multiple sclerosis
NK	Natural killer
NKG2D	NKG2 family of C-type lectin-like receptor D
NOD	Non-obese diabetic mouse model
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death 1
PMA	Phorbol myristic acid

PPI	Preproinsulin
RA	Rheumatoid arthritis
RORγt	Retinoid orphan receptor gamma t
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
TBX21	T-box transcription factor 21
TCF-1	T cell factor 1
TCR	T cell receptor
Teff	T effector
TEM	T effector memory
TEMRA	Terminal effector memory re-expressing CD45RA
Tfh	T follicular helper
TGF	Tumor growth factor
Th	T helper
T1D	Type 1 diabetes
TIM3	T-cell immunoglobulin and mucin domain 3
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TLO	Tertiary lymphoid organ
TNF	Tumor necrosis factor
Tph	T peripheral helper
Treg	T regulatory
Trm	Tissue resident memory
Tscm	Stem cell -like memory T
TOX	Thymocyte Selection Associated High Mobility Group Box

1 INTRODUCTION

Type 1 diabetes (T1D) got its first disease-modifying immunotherapy treatment, teplizumab, approved in 2022 in the United States (1). This therapy was shown to delay the onset of T1D in at-risk individuals for several years (2). This is a remarkable outcome for the vast effort for which years, even decades of work was put into. Teplizumab delays the onset of T1D by specifically targeting T cells. Now more than ever, it would be of utmost importance to be able to identify the individuals who might benefit for these types of immunotherapy treatments. To achive this, a deeper understanding of the T-cell phenotypes associated with T1D progression and the development of robust T-cell related biomarkers would be helpful.

T1D is an autoimmune disease that commonly develops during the childhood, but can also manifest during the adulthood (3). Autoimmune diseases are, in general, considered quite uncommon, but increasing evidence suggests that at least some autoimmune diseases have become more prevalent (4). T1D is not an exception. The incidence rates of T1D appear to be increasing worldwide, Finland being one of the countries where highest incidence rates are observed (5). Around 60 cases per 100 000 children per year are affected in Finland (6), making T1D one of the most common chronic diseases of childhood in the country. Hence, managing T1D is a considerable societal and medical endeavour in Finland (7). As T1D onset commonly occurs during childhood, a considerable burden is also placed upon the affected families.

The symptomatic onset of T1D results from progressively declining levels of endogenous insulin production due to the autoimmune process that targets insulin-producing β cells at pancreas. β-cell destruction is largely considered to be T-cell mediated. Insulin-producing β cells are targeted by the autoreactive T cells, and directly killed as a result of breach in the immune tolerance. As endogenous insulin production is progressively decreased, the hallmark symptoms associated with glucose metabolism impairment manifest in the affected individuals and eventually

the diagnosis is set. Individuals with T1D require a lifelong insulin replacement therapy after the clinical onset of the disease. (3)

Major efforts for investigating T1D pathophysiology have been carried out over the years. Major genetic risk loci are known, and individuals at different genetic risk levels can be identified. However, not all subjects even with high genetic risk develop T1D. Therefore, it has been proposed that environmental factors very likely have an impact to the development of the disease as potential triggering events. As T cells are considered the main mediators of the disease, a lot of effort has been placed upon finding potential T-cell subsets that may have a major contribution to the disease process. (8) Alterations in T-cell subset composition between healthy and affected individuals could help identify the potential biomarkers for disease development or potential targets for immunotherapies.

Recently, several immunotherapy trials to delay the onset of T1D have been carried out. As mentioned above, one of the most recent successes is related to the trial where T cells were targeted with humanized Fc nonbinding anti-CD3 monoclonal antibody teplizumab. Teplizumab was used to treat individuals at-risk for T1D before the onset of clinical T1D and as a result, the immunotherapy delayed the onset of the T1D approximately an average 2 years. (2,9) Currently, it is not possible to prevent the onset of T1D fully or cure the disease, but also the obtained delaying the disease onset is an important progress in the field. By delaying the onset, the burden associated with insulin medication and common medical complications associated with T1D are also delayed. Promisingly, in the United States, teplizumab gained the approval from U.S. Food and Drug Administration (FDA) agency in 2022 for delaying the onset of T1D in pre-diabetic individuals (1). It is remarkable that this immunotherapy can be now offered to individuals at-risk for T1D, i.e. individuals without clinical disease. These findings underline the importance of studying immune changes associated with the development of T1D before disease onset.

This doctoral thesis is based on three subprojects aiming to characterize T-cell signatures during the development of human T1D. These projects elucidated the function and phenotype of $CD8^+$ and $CD4^+$ T

cells during the disease progression and the timing of the alterations within these T-cell subsets. The findings shed light to T-cell landscape at different stages of T1D and have potential implications for developing biomarkers to identify individuals who are most at-risk to develop the disease and who would benefit from the immunotherapeutic approaches to delay the onset of the disease.

2 REVIEW OF THE LITERATURE

2.1 DEVELOPMENT OF T-CELL RESPONSES

To understand the origin of the autoimmune diseases, the key aspects of immune cell responses in health are first described. Lineage commitment of the cells of the immune system and the cascade of the cellular responses upon antigen challenge are well-conserved developmental traits, described shortly in the following chapters 2.1.1 and 2.1.2. Development of immune traits, the composition of immune cell subsets and their functionality are, however, heavily influenced by various environmental factors. While the effect of the environmental factors to the developing immune system are not the topic of this thesis, it has to be noted that many autoimmune diseases have been associated with certain environmental exposures in early life. These aspects are shortly described in the context of autoimmune mechanisms and T1D development in chapters 2.3.1 and 2.4.3, respectively.

2.1.1 Lineage commitment of immune cell subsets

Cells of the immune system are collectively called leukocytes. These cells originate from the progenitor cell called hematopoietic stem cell. The daughter cells of hematopoietic stem cells commit either to common lymphoid or myeloid progenitors. Common myeloid precursors subsequently commit either to granulocyte-macrophage or megakaryocyte/erythroid lineages that give rise to different granulocyte, monocyte, dendritic cell (DC) or erythroid progenitors. Myeloid cells are integral part of the innate immune system and erythroid progenitors give rise to blood components; erythrocytes, and megakaryocytes that shed into platelets. (10) Development of the cells of the myeloid or erythroid origin are not discussed any further in this thesis. Instead, the focus in the following chapters, is on the lymphoid components of the immune system, and adaptive immunity, especially T and B cells. (Fig. 1)

Common lymphoid progenitor cells give rise to B, T, natural killer (NK) and innate lymphoid cell (ILC) progenitors that further commit to B, T, NK cells or ILCs respectively. These cell types are together called lymphoid cells. NKs and ILCs are considered conventionally as parts of innate immune system, whereas T and B cells are the key players of adaptive immune system that function in cellular and humoral defences, respectively. (10) As this thesis concerns mainly T cells, only the development of T-cell responses will be described in detail in the following chapters. (Fig. 1)

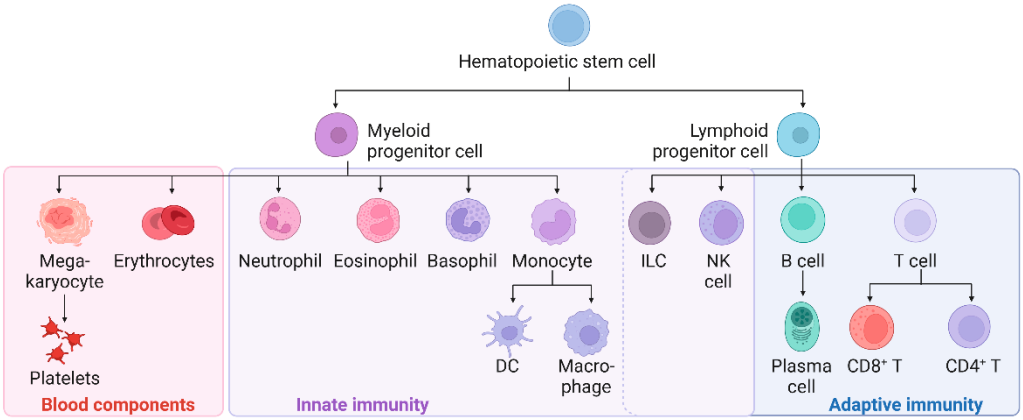


Figure 1. Immune cell landscape and blood components. Hematopoietic stem cell is the progenitor cell for all blood and immune cell components. Blood components and cells of innate and adaptive immunity originate from myeloid or lymphoid progenitors, respectively. ILC = innate lymphoid cell, NK = natural killer. Adapted from “Stem Cell Differentiation from Bone Marrow”, created by "Akiko Iwasaki " using BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates.

2.1.2 Development of conventional T-cell responses

T cells are part of adaptive immune system, and they have a capacity to recognize a diverse array of antigens. As such, they are responsible for establishment and maintenance of cellular, target-specific immune responses. (11) Two major subsets of T cells exist, $CD4^+$ T helper and cytotoxic $CD8^+$ T cells (10).

T-cell progenitors develop first in bone marrow, but soon traffic to thymus for maturation. In thymus, the progenitor cells mature into $CD4^+CD8^+$ double-positive thymocytes which further differentiate into single-positive $CD4^+$ and $CD8^+$ thymocytes that eventually give rise to $CD4^+$ T helper cells and cytotoxic $CD8^+$ T cells. (11) As thymocytes go through these maturation step in thymus, they go through two selection processes that ensure adequate T-cell responses. In positive selection, thymocytes with T cell receptor (TCR) recognizing self antigens weakly are kept, whereas TCRs that do not recognize self antigens die through apoptosis. In contrast, during the negative selection, thymocytes that recognize self antigens with high avidity TCRs are eradicated or the thymocytes differentiate into T regulatory cells (Tregs). (11) When these selection processes have ensured optimal performance of the TCRs and highly autoreactive cells are eradicated, the thymocytes exit thymus as naïve $CD4^+$ or $CD8^+$ single-positive T cells. (10)

Naïve $CD4^+$ and $CD8^+$ T cells circulate through periphery and secondary lymphoid organs (SLOs), such as lymph nodes, and use TCR-scanning to find their target, which is called an antigen (10). Lymph nodes are integral anatomical niches where naïve T cells can encounter their antigen. Within lymph nodes, naïve T cells reside in the T-cell zones. When pathogens or their antigens arrive to the lymph nodes through the lymphatics, antigen presenting cells (APCs) and naïve T cells in the lymph node relocate to the same areas to enable the antigen presenting and recognition, respectively. (10) The antigen presentation occurs mainly via DCs that present antigen-derived peptides bound by Human Leukocyte Antigen (HLA) class II or I molecules to naïve $CD4^+$ and $CD8^+$ T cells, respectively. (10)

The antigens presented to $CD4^+$ T cells via HLA class II molecules originate from extracellular proteins, such as microbial proteins (10). Conversely, antigens presented through HLA class I molecule to $CD8^+$ T cells originate from endogenous proteins (i.e. self-proteins, or viral proteins in infected cells). In addition, an important process called cross-presentation through which DCs can activate naïve $CD8^+$ T cells by presenting exogenous antigens through HLA class I molecules, exists. (12)

When the antigens are presented to naïve T cells at SLOs, antigen recognition is mediated through a TCR specific to the antigen-derived peptide. The activation of antigen-specific T cells occurs through an interaction with TCR-peptide-HLA-complex and costimulatory molecules (CD80, CD86) on APCs (and their ligands (CD28) on T cells), and through IL-2 production (10). (Fig. 2)

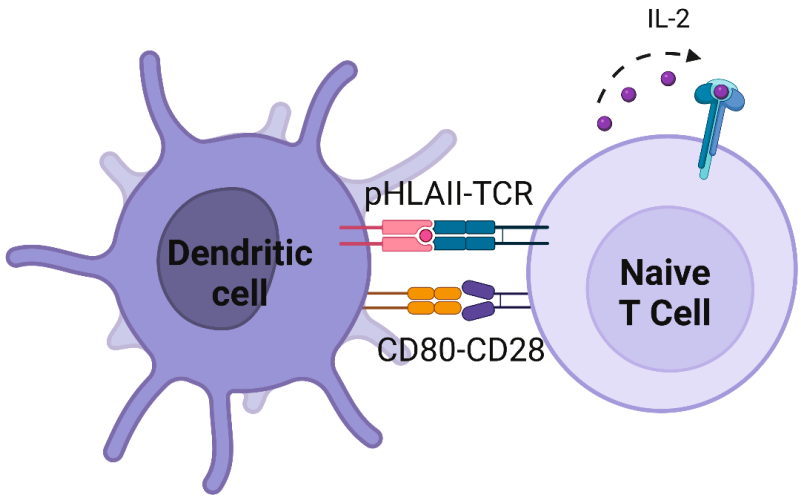


Figure 2. Antigen presentation to naïve T cells. DC-CD4^{+} T cell interaction presented as an example. DCs present antigens throught HLA class I or II molecules to naïve T cells that can recognize the HLA bound peptides with their TCR (pHLAII-TCR complex in the figure). Co-stimulatory signaling is mediated through interaction between CD80 (or CD86) on APCs and CD28 on T cells. IL-2 is secreted as a response to the recognition of the antigen, and it supports the activation and proliferation of antigen-specific T cells. Created with BioRender.com.

T-cell activation initiates a cascade, where first, a clonal expansion of T cells occurs to provide sufficient number of players (antigen-specific T cells) for the given antigen challenge. Second, naïve cells maturate into effector T cells and third, the clearance of the source of the antigen is pursued by these cells. Finally, during the contraction phase, which occurs after the antigen has been eliminated from the body, most of the effector cells go through apoptosis while a small minority of the effector cells will

differentiate into long-lasting memory T cells. Memory T cells can mediate faster response during a re-encounter with the same antigen. In general, this process applies to T-cell responses against microbial pathogens and establishment of a specific memory against given microbe. (11) A proper cocktail of context dependent cytokines and adequate balance of activating and inhibiting signals are also necessary cues for the T-cell activation and subsequent differentiation into appropriate effector cells. Keeping these paradigms of T-cell responses in health in mind, they can also be applied to the settings of autoimmunity, described in chapter 2.3.

2.2 HUMAN HELPER AND CYTOTOXIC T-CELL SUBSETS

T cells are the key players in the cellular responses of the adaptive immune system. They come in several flavors and variety of differentiation status. The following chapters focus on the most important effector T-cell subsets that are relevant in the context of this thesis.

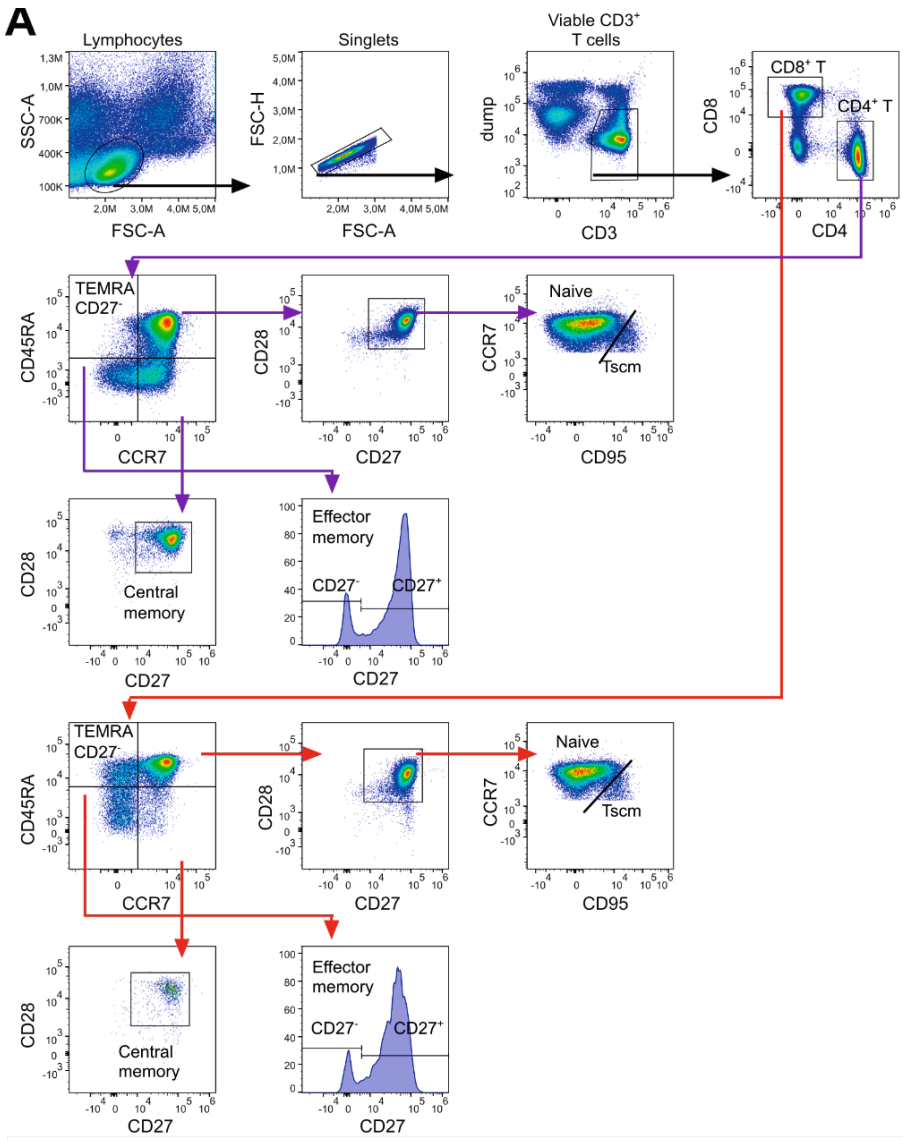
2.2.1 Delineation of T-cell subsets in the circulation

To be able to study specific T-cell responses, a delination of T cells into different subsets is required. This is conventionally performed by utilizing expression pattern of various surface receptors that are expressed at different stages of T-cell maturation (Fig. 3A). T cells express CD3, and CD4 or CD8 molecules as described before. Phenotypically naïve cells express CD45RA, CCR7 and the co-stimulatory molecules CD28 and CD27 ($CD45RA^{+}CCR7^{+}CD28^{+}CD27^{+}$) (Fig. 3B) (13,14). CCR7 is a chemokine receptor that mediates the homing of T cells to SLOs and retain cells in them (10). The co-stimulatory molecule CD28 mediates important signaling between APCs and T cells. CD27 also mediates co-stimulatory signaling, as it interacts with CD70 which, in turn, is expressed on DCs or B cells. Signaling through CD70-CD27 interaction activates T cells, induces proliferation and survival of T cells. (15)

Memory T cells can be categorized into a few subgroups. Namely, the most important subgroups are (in the order of maturation status) central memory T cells (TCM, $CD45RA^{-}CCR7^{+}CD28^{+}CD27^{+}$), effector memory T cells

(TEM, CD45RA$^-$CCR7$^-$CD27$^{+/-}$), and terminal effector memory T cells re-expressing CD45RA (TEMRA, CD45RA$^+$CCR7$^-$CD27$^-$) (Fig 3B) (13). Both blood CD4$^+$ and CD8$^+$ T cells exhibit these subsets at varying frequencies. TCM cells re-circulate between blood and SLOs and have still retained some proliferative capacity, whereas TEM and TEMRA cells have reduced proliferative capacity but increased effector functions, such as cytokine production capacity and/or cytotoxicity. They are capable of migrating into and mediating effector functions in non-lymphoid tissues upon antigen challenge. (13) Additionally, a small subset of stem cell-like memory T cells (Tscm, CD45RA$^+$CCR7$^+$CD28$^+$CD27$^+$CD95^{hi}) can be identified within the T-cell memory compartment (Fig. 3B). These cells represent long-lived memory cells with capacity for stemness. (14,16) They can potentially give rise to TCM and TEM cells (17) and participate in self-renewal of T cells (18). (Fig. 3–4)

Different memory subsets represent, in reality, a relatively heterogenous continuum of cells with varying differentiation status. Surface receptor expression of various markers can be used to distinguish less or more differentiated status of the cells. In this thesis (project I), CD27 expression is largely used to seperate less differentiated (CD27$^+$) and highly (i.e. terminally) differentiated (CD27$^-$) memory T-cell subsets. Expression of CD27 in TEM cells appears to be a feature of peripheral blood T cells that likely represent an intermediate population between TCM and TEM (and TEMRA). Indeed, TEM and TEMRA-like CD27$^+$ T cells appear to be able to proliferate and recirculate, whereas CD27 downregulation seems to indicate more terminally differentiated subset that is especially evident within the CD8$^+$ T-cell compartment (18). TEMRAs represent a minor, almost neglible, fraction in CD4$^+$ T cells in healthy individuals. In contrast, CD27$^-$ TEMRA represent a more common subset in CD8$^+$ T-cell compartment but, nevertheless, high interindividual variation is observed (14).


A
Lymphocytes
SSC-A
FSC-A
Singlets
FSC-H
FSC-A
Viable CD3+ T cells
dump
CD3
CD8+ T
CD4+ T
CD8
CD4
TEMRA CD27-
CD45RA
CCR7
CD28
CD27
Naive
Tscm
CCR7
CD95
Central memory
CD28
CD27
Effector memory
CD27-
CD27+
CD27
TEMRA CD27-
CD45RA
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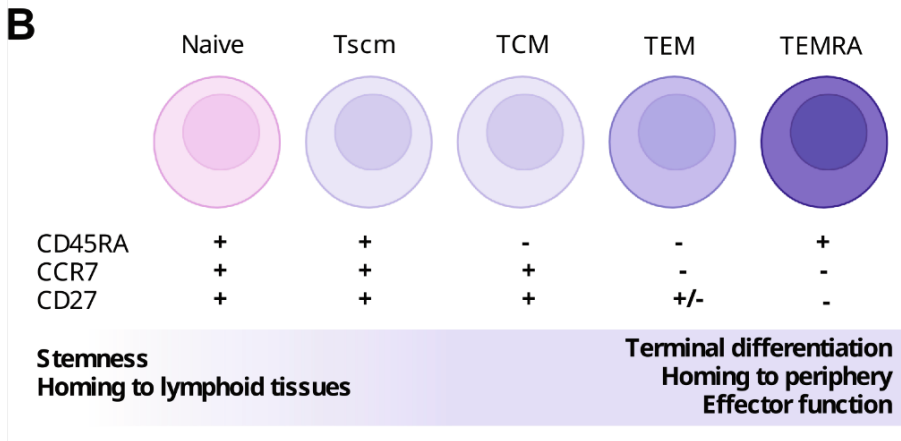
B
Naive
Tscm
TCM
TEM
TEMRA
CD45RA + + - - +
CCR7 + + + - -
CD27 + + + +/- -
Stemness
Homing to lymphoid tissues
Terminal differentiation
Homing to periphery
Effector function

Figure 3. Delination of conventional human $CD4^+$ and $CD8^+$ memory T-cell subsets in circulation. (A) An illustrative flow cytometry approach of delinating different memory T-cell subsets. (B) Naïve and memory T-cell subsets, and expression patterns of CD45RA, CCR7 and CD27. Adapted from Mahnke et al. (2013) (13). Tscm = Stem cell-like memory T, TCM = central memory, TEM = terminal effector memory, TEMRA = terminal effector memory re-expressing CD45RA. B panel is Created with Biorender.com.

Especially cytomegalovirus (CMV) seropositivity has a considerable positive correlation with the frequency of TEMRA $CD8^+$ T cells (19). The effect of CMV seropositivity to $CD8^+$ T-cell compartment is shortly discussed in chapter 2.2.4.

Of note, T cells are easily obtained through a blood sample and as such, they are often discussed in this context. It is important to realize, that T cells in circulation do not necessarily reflect T cells in tissues. In the tissues T cells are mainly tissue resident memory (Trm) T cells that mediate their actions in the tissues by participating in the tissue surveillance. During antigen encounter in peripheral tissues, Trm cells recruit and induce the activation of other immune cell types (18). They persist at specific anatomical niches. Especially the co-expression of CD69 and CD103 are often considered as hallmark indications of tissue residency. (18) The main site of action for the effector T cells (Teff) is typically in the inflamed tissues where they travel from periphery (10). In many cases, however, peripheral blood T cells are the next best thing available if sampling specific organs is difficult or clinically contraindicated. T cells from circulation may, in some cases, reflect the phenomena ongoing in the tissues. In this thesis, the circulating T-cell compartment is studied, as the sampling of pancreatic tissue and/or lymph nodes cannot be routinely performed in childen at-risk or with T1D.

2.2.2 $CD4^+$ T helper cell subsets - the Th nomenclature

Above, different naïve, effector and memory subsets of T cells in circulation were described. $CD4^+$ T-helper effector and memory T cells can be further

divided into functional subsets that orchestrate the responses of other immune cells by producing a variety of effector cytokines to promote context-dependent immune responses (20).

Several $CD4^+$ T-helper cell subsets (Table 1) exist, and they arise in context dependent manner - different pathogens require different means to fight them. To do this, $CD4^+$ T cells show a remarkable plasticity. $CD4^+$ T helper cells with varying functions can be distinguished by the cytokine production profile and/or chemokine receptor expression patterns. Most commonly, $CD4^+$ T cells are divided into T helper (Th) 1, Th2, Th17, Th1/Th17 subsets, as well as into follicular T helper (Tfh), Tregs and cytolytic (CTL) $CD4^+$ T cells. (21,22) Their hallmark surface receptors, transcription factor and cytokines are summarized in Table 1 and Fig. 4. Other subsets, such as Th9 and Th22, also exist but will not be discussed further here. (21)

Table 1. Summary of hallmark features of the $CD4^+$ T-cell subsets.

Cell subset	**Chemokine receptors/ surface receptors**	**Transcription factors**	**Cytokines**
Th1	CXCR3	TBX21 (Tbet)	IFN- γ
Th2	CCR4, CrTh2	GATA-3	IL-4, IL-13
Th17	CCR6, CD161	RORγt	IL-17, IL-21
Th17/Th1	CXCR3, CCR6	TBX21, RORγt	GM-CSF, IFN-γ
Tfh	CXCR5	BCL6	IL-21, CXCL13
Treg	CD25	FOXP3, CTLA-4	IL-10, TGF-β
CTL	CX3CR1	TBX21, EOMES	GZMB, PRF

Reviewed in (13,20,23).

Th1 cells fight against intracellular pathogens, such as viruses. IFN-γ production also activates other immune cell subsets so that they can direct their responses in an adequate manner. For example, IFN-γ promotes the cytotoxic capacity of $CD8^+$ T cells and NK cells, and phagocyte function of the innate cells. (13,21,24,25)

Th2 cells mainly produce interleukins IL-4 and IL-13 that induce the production of immunoglobulin IgE by B cells and activate innate cells, such as eosinophils, basophils, and mast cells, to fight the helminth infections. (13,21,24,25)

Th17 cells mainly produce IL-17 to guide the fight against extracellular pathogens. Th17 cells are also able produce IL-21. There appears to be plasticity between Th17 and Th1 cells, and it has been considered that context-dependently Th17 cells can differentiate into Th1/Th17 cells. (13,21,24,25)

Tfh and Treg cells are also vital components of the helper T-cell landscape, of which Tfh cells, central to the topic of this thesis, are described in the following chapter 2.2.3. Tregs are key players in the peripheral tolerance mechanisms and they produce IL-10 and TGF-β which inhibit the action of other immune cell subsets. (10) Of note, both Treg and Tfh cells demonstrate plasticity and can acquire features of other helper T-cell subsets according to the immunological milieu. For example, Tregs can differentiate into Th1-type Treg, and Tfh cells to T follicular regulatory cells (25). It must be noted that assigning $CD4^+$ T cells under distinct labels helps us define the flavor of T cells to classify general differentiation states of T cells. The reality, however, is more likely to be a continuum of differentiation states and fluid phenotypes that arise in a context-dependent manner.

In addition, $CD4^+$ T cell landscape also contains a small subset of cells with cytotoxic capacity ($CD4^+$ CTL), and they are associated with viral infections, cancer, and autoimmunity (22). They have similar granule-mediated cytotoxic function as the cytotoxic $CD8^+$ T cells described in the chapter 2.2.4. $CD4^+$ CTLs have a capacity to identify and kill their target cells in an antigen-specific manner (22). It has to be noted, however, that $CD4^+$ CTLs recognize their antigens in the context of HLA class II molecules, and these are expressed only by professional APCs. Therefore, the activity of $CD4^+$ CTLs is more confined compared to $CD8^+$ T cells. (26)

In the context of T1D and autoimmunity, of the Th subsets depicted here, Th1 and Th17 cells likely play a role in the disease process. T1D is especially considered to be Th1-mediated disease (27), but this is likely a

slight undermining of the complex interplay between several different subsets of T cells that are affected, and the remarkable plasticity that $CD4^+$ T cells exhibit. In addition, $CD4^+$ B-cell helper T-cells and Tregs play a major role in the context of T1D pathophysiology. The role of T-cell subsets central to this thesis are described in chapter 2.5 in the context of T1D.

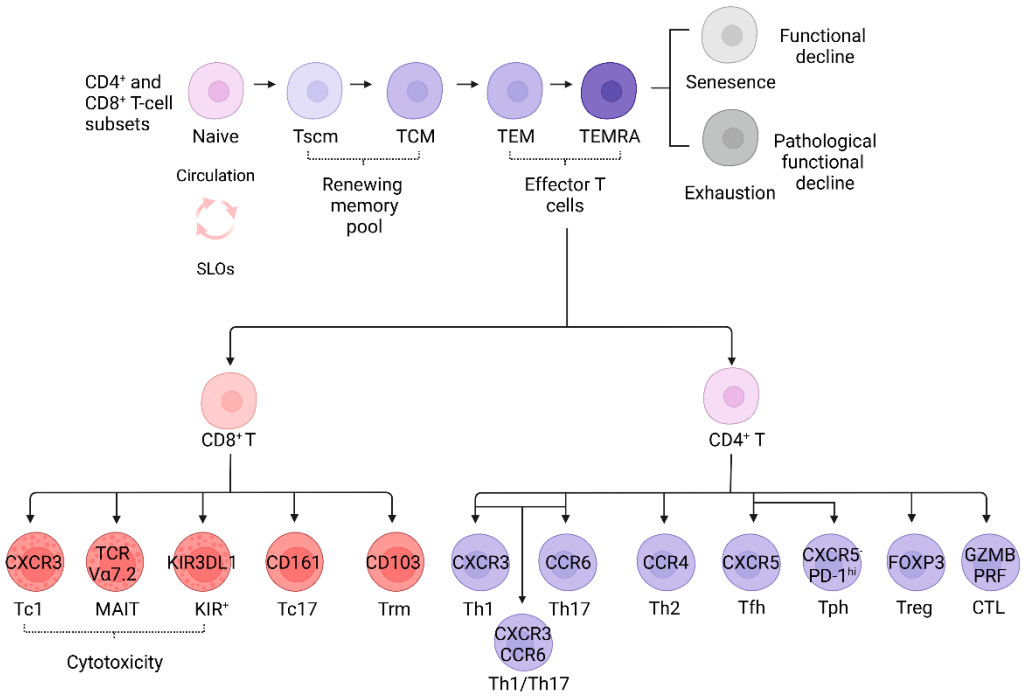

Figure 4. T-cell landscape. Effector T cells can be divided into distinct subsets that arise in a context-dependent manner. Each subset can be distinguished either by certain surface marker combinations, hallmark transcription factors and/or production of soluble mediators. Tscm = stem cell-like memory T cell, TCM = central memory, TEM = T effector memory, TEMRA = Terminal effector memory cell re-expressing CD45RA. Tc = T cytolytic, Th = T helper, MAIT = mucosal associated invariant T cell, KIR = killer lectin inhibitory receptor, Tfh T follicular helper, Tph = T peripheral helper, Treg = T regulatory, CTL = cytolytic $CD4^+$ T cell. Adapted from "Cell Differentiation Pathway", by BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates

2.2.3 $CD4^+$ T follicular helper cells

In addition to the different $CD4^+$ Th subsets described in the previous chapter, $CD4^+$ Tfh cells are a fundamental subset of T cells of the adaptive immune system (Fig. 4). Tfh cells are professional B-cell helper T cells. They help B cells to activate, proliferate and produce immunoglobulins. Hence, Tfh cells are a key T-cell helper component in the context of establishing B-cell-mediated humoral immunity. (28)

Tfh cells are differentiated in the SLOs, such as lymph nodes (10), in a multistep manner which have been simplified in the following paragrahps. DCs prime naïve $CD4^+$ T cells in the T-cell zones of SLOs in an antigen-dependent manner. Naïve $CD4^+$ T cells that receive a proper cocktail of activating and inhbiting signals for Tfh differentiation at SLOs are able to upregulate B-cell lymphoma 6 (BCL6) expression, which is the hallmark lineage-defining transcription factor of the Tfh cells. (28)

Upon BCL6 upregulation, expression of chemokine receptor CXCR5, a hallmark surface receptor of Tfh cells, is induced and on the other hand, expression of receptors associated to migration toward circulation are downregulated (28). CXCR5 expression aids Tfh cells to home into CXCL13-rich areas at SLOs (28). Tfh cells migrate to the T-B cell border at SLOs and can there interact with B cells. B cells specific to a particular antigen can present peptides that Tfh cells of the same antigen specificity can recognize. (10) This is the basis for a meaningful, prototypical adaptive immune response – the cell components of the adaptive immune system responding to the same target. Tfh cells require signals from B cells to differentiate into mature Tfh cells that can, in turn, facilitate the B-cell help functions (28).

The peak of B-cell helper functions occurs, in the context of Tfh cells within the germinal centers (GCs) (10). GCs are organized in structures consisting of a light zone (LZ) and a dark zone (DZ). Differentiated Tfh (GC-Tfh) cells provide B cell help in LZs via IL-21, CXCL13, IL-4 and CD40L. B cells that acquired help from GC-Tfh cells, migrate then to DZ. Proliferation and somatic hypermutation - editing of variable region of immunoglobulin genes to enhance the response to antigens - occurs in DZ. (28)

Tfh cells are considered the prototypical B-cell helper T cells. Recent discovery of T peripheral helper (Tph) cells (29) has expanded B-cell helper T-cell paradigm as these cells appear to mediate B-cell help in inflamed peripheral tissues, in contrast to Tfh cells that conventionally operate in SLOs. As the discovery of Tph cells is relatively recent, and is associated with autoimmunity, these cells will be discussed in detail in chapter 2.5.2.

2.2.4 Cytotoxic $CD8^+$ T cells

The main function of $CD8^+$ T cells is to mediate cytotoxic functions that drive target cells into apoptosis. Typically, the target cells of $CD8^+$ T-cell mediated cytoxicity are cells infected by viruses, or cancer cells. (30) $CD8^+$ T cells can be activated independent of $CD4^+$ T-cell help in specific circumstances, but T-cell help provided by $CD4^+$ T cells is, nevertheless, often a prerequisite for the adequate activation of $CD8^+$ T cells. Once again, $CD4^+$ and $CD8^+$ T cells responding to same antigen form the basis of a meaningful adaptive immune response during microbial infections, for instance.

As described above, naïve $CD8^+$ T cells must be activated through antigen recognition. The immunological milieu can then drive the differentiation of $CD8^+$ T cells into various effector and memory subsets with distinct differentiation states (Fig. 3–4) (30). IL-12 and type 1 interferons (T1-IFNs) guide the $CD8^+$ T-cell differentiation towards effector cells by induction of transcription factors TBX21 and EOMES (10). Activated effector cells also need to migrate to the site of infection to mediate their effector functions. The expression of the chemokine receptor CXCR3 is induced on $CD8^+$ T cells by inflammatory states, and it helps in migration towards peripheral tissues where ligands of CXCR3 are expressed during inflammation. (30)

Cytotoxicity is arguably the most important $CD8^+$ T-cell function. It is mediated through cytolytic molecules that are packed within cytolytic granules in cytotoxic $CD8^+$ T cells. (10) Perforin and the serine proteases granzymes A and B mediate pore-formation and apoptosis of the target cells, respectively. Cytotoxic granules are released via exocytosis upon

$CD8^+$ T cells recognizing their target cells. $CD8^+$ T cells and target cells create an immunological synapse where the contents of the granules are released (degranulation). Perforin can bind to the plasma membrane of the target cell, where it can oligomerize and induce pores on target cells. As pores are formed, granzymes can get into the target cell. Granzymes are proteases that activate caspases, which, in turn, activate the cascade of apoptosis in granule-independent-manner. (10) In addition, cytolytic granules contain, for example, granulysin, which has antimicrobial and cell-membrane damaging activities (31). $CD8^+$ T cells are also avid producers of IFN-γ, which conventionally mediates pathogen clearance by inducing the phagocytic function of macrophages, and aid in the establishment of inflammatory milieu (10,32). Circulating cytotoxic $CD8^+$ T cells have often downregulated CD27 indicating a terminal differentiation status, and they express chemokine receptor CX3CR1, that mediates the homing to the inflamed tissues (18).

One particular curiosity should be mentioned in the context of cytotoxic $CD8^+$ T cells. CMV infection elicits permanent alterations in the circulating memory $CD8^+$ T-cell compartment (19,33). An atypically high frequency of CMV-specific $CD8^+$ T cells remain after the infection has contracted, and the antigen-specific cells exhibit a phenotype of advanced terminal differentiation status (19,30). During infection contraction, CMV-specific cells start to *re*-express CD45RA, and memory cells re-expressing this isoform of CD45 are together called TEMRA (Fig. 3) as described in chapter 2.2.1 (19). TEMRA $CD8^+$ T cells that have a high potential for cytotoxicity as indicated by the expression of granzyme B, and these cells are also proinflammatory as indicated by the robust production of IFN-γ and TNF-α. (19) It is beyond the point of this thesis to further discuss this phenomenon for the overall immune landscape, but it has to be noted that the effect of CMV infection on $CD8^+$ T-cell subsets needs to be taken into account in $CD8^+$ T-cell analyses as CMV infection elicits such drastic changes in the $CD8^+$ T-cell subset composition. The visual representation of the increase in the $CD27^-$TEMRA frequency in CMV+ individual is depicted in the Fig. 5.

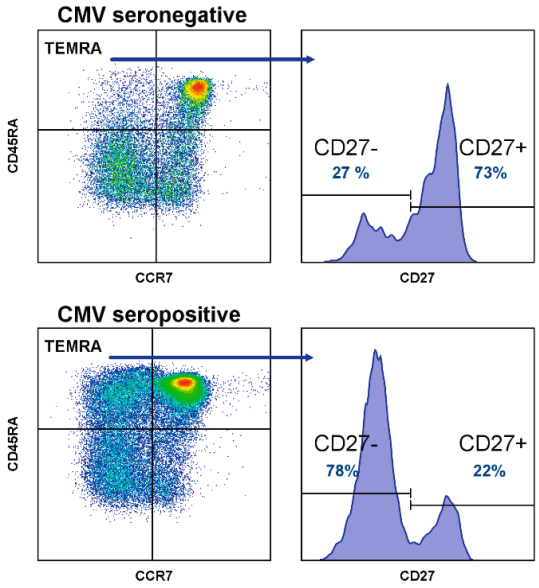

Figure 5. CMV infection drastically alters circulating $CD8^+$ T-cell subset composition. Representative flow cytometry plots depicting the CD27 expression pattern within $CD45RA^+CCR7^-$ TEMRA subset in a representative CMV-negative and CMV-positive individuals. Considerably higher frequency of $CD27^-$ cells are observed in CMV-seropositive individuals.

2.2.5 Nuances of the $CD8^+$ T-cell phenotypes

One could argue that the story of specific $CD8^+$ T-cell subsets is not as well-established as for $CD4^+$ T helper cells (i.e. various Th subsets, Tfh cells and Tregs). However, to a no surprise, various cytotoxic $CD8^+$ T-cell subsets also exist in a context dependent manner. (Fig. 4) $CD8^+$ T cells target typically virus infected and cancerous cells in various anatomical niches. Expanding on this theme, different $CD8^+$ T-cell subsets are described next.

$CD8^+$ T cells can be broadly categorized into Tc1 and Tc17 subsets, analogous to $CD4^+$ Th1 and Th17 cells (23). Tc1 cells produce IFN-γ and are considered to be "classical" cytotoxic effector cells that participate in Th1/Tc1-type T-cell responses (32). Tc17 cells produce IL-17 and, in contrast to Tc1 cells, are considered more of an inflammatory rather than a cytotoxic cell type. Similar to Th1/Th17 cells within the $CD4^+$ T-cell compartment, Tc17 cells are expected to have plasticity and indeed, dual

producers of IFN-γ and IL-17 have been described, at least in mice (34). It also appears that IL-12 (interleukin associated with Th1/Tc1 development) can induce a cytolytic capacity in Tc17 cells - one more indication of the complex nature and remarkable plasticity of the T cells. (34) Of note, Tc17 also share features, such as expression of CD161 and RORγt, with a specialized subset of mucosal associated invariant T (MAIT) cells. Tc17 cells are considered conventional T cells whereas MAIT cells are unconventional T cells expressing semi-invariant TCRs that are restricted to recognize microbial antigens presented through non-classical HLA class I molecule MR1 rather than classical HLA class I molecule (35). In contrast to inflammatory Tc17 cells, circulating MAIT cells in human appear to be a subset of cytotoxic $CD8^+$ T cells as they express high levels of cytolytic proteins. (35)

Cytotoxic function of "conventional" $CD8^+$ T cells is a rather well-described phenomenon. However, the $CD8^+$ T-cell paradigm might be expanding since more appreciation is given to the nuances in the $CD8^+$ T-cell functionality. Recent report by Jonsson et al. (36) suggest that in inflamed tissue, $CD8^+$ T cells expressing low levels of Granzyme B, but co-expressing granzyme K and IFN-γ appear to have a role in driving inflammation instead of mediating robust cytotoxicity. However, it has to be noted that Granzyme K can induce apoptosis of target cells, but not via activating caspases like Granzyme B (37).

Another more recently described $CD8^+$ T-cell subset worth mentioning would be a subset that is denoted by the expression of inhibitory killer cell immunoglobulin-like receptors (KIRs) (38). These $CD8^+$ T cells express also other co-inhibitory molecules such as TIGIT. These cells are suggested to possess inhibitory or even regulatory capacity towards autoreactive cells, i.e. they are suggested to participate, to an extent, in maintaining peripheral tolerance alongside $CD4^+$ Treg cells. (38) In the study by Li et al. (38), it was shown that KIR^+ $CD8^+$ T cells suppressed the action of autoreactive $CD4^+$ T cells. The proposed mechanism is that during microbial infection, autoreactive T cells cross-reactive to microbial proteins can emerge and erroneously cause autoimmune process after viral infection. The role of KIR^+ $CD8^+$ T cells would be to limit these potentially

autoreactive T cells by driving them to the apoptosis in a contact dependent manner. KIR^+ $CD8^+$ T cells expressed granzyme B, perforin and CX3CR1 which suggest that the suppressive mechanism is linked with the cytotoxic capacity, nevertheless. (38)

In conclusion, arguably the most fundamental function of $CD8^+$ T cells is to mediate target-specific cytotoxicity that drives the target cells into apoptosis. Nonetheless, through more advanced methodologies, we might soon characterize also other distinct nuances in $CD8^+$ T-cell functionality in the T-cell landscape.

2.2.6 T-cell exhaustion

Expanding on the theme of T-cell responses, it is essential to consider the phenomenon of T-cell exhaustion. T-cell exhaustion is a term describing T-cell phenotype arising in the context of chronic antigen stimulation that likely arises from an effort to contain chronic infection or tumor challenge leading eventually to T-cell dysfunction as the antigen exposure persists. Exhausted T cells have been detected first in the context of chronic viral infections and cancer, but it may also play a role in the establishment of autoimmunity as chronic antigen stimulation is also a feature of autoimmunity. (39,40)

Conventionally, effector T cells arise in the context of immunological challenge, and when the challenge, for example viral infection, is cleared, effector T cells die leaving only a small proportion of memory T cells behind that are long-lived. They can expand during re-infection. (10) In contrast, T-cell exhaustion represents a persisting continuum of T-cell phenotypes during chronic antigen exposure (Fig. 6) (41). T-cell exhaustion arises from a distinct transcription factor TOX-mediated transcriptional program, and consists of cells with various functional capacities. Transcriptional program for T-cell exhaustion leads to the emergence of precursor exhausted T cells which represents a phenotype still capable of self-renewal. (41–44) Transcription factors TBX21 and EOMES are expressed at the earlier and later stages of exhaustion contimuun, respectively. Cell states between less and more exhausted T cells likely contain effector cells that aid in containing the chronic antigen exposure.

The “in-between” exhausted cells express CX3CR1, proinflammatory cytokines and an increasing rate of various co-inhibitory receptors such as PD-1, TIGIT and LAG-3. Finally, the terminally exhausted cells eventually downregulate the expression of CX3CR1, the cytokines IL-2, IFN-γ and TNF-α, cytotoxic compounds, and exit the cell cycle. These cells, however, continue to express high levels of co-inhibitory receptors. T cells expressing high levels of TIM3 are considered to represent the highest degree of dysfunction. (41–44)

It seems to be indisputable that exhausted cells are a distinct T-cell phenotype. It has to be noted, however, that the features associated with exhausted T cells are also evident in conventional Teffs during acute infections or in physiological maintenance of self-tolerance (39,40). Next, some details on the similarities of conventional Teffs and exhausted T cells. During acute infection, effector T cells also exhibit a transcriptional program consisting of TBX21 and EOMES, which results in upregulation of proinflammatory cytokines and cytotoxicity (45). The transciption factor TOX is also expressed, but only transiently (42). Co-inhibitory receptors are upregulated during the activation of T cells as there is a delicate balance of co-inhibition and co-activation. During terminal differentiation of conventional memory T cells, T cells also gradually lose polyfunctionality. In other words, conventional terminally differentiated T cells get also eventually hypofunctional and senescent. (18)

It has to be noted that immune system functions in an intricate manner. It consists of a coordination of several subsets of different cell types, meaning that the immunological milieu has a major impact on T-cell fates. There is also a duality in the nature of T-cell exhaustion. In chronic viral infections and in cancer the exhausted T cells are often hypofunctional, unable to contain chronic infection or participate in adequate tumor control, respectively (41). In the case of autoimmunity, however, it is not as clear whether T-cell exhaustion actually results in slower progression of the clinial disease as seen for example in T1D where autoreactive $CD8^+$ T cells have been shown to express markers associated with T-cell exhaustion in individuals with slow progression to T1D (46).

In conclusion, there are similarities and differences in phenotypes of conventional and exhausted T cells. Often several markers, transcription factors and functional output is required to distinguish between conventional and exhausted T cells, necessitating a careful consideration whether to label cells expressing these features exhausted or not. Importantly, further investigation needs to be carried out to understand the relevance of T-cell exhaustion in different chronic conditions and to detect the events that tip the balance over from maintaining the chronic antigen exposure towards hypofunctionality during chronic viral infection, in cancer and in autoimmunity.

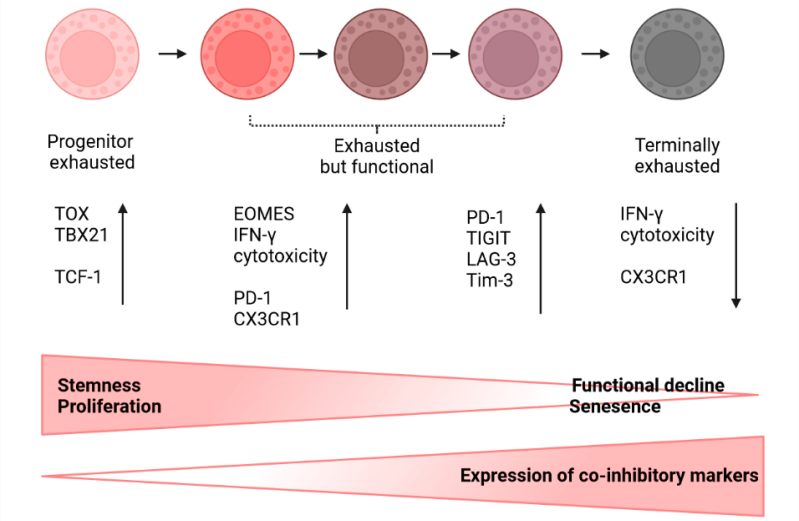

Figure 6. T-cell exhaustion continuum. Exhausted T cells arise from a distinct transcriptional program and exhibit a continuum of effector T cells that eventually get hypofunctional and terminally exhausted. Created with Biorender.com.

2.3 AUTOIMMUNITY

2.3.1 Mechanisms of autoimmunity

Autoimmune diseases are generally considered rare, but already for decades at least some autoimmune diseases have become more prevalent. There are approximately 100 different autoimmune diseases, the most

common ones being T1D and autoimmune thyroiditis. (47) In the following section, general mechanisms of autoimmune diseases are described before assimilating the paradigms into the setting of T1D in chapter 2.4.3.

As described in chapter 2.1.2, normal T-cell development involve phenomena called positive and negative selection, where T-cell progenitors called thymocytes with low-avidity for self-peptides and high-avidity for self-peptides are either preserved or eradicated, respectively. Negative selection forms the basis of central tolerance. Thymocytes surviving negative selection differentiate and enter circulation as naïve T cells. Even in healthy individuals some self-reactive or autoreactive T cells can remain, but peripheral tolerance mechanisms limit their activity in health. (10,14) Tregs, described briefly in chapter 2.2.2, are the main cellular mechanism that maintain the tolerance in periphery (10).

Several key events lead to to the development of autoimmunity. First, genetic predisposition is often required. However, monogenic autoimmune diseases are rare. Instead, multiple genetic factors usually are involved. (47,48) In T1D, coeliac disease (CeD), rheumatoid arthritis (RA) and multiple sclerosis (MS), to mention a few, several HLA variants leading to increased risk for disease development have been identified (47). Genetic variants in the HLA class II loci have the strongest association with autoimmunity, but also other gene variants have been also identified. Many of these risk variants are associated with potential functional alterations of the immune cell components. (47,48) Genetic risk factors of T1D are briefly described in chapter 2.4.3.

Secondly, usually a triggering event occurs before pathological autoimmunity. These triggering events are largely, at the moment, associated with environmental factors, and more specifically to microbial infections (47). Strongest evidence has thus far accumulated in the context of MS, where Epstein-Barr virus infection was shown to be a predisposing event for the development of the MS pathology (49).

Several mechanisms have been proposed in the context of infection-related breach of tolerance and autoimmunity. Some of the precipitating events in autoimmunity likely result from microbial peptides resembling human (self) peptides, a phenomenon called molecular mimicry. (50) In

microbial infection, microbial proteins/peptides end up in the SLOs, and T cells specific to these microbial antigens react to them when presented by APCs. If microbial antigens resemble self-antigens, T cells originally reacting to microbial antigen can then cross-react with the resembling self-antigen and elicit an autoimmune reaction. In addition, epitope spreading is a phenomenon where immune response develops toward epitopes that are, in a way, secondary to the ongoing immune response. In autoimmunity, epitope spreading can manifest so that a T-cell response emerges towards another epitope within same antigen or towards (self)antigens in close proximity to the ongoing autoimmune response. (50) Additionally, the generation of neoepitopes by post-translational mechanisms is well-known phenomenon for example in CeD, where modified gluten peptides are recognized as foreign by gluten-specific T cells (51).

Third, a breach in the peripheral tolerance can occur via Treg dysfunction and/or reduced frequency (47). In addition, autoreactive Teff cells may get resistant to Treg suppression, as seen in T1D (17, 18). These effects enable the loss of Treg control over the autoreactive T cells. The activation of autoreactive T cells can then directly cause the autoimmune disease. (47)

In autoimmunity, the following humoral or cellular mechanisms have been additionally identified. In certain autoimmune diseases autoantibodies produced by B cells play a major role in the autoimmunity. Autoantibodies can create immune complexes that mediate tissue damage; this is an important pathogenic mechanism for example in systemic lupus erythematosus (SLE) and RA. Autoantibodies can also cause NK-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). When autoantibodies are deposited on their target cells, NK cells can recognize the Fc region of autoantibodies. This pattern recognition elicits NK cells to release the cytotoxic compounds that then kill the target cells that are host's own cells in the case of autoimmunity. (47)

The basis of a meaningful immune response was that cells of the immune system recognize common targets. Unfortunately, this holds also true in the case of the autoimmunity. Autoreactive $CD4^+$ T cells can aid autoreactive B cells to produce autoantibodies, and autoreactive $CD8^+$ T

cells to directly kill their target cells. The role of $CD4^+$ T and $CD8^+$ T cells in T1D is described in detail in chapter 2.5 as they play a major role in the development of T1D.

2.4 TYPE 1 DIABETES

2.4.1 Symptoms, clinical diagnosis and treatment

In T1D, the autoimmune process progressively destroys insulin-producing β cells. In health, insulin plays a key role in managing glucose levels. When insulin production progressively diminishes, glucose levels start to rise leading to hyperglycemia. Eventually insulin deficiency causes the hallmark symptoms of T1D: thirst, excessive urination and weight loss. Also, abnormally high plasma glucose levels can be detected as an indication of hyperglycemia. (7,54)

During the late asymptomatic pre-diabetic stage, when clinical diagnosis has not yet been set, glucose metabolism defects can be detected close to the diagnosis. These defects are called impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) according to the plasma glucose measurements and oral glucose tolerance tests, respectively. (55) In Finland, T1D is diagnosed by the impairment in the glucose tolerance (2h value in glucose tolerance test >11 mmol/l), high fasting plasma glucose levels (≥7.0 mmol/l) and/or increased glycated heamoglobin (HbA1c) levels (≥ 48 mmol/mol). (7)

Features indicative of T1D commonly are young age at disease onset (under 30 years of age), multiple persisting T1D-related islet autoantibodies (AAb) in the circulation, low C-peptide level (a surrogate marker for insulin production) in the circulation, as well as ketoacidosis. Ketoacidosis is a serious medical condition that requires hospital care. (55)

T1D is a fatal disease unless it is treated using exogenous insulin. Glucose levels have to be maintained with care as stable glucose levels also decrease the risk of complications that are usually associated with T1D. (55)

2.4.2 Staging and progression patterns of type 1 diabetes

Three stages of T1D development can be distinguished (Fig. 7). Stage 1 is a pre-diabetic stage characterized by the presence of two or more persisting AAbs in the circulation, but the individuals remain euglycemic. AAbs are the first evidence of autoimmunity, and at this stage there is no overt β-cell damage yet. (56) Insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), IA-2 antigen autoantibodies (IA-2A) and zinc transporter A antibodies (ZnT8A) are autoantibodies associated with T1D, and the antigens for these are mostly β-cell granule proteins (8,57). In the context of this thesis, children with persisting AAbs are called autoantibody-positive (AAb^+) children.

Stage 2, another pre-diabetic stage, describes a stage where AAb^+ individuals develop dysglycemia (IFG and/or IGT) indicating that the autoimmunity has already caused β-cell damage that disrupts the glucose metabolism. 5-year risk for the symptomatic disease rises as high as 75%. (56)

In stage 3, T1D diagnosis can be set as the clinical manifestation of the disease is observed as described in chapter 2.4.1. Individuals with T1D are required to use lifelong exogenous insulin treatment that is a considerable medical burden to the individuals and affected families. (56)

It has to be noted that pathogenetically T1D is likely a heterogenous disease and not a singular entity. It is possible that slightly different pathways result in the insulin deficiency caused by an autoimmune process targeting and killing insulin producing β cells. (8) Indeed, it appears that there are at least two major pathways to clinical T1D, rapid progression and slow progression. These progression patterns have been associated with the emergence of insulin autonantibody (IAA) or glutamic acid decarboxylase 65 (GADA) as the first AAb, respectively. (8) Rapid progression occurs during early life, and IAA emerges already usually at the age of 1–2 years. Slower progression is usually observed if GADA emerges as first AAb, and this occurs typically later in childhood (age 4–5 years) or even during adulthood. (Fig. 7) “IAA first” or “GADA first” have been tentatively labeled as different T1D endotypes. (8,58) The IAA or GADA first

endotypes appear not differ in the overall risk of developing T1D, the disease just develops at a different pace. (59)

In summary, different stages in T1D development can be distinguished. The pace that the T1D develops seem to follow at least two trajectories: with fast and slow progression, and likely these trajectories are related with different disease endotypes where slightly different genetic predisposition and environmental factors together play a role in the initation of the T1D. (Fig. 7)

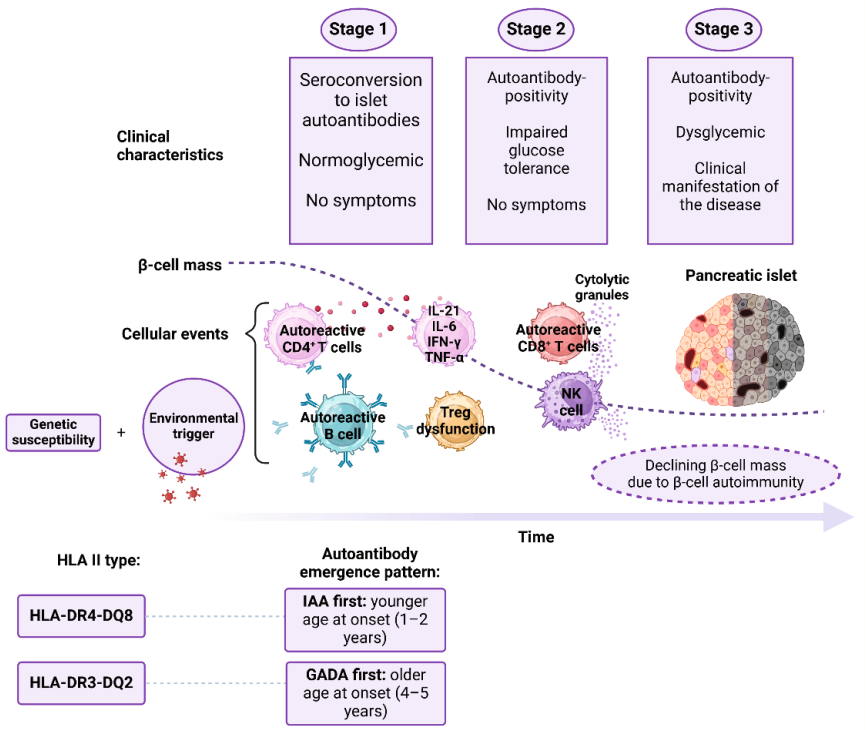

Figure 7. Summary of the events during the progression to T1D. Genetic susceptibility together with environmental trigger initiate the progression to T1D. Stage 1 begins at the seroconversion to AAb positivity, i.e. the earliest time point where autoimmunity can be detected. During stage 1 individuals are normoglycemic. Near the end of stage 1 β-cell mass starts to diminish due to the autoimmune process and stage 2 depicts the stage where IGT can be detected as a sign of insufficient insulin production by β cells. Finally, stage 3 corresponds to the onset clinical T1D where autoimmunity has progressively destroyed β cells so that there is not enough insulin production to cater the needs of the glucose metabolism. Adjusted from Insel et al. 2015 (56). Created with BioRender.com.

2.4.3 Autoimmune process in type 1 diabetes

T1D is a T-cell mediated, organ-specific autoimmune disease. Genetic predisposition and environmental factors together contribute to the risk for developing T1D. (8) The strongest genetic associations with the T1D are certain HLA class II haplotypes and their combinations. Also single nucleotide polymorphisms (SNPs) in multiple non-HLA genes, including the insulin (*INS)* gene have an impact on T1D risk. (60–62) In the Caucasian population, over 90% of patients that develop T1D during childhood exhibit HLA-DRB1*03-DQA1*05-DQB1*02 (HLA-DR3-DQ2) and/or HLA-DRB1*04:01/02/04/05)-DQA1*03-DQB1*03:02 (HLA-DR4-DQ8) haplotypes. Individuals heterozygous for DR3/DR4 haplotypes have the highest risk for developing T1D. Of note, there are also HLA class I risk haplotypes associated with the risk for developing T1D. (63) The investigation of the functional consequences of non-HLA polymorphisms in coding and non-coding regions of the genome is an active field of study where investigators try to identify mechanisms that eventually tip the balance towards autoimmunity rather than immune regulation and/or functional consequences that render β cells more susceptible to inflammatory stress.

Various environmental factors have been suggested to play a role in the susceptibility for developing T1D in genetically predisposed individuals. The immunological initiation of autoimmunity is likely caused by a virus as signs of viral infection can be detected in the islets. For example, insulitic islets show a high expression IFN-stimulated genes (64) and hyperexpression of HLA class I molecules (65). The strongest evidence of viruses is currently against enterovirus B. Enterovirus B is known to have tropism to pancreas and able to infect pancreatic β cells. (66) In addition, the damage or stress caused by viral infection in the β cells would induce presentation of viral proteins by HLA class I molecules, together with atypical (auto)antigen presentation. (8) Additionally, β-cell stress may lead to generation of neoepitopes when self-proteins are post-translationally modified and as such, can be perveiced as foreign. (67)

Autoreactive $CD4^+$ T cells are thought to be the main mediators of autoimmunity towards β cells. Why autoreactive $CD4^+$ T cells recognize β

cells as foreign? There are a multitude of potential reasons: specific HLA class II molecules could present T1D-associated (auto)antigens or neoepitopes to autoreactive $CD4^+$ T cells (67). It has been also speculated that central tolerance mechanisms within thymus work inadequately in T1D (68,69). Additionally, autoreactive T cells may be also released from the harness of peripheral tolerance mechanisms in T1D as it appears that the effector T cells become resistant to Treg mediated suppression (52,53).

Furthermore, $CD4^+$ T-cell help is often a prerequisite for the activation of other immune cell subsets indicating that the initiation and orchestration of autoimmunity is likely starting from $CD4^+$ T cells. The activation of autoreactive B cells and subsequent production of AAbs requires stimulatory signals coming from autoreactive $CD4^+$ T cells. Therefore, there is considerable evidence that B cells do not actually initiate the autoimmune process. (70) Aabs are not cytolytic (i.e. do not mediate ADCC) (70), but presumably AAbs and B cells contribute to the overall autoimmune process indirectly by enhancing the T-cell activation (8).

Importantly, autoreactive $CD4^+$ T cells likely enhance the activity of autoreactive $CD8^+$ T cells which can then contribute to the direct killing of β cells by directly targeting insuling-producing β cells and killing them in a progressive manner. This leads to the decline in insulin production and eventually to the symptomatic onset of T1D. (8)

Intriguingly, there are also some evidence that during inflammatory conditions, β cells may also exhibit an aberrant expression of HLA class II molecules, which in turn would enable autoantigen recognition by autoreactive $CD4^+$ T cells directly within the islets (71). An aberrant expression of HLA class II molecules within the islets in individuals with T1D-related autoimmunity might also promote the action of CTL $CD4^+$ T cells (described in chapter 2.2.2) which could also contribute to the killing of β cells (72).

2.5 T CELLS IN TYPE 1 DIABETES

Blood T cells have been extensively studied in T1D, and several T-cell subsets have been implicated to play a role in the disease process due to

alterations observed in circulation. Table 2 summarizes the findings central to this thesis. In contrast, T cells at the site of autoimmunity within pancreas have been studied largely in murine models (73) and, only to a lesser extent using rare samples obtained from human pancreata (74–76). In humans, the insulitic lesions and the immune cell infiltration are less prominent than in the Non-obese diabetic (NOD) mouse model, which is the most widely used animal model to study T1D. Differences in the presentation of insulitis might be related to the differences in the anatomical composition of pancreas between mice and human. (65,77) Additionally, interindividual heterogeneity, T1D disease stage where the tissue sampling has been performed and the potential temporal nature of the insulitic lesions, may play a major role in the differences observed between humans and mice.

The studies on pancreata of individuals with T1D have revealed that autoreactive T cells can be found within the pancreas (78,79) and a recent histological study, where pancreata of AAb^{+} organ donors or organ donors with recent-onset T1D were examined, revealed also tertiary lymphoid organs (TLOs) within pancreas. TLOs contained memory T cells, B cells and plasma cells giving a strong support for an autoimmune process where the interplay between T and B cells seems to play a role in the development of T1D. (74)

The samples from pancreas are rare due to the difficulty in obtaining pancreatic samples as pancreas is not routinely sampled, and more typically, circulating T cells have been studied in T1D. However, the exact phenotypes of the T-cell subsets contributing to the disease process have remained elusive.

Table 2. Summary* of circulating polyclonal T-cell alterations in (pediatric) T1D.

T-cell subset	Finding	Reference
Granzyme B$^+$ Perforin$^+$ memory CD8$^+$	More frequent in T1D than controls.	(80)
KLRG1$^+$TIGIT$^+$ CD8$^+$	More frequent in teplizumab treatment responders.	(2)
Tfh	More frequent in AAb$^+$ children with IGT, and in children with T1D than controls.	(81), (82)
Tph	More frequent in AAb$^+$ children and in children with T1D than controls.	(83)
Th17	More frequent in children with T1D than controls**. Also contrasting findings***.	**(84–86), ***(87)
Treg	Various contrasting findings in the phenotyping experiments.# Effector cells resistant to Treg mediated suppression in T1D.##	#See discussion. ##(52,53)

*Central to this thesis.

2.5.1 CD8$^+$ T cells in type 1 diabetes

CD8$^+$ T cells are a major component of the immune infiltrates in islets with insulitis (88). The frequency, phenotype and function of autoreactive CD8$^+$ T cells have been studied in the human samples obtained from pancreas and from circulation, whereas less focus has been placed upon studying global, polyclonal CD8$^+$ T cells. It is also evident that CD8$^+$ T cells may have been overlooked as CD4$^+$ T cells are considered as the main event for the development of autoimmunity.

The studies on autoreactive CD8$^+$ T cells have revealed that they can be detected both within the pancreas and blood of healthy individuals, too, but the cells appear to be either less frequent or phenotypically different from the individuals with T1D (69,89). The autoantigen targets of autoreactive CD8$^+$ T cells have been shown to be similar in the samples

obtained from pancreas and from the circulation (79,89,90). As immune responses need to be orchestrated towards a common target, it is expected that autoreactive $CD8^+$ T cells recognize similar targets as autoreactive $CD4^+$ T cells. Insulin or its precursor preproinsulin (PPI) is an important autoantigen for $CD4^+$ T cells in T1D (90). Indeed, certain proinsulin peptides have been shown to also be the autoantigen targets of $CD8^+$ T cells (91). These findings support the idea of common targets of autoreactive $CD4^+$ and $CD8^+$ T cells.

The frequency of autoreactive $CD8^+$ T cells has been shown to be slightly higher in the pancreas of individuals with T1D compared to healthy controls (79). However, when frequency of autoreactive cells has been studied from the circulation, results have been varying: some report higher frequency of autoreactive $CD8^+$ T cells in individuals with T1D compared to healthy indviduals (92–94), while some report comparable frequencies (79). This variation is likely partially due to the extremely low number of antigen-specific $CD8^+$ T cells that can be detected. Culina et al. (79) reported approximately 1 to 50 β-cell reactive $CD8^+$ T cells per 10^6 total $CD8^+$ T cells (0.000001 to 0.00005%) for the context of various autoantigens.

The cytotoxic potential and phenotype of autoreactive $CD8^+$ T cells have been examined in several studies. To mention a few, proinsulin-specific $CD8^+$ T-cell lines were shown to produce proinflammatory cytokines IFN-γ and TNF-α, and to express chemokine receptors CXCR3 and CCR4, alongside with effector molecules, granzyme B and perforin in a study by Skowera et al. (91), supporting the view of Th1/Tc1-type inflammatory response mediating T1D. Culina et al. (79) reported that ZnT8-specific $CD8^+$ T cells from individuals with T1D responded more frequently by producing IFN-γ in ELISPOT assays compared to healthy individuals eventhough the absolute frequencies of these cells were comparable.

Global $CD8^+$, i.e. not antigen-specific, T-cell responses, phenotypes and functionality have been studied in a lesser extent in T1D. Hamel et al. (80) reported a higher frequency of circulating granzyme B- and perforin-expressing memory $CD8^+$ $CD45RA^-$ T cells in children with T1D indicating an enrichment of $CD8^+$ T cells with cytotoxic capacity. Importantly, recently a global $KLRG1^+TIGIT^+$ $CD8^+$ T-cell signature has been associated with a

beneficial treatment response in teplizumab immunotherapy trials, where an increased frequency of these cells have been observed in the treatment responders (95,96). In addition, it has been observed that individuals with slower progression of T1D after disease onset, exhibited a higher frequency of autoreactive $CD8^+$ T cells that expressed a highly similar co-inhibitory signature than seen in the teplizumab trials – a signature that has been labeled as "exhausted" (46).

To conclude, proinflammatory autoreactive $CD8^+$ T cells and global cytotoxic $CD8^+$ T cells have been associated with T1D development and co-inhibitory receptor expressing $CD8^+$ T cells also with immunotherapy efficacy (2,95,97) and disease progression (98).

2.5.2 $CD4^+$ B-cell helper T-cells in type 1 diabetes

Tfh cells represent the classical B-cell helper T cells. Several lines of evidence suggest that IL-21-producing Tfh cells play a role in the T1D disease process (81,82). As described in chapter 2.4.2, during early, asymptomatic, stage of T1D-related autoimmunity, AAbs can be detected months to years before the clinical diagnosis. As Tfh cells are critically required for the antibody production, Tfh cells must play a role in the T1D disease process. B cells are dependent on IL-21 in order to expand and mediate their effector function that is to produce antibodies. (28,99) Indeed, a higher frequency of IL-21 producing T cells and circulating Tfh (cTfh) cells in T1D patients (82), and circulating activated $PD\text{-}1^+ICOS^+$ Tfh cells in AAb^+ children with IGT and children with newly diagnosed T1D (81) have been detected. In addition, autoreactive Tfh cells have been found in AAb^+ children (100). Tfh cells have also been shown to be expanded in various other autoimmune diseases (101).

Additional T-cell subsets have been also suggested to be able to provide B-cell help. The most prominent finding in the recent years has been the identification of $CXCR5^-PD\text{-}1^{hi}$ Tph cells that were shown to produce IL-21 in the synovial fluid in seropositive RA (102). Circulating $CXCR5^-PD\text{-}1^{hi}$ Tph cells are also increased in children with newly diagnosed T1D and in AAb^+ children who later progressed to T1D (83). Similar reports have been

obtained from other autoimmune diseases, such as CeD (103), SLE (104) and juvenile idiopathic arthritis (105).

Tph cells express various chemokine receptors associated with migration into tissues rather than secondary lymphoid organs. Indeed, Tph cells lack CXCR5 expression in contrast to $CXCR5^+$ Tfh cells. Importantly, Tph cells have been shown to be capable of producing high levels of CXCL13 alongside IL-21. (102,106) These cells also exhibit a higher expression of *IFNG* and *IL10* compared to PD-1 low-expressing, $PD\text{-}1^{lo}$, T cells. In addition, *in vitro* experiments of Tph and B co-cultures have indicated that Tph cells are able to induce B-cell differentiation into plasma cells, consistent with the idea of their potential B-cell helper functions. (83,102)

It has been suggested that Tph cells could especially contribute to the formation of TLOs in inflamed tissues, and further, TLOs could sustain the autoimmune response at peripheral tissues (107,108). TLO organization have been observed in many autoimmune diseases, such as in seropositive RA (29) and MS (109). Lately, the TLO organization has been shown also in the pancreas of human AAb^+ individuals and patients with T1D as mentioned in the intro of the chapter 2.5 (74). Of note, interestingly, there is also some evidence that Tfh cells may not always be confined solely to SLOs. For example, in MS, Tfh cells have been shown to exist in CNS (109). This could be due to the establishment of TLOs at the sites of autoimmunity.

There is strong rationale that the T cells in TLOs could be $CD4^+$ B-cell helper T-cells as both T and B cells are observed within them. The study by Korpos et al. (74) showed that there were both less mature TLO structures that had intermixed T and B cells, and also cases where highly structurized, mature TLOs could be observed within pancreas. These mature structures appeared similar to GCs, which are seen in SLOs, described in chapter 2.2.3. Within GCs, B cells can undergo differentiation into plasma cells that produce antibodies (28). In the context of TLOs in T1D, these would likely be AAbs. It has been envisioned that T cells would provide B-cell help in these areas to enable B-cell differentiation. To produce a meaningful immune response, T and B cells respond to same antigens (28) – in the

context of T1D, these are likely the autoantigens. Currently, it is unclear, what the differentiation pathway of Tph cells is. It has been suggested that they could be differentiated from GC Tfh cells in context-dependent manner (106). Additionally, Tph cells also exhibit similarities to Th1 and Th17 cells, so the origin story of Tph cells is still slightly unclear.

Expanding on the theme of IL-21-producing $CD4^+$ T cells in T1D, Th17 cells have also been implicated to be associated with the disease process. Th17 cells produce mainly IL-17, but also IL-21. Th17 cells have been shown to be increased in the blood and lymph nodes of patients with T1D (84–86). Contrasting findings have also been reported, where Th1 $CD4^+$ T cells have been expanded in children with T1D, whereas Th17 $CD4^+$ T cells were shown to be reduced (87). Functional studies in mice have also shown that IL-21-producing Th17 cells are able to promote B-cell help and promote GC formation (110), implying that these cells may also be part of the complex continuum of B-cell helper T-cells.

In conclusion, several lines of evidence suggest that IL-21-producing T-cell subsets play a role in the development of T1D. It is currently unclear whether the expansions of IL-21-producing circulating Tfh and Tph cells reflect ongoing autoimmunity in SLOs or TLOs, or whether the expansion detected in circulation is already a sign of a reminiscent, diminishing autoimmunity during a period where most of the pancreatic β cells are already destroyed.

2.6 FUTURE PERSPECTIVES FOR PREVENTION OF TYPE 1 DIABETES

Promising immunotherapies of T1D have emerged in order to prevent or delay the onset of the disease. The common theme for future perspectives for treating T1D are better targeting the individuals who would benefit from the treatments. As such, the envisioned approach for delaying the onset of T1D, would be population-wide AAb screening for children, as children multipositive for AAbs are very likely to eventually develop the disease. Ghalwash et al. (111) suggest that sampling at 2 and 6 years of

age would capture well the two waves of AAb emergence, i.e. fast and slow progression patterns, in children. It could be envisioned that sampling performed routinely in children at those ages would be beneficial in order to detect the children in stage 1 or stage 2 T1D, where delaying the onset of the disease would still be therapeutically possible. (111)

Immunotherapies of T1D can be divided into primary and secondary prevention of the disease. Primary prevention refers to preventing the start of the autoimmune process and secondary prevention to delaying the onset of T1D by abrogating the already ongoing autoimmune process, at least transiently. It is easy to understand that in a relatively rare, heterogenous and multifactorial disease secondary prevention is more easily achvievable in comparison to primary prevention. (112) Current immunotherapeutic efforts are target individuals with dysglycemia or recent onset disease. As T cells are heavily involved in the disease process, many of the immunotherapies under investigation are targeted towards T cells. (113)

Teplizumab trials have been a major breakthrough in the field. In the seminal phase 2 trial, it was shown that dysglycemic individuals at high-risk for developing T1D benefitted from a 2-week teplizumab treatment by a mean 2-year delay of T1D diagnosis (2). Teplizumab appears to induce a transient inhibition of T-cell activation and depletion of T cells. As an anti-CD3 antibody, teplizumab binds to the CD3 molecule on T cells. CD3 is a protein complex that associates with the TCR and is required for T-cell activation. Binding of teplizumab to CD3-ε chain induces T-cell anergy and can lead to later apoptosis of T cells. (114) Encouragingly, teplizumab was FDA-approved in 2022 in USA for individuals ≥8 years old with stage 2 T1D i.e. individuals multipositive for several AAbs (1). European medical agency will likely start their own investigation to determine whether to approve teplizumab also in Europe to delay the onset of stage 3 T1D. Of note, also other immunotherapies modulating T cells, such as Abatacept (CTLA-4-Ig) and Alefacept (LFA-3-Ig), are investigated for delaying the onset of T1D. (115,116) One additional therapy could be briefly discussed in the context of this thesis. Anti-IL-21 antibody combined with β-cell preserving agent liraglutide has shown potential in preserving C-peptide levels (117). As

discussed earlier, the IL-21 pathway has been indicated as one of the key pathways that have been directly linked with development of T1D. Anti-IL-21 immunotherapy is believed to inhibit the migration of autoreactive $CD8^+$ T cells into pancreas (117), but could also inhibit the function of $CD4^+$ B-cell helper T cells.

In addition to immunotherapies, active investigation of developing vaccination for enteroviruses, potential environmental triggers of T1D, is ongoing (118). Krogvold et al. (119) treated individuals with stage 3 T1D with a combination treatment with pleconaril and ribavirin, an enterovirus-specific and broad-spectrum antiviral drug, respectively. Higher residual insulin production persisted in individuals treated with the antivirals compared to placebo group after a 12 month of treatment period (119). An enterovirus vaccination study against coxsackie B virus has also been started in Finland, where the aim is to find a primary prevention strategy for T1D by eliminating the effect of coxsackie B virus infection as a potential environmental trigger of autoimmunity (120).

3 AIMS OF THE STUDY

T1D is a autoimmune disease that remains without a curative treatment, but recent successess in immunotherapies targeting T cells have shown the potential to delay the destruction of the insuling-producing β cells. This gives a strong rationale to focus on studying T cells in T1D as described in the sections above. A better characterization of T-cell responses in T1D to develop new biomarkers and therapeutical targets is urgently needed.

In this thesis, characterization of T cells and the timing of the known T-cell expansions associated with T1D pathophysiology were examined. The aim was to identify T-cell signatures in $CD8^+$ T cells and IL-21-producing CD4 B-cell helper T cells that might shed light to the T-cell landscape at the different stages of T1D development, and could be potentially utilized in the development of biomarkers for the disease progression.

The specific aims of the study were:

I: To characterize $CD8^+$ T-cell signatures at different stages of T1D progression

II: To examine the timing of $CD4^+$ B-cell helper T-cell expansion and phenotypes during the progression of T1D

III: To evaluate the potential of plasma IL-21 levels as a biomarker of T1D progression

4 SUBJECTS AND METHODS

4.1 STUDY SUBJECTS (I–III)

All participants and/or their legal guardians had given written informed consent as mandated by the Declaration of Helsinki. The study was approved by the local ethics committees at the Turku University Hospital and Kuopio University Hospital. The Type 1 Diabetes Prediction and Prevention (DIPP) study was approved by the ethics committee of the Hospital District of Northern Ostrobothnia. Coded samples were used throughout the study.

4.1.1 Pediatric samples (I–III)

Blood samples from the pediatric study subjects were collected through Turku University Hospital and the DIPP study. In all of the projects, the pediatric cohorts consisted of children with newly diagnosed T1D (0–7 d after clinical diagnosis), AAb^+ at-risk children and healthy age-matched controls. In addition, a rare sample set consisting samples from 17 children before and after seroconversion to AAbs was studied. The characteristics of the study cohorts are described in detail in articles I–III. Autoantibody-positivity was determined based on positivity for one or more biochemical AAbs (IAA, IA-2A, Znt8A and GADA). Autoantibody-positivity was analysed as indicated in (121). All participants had HLA class II genotypes associated with T1D risk. (Fig. 8)

4.1.2 Adult samples (I, III)

Blood samples from adult study subjects were collected through the T1D-IMMUNO study at the Kuopio University Hospital. All adults with T1D were considered as individuals with longstanding or established T1D. Healthy controls were age-matched. The characteristics of the study cohorts are described in detail in articles I and III. (Fig. 8)

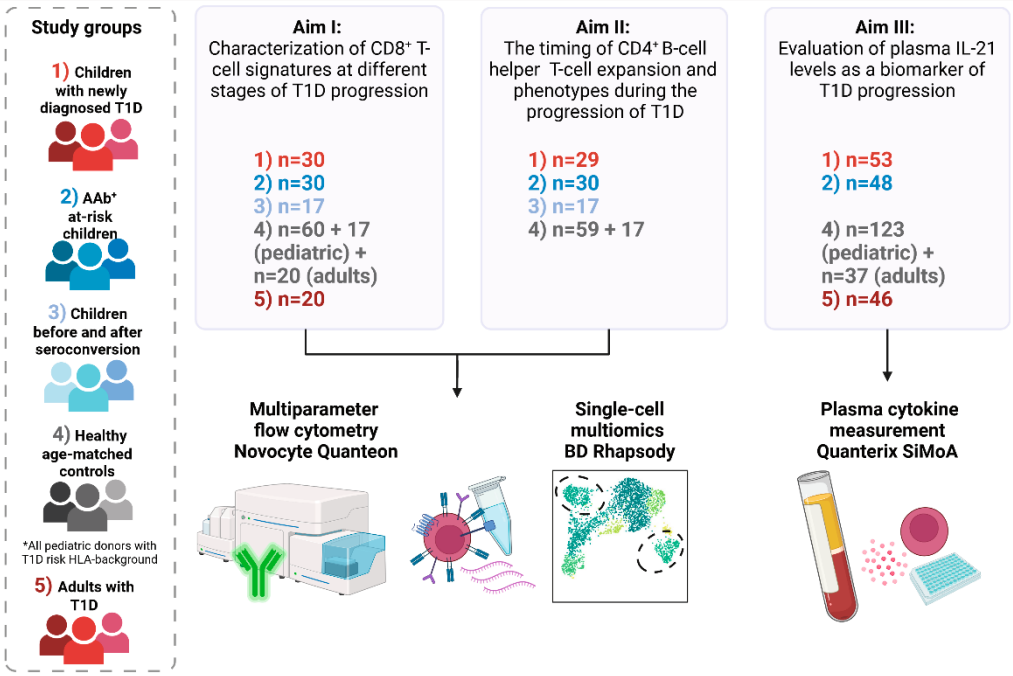

Figure 8. Study groups, objectives and utilized methods. Created with Biorender.com.

4.2 SAMPLE COLLECTION (I–III)

4.2.1 Peripheral blood mononuclear cells (PBMCs) (I–III)

PBMCs were collected using Ficoll density gradient centrifugation from heparinized blood samples. Pediatric samples were processed at Turku University Hospital and adult samples at the Department of Clinical Microbiology, University of Eastern Finland.

After Ficoll density gradient centrifugation, PBMCs were counted and frozen. PBMCs from pediatric samples were frozen in a mixture of RPMI-1640 medium, human AB serum and 10% DMSO, and adult samples in a mixture of FBS and 10% DMSO. Samples were stored in liquid nitrogen until use.

4.2.2 Plasma samples and measurements (I, III)

Plasma samples were collected after centrifugation in 700 x g, 10 min, and stored in -80°C.

CMV-specific IgG antibodies (I) were measured with the Vidas CMV IgG assay (bioMérieux S.A.), according to manufacturer's instructions. C-peptide levels were measured with electrochemiluminescence immunoassay and high-sensitivity C-reactive protein with particle enhanced immunoturbidimetric assay (both Cobas, Roche Diagnostics, III).

4.3 MULTIPARAMETER FLOW CYTOMETRY (I–II)

4.3.1 Surface marker staining (I–II)

0.5–1x10^6 PBMCs were aliquoted and labeled with fluorochrome-conjugated monoclonal anti-human antibodies for 20–30 min as indicated in the original publications (Article I, Suppl. Table 3; Article II, Suppl. Table 3). Brilliant Stain Buffer Plus (BD Biosciences) was added in each immunostaining where multiple Brilliant Violet or Brilliant Blue dyes were used according to the manufacturer's instructions. All samples were acquired with the Novocyte Quanteon flow cytometer (Agilent).

4.3.2 Stimulations and intracellular cytokine staining (I)

For intracellular cytokine analysis PBMCs were stimulated with 50 ng/mL Phorbol myristic acid (PMA, Sigma) and 1 µg/mL ionomycin (Sigma) for 5 h in 37°C.). PE-labeled anti-human CD107a antibody (Biolegend, dilution 1:10) was added at the beginning of the stimulation with PMA and ionomycin. Brefeldin A (5 µg/mL, eBioscience) and/or Monensin (2 µM, Biolegend) were added after the first incubation hour as indicated in the original publication (I). Surface stainings were conducted as detailed above. Subsequently, the cells were fixed and permeabilized with the Fixation and Permabilization kit (eBioscience). After fixation and permeabilization, the cells were stained with a mixture of fluorochrome-conjugated monoclonal anti-human antibodies for 30 min, as indicated in the original publication

(Article I, Suppl. Table 1). All samples were acquired with the Novocyte Quanteon flow cytometer (Agilent).

4.3.3 Magnetic cell isolation and flow sorting (I–II)

Briefly, for the single-cell multiomics experiments (I–II), magnetic isolation of $CD8^+$ T cells (I) (human CD8+ T cell isolation kit (negative selection), Miltenyi) or memory $CD4^+$ T cells (II) (human Memory CD4+ T cell Isolation Kit (negative selection), Miltenyi) were performed before sorting as indicated in the original publications (I–II) to enrich for the T-cell subtypes of interest and to shorten the sorting time.

Next, surface immunostainings required for sorting (Article I, Suppl. Table 4; Article II, Suppl. Table 3) were performed as indicated above and finally, the cells were resuspended in PBS supplemented with 0.5% Bovine Serum Albumin. Sorting was performed with Sony MA900 cell sorter (Sony Biotechnology, Article I, Suppl. Fig.1; Article II, Suppl. Fig. 1), and purity of the sorted samples were confirmed after sorting through flow cytometric analysis.

4.4 SINGLE-CELL MULTIOMICS (I–II)

4.4.1 Single-cell capture

Single-cell multiomics analyses were performed with the BD Rhapsody Single-Cell Analysis platform (BD Biosciences). Samples obtained from different individuals were labeled with the Single Cell Sample Multiplexing Kit (BD Biosciences) for multiplexing. In addition, the cells were labeled with panels of BD Abseq oligo-nucleotide conjugated monoclonal antibodies (BD Biosciences, Article I, Suppl. Table 3; Article II, Suppl. Table 3) according to manufacturer's instructions. Single-cell capture was performed with the BD Rhapsody Express System (BD Biosciences).

(I) Cells were rested overnight at +4°C in complete RPMI-1640 after $CD8^+$ T-cell isolation described in the chapter 4.3.3. $CD27^+$ and $CD27^-CD8^+$ T cells were sorted individually from five children with T1D and five healthy controls, and $CD27^+$ and $CD27^-CD8^+$ T-cell subsets were pooled at 1:1 ratio

to enrich the rare $CD27^{-}CD8^{+}$ T cells. Single cells were captured directly or following a 90 min stimulation with PMA (50 ng/mL) and ionomycin (1 µg/mL).

(II) PBMCs were rested overnight at +37°C, 5% CO_2 in RPMI-1640 supplemented with supplemented with 2 mM L-glutamine (Lonza), 20 mM 2-ME (Sigma), 1 mM sodium pyruvate (Lonza), nonessential amino acids (Lonza), 100 IU/mL penicillin (Lonza), 100 mg/mL streptomycin (Lonza), 10 mM HEPES (Lonza) and inactivated 5% human AB serum (Sigma) before memory $CD4^{+}$ T cell isolation described in the chapter 4.3.3. PD-1^{hi} and PD-1^{lo} memory $CD4^{+}$ T cells were sorted individually from three T1D patients and three healthy controls. Samples were subjected to a 90 min stimulation with PMA (50 ng/mL) and ionomycin (1 µg/mL). Single cells were separately captured for PD-1^{hi} and PD-1^{lo} subsets.

4.4.2 Library preparation and sequencing

The library preparation and sequencing was conducted according to manufacturer's instructions and are characterized in detail in Article I and Article II. (I) The libraries were prepared to study the mRNA expression of 473 genes (targeted approach, 399 genes in human Immune Response panel and 76 supplemental genes, BD Biosciences, Article I, Suppl. Table 5), surface protein expression of 14 surface proteins (BD Abseq oligonucleotide-conjugated monoclonal antibodies, BD Biosciences, Article I, Suppl. Table 3) and for Sample Tags (BD Single Cell Sample Multiplexing Kit) to enable multiplexing in the data analysis. (II) Libraries were prepared separately for PD-1^{hi} and PD-1^{lo} samples to study the mRNA expression of 261 genes (targeted approach, 259 genes in Human Targeted T cell Expression panel and two supplemental genes, BD Biosciences, Article II Suppl. Table 4), surface protein expression of 15 surface proteins (Abseq antibody-oligonucleotide conjugates, BD Biosciences, Suppl. Table 3), CDR3 VDJ profiling and for Sample Tags (BD Single Cell Sample Multiplexing Kit) to enable multiplexing in the data analysis.

The preparation of libraries to each modality were performed as indicated in the articles I–II and the libraries were sequenced on Novaseq 6000 (Illumina) at Finnish Functional Genomics Centre Turku Bioscience.

4.5 DATA ANALYSIS

4.5.1 Flow Cytometry (I–II)

All flow cytometry data were analysed using FlowJo (BD Biosciences). Stimulated samples were gated with the help of unstimulated samples, and phenotypical data was analysed with the help of fluorescence minus one (FMO) controls where applicable. Representative gating strategies of individual panels are shown in the original publications (Article I, Suppl. Fig. 2-3, 9; Article II, Suppl. Fig. 1).

4.5.2 Single-cell multiomics data preprocessing (I–II)

T-cell response and Immune cell response panel FASTA files (obtained through https://scomix.bd.com/) were used to align the reads into human genome version 38 at SevenBridges (Seven Bridges Genomics) servers. Subsequently, samples were preprocessed using BD's Rhapsody pipeline (122).

4.5.1 Single-cell multiomics analyses (I–II)

Single-cell analyses were conducted using R (123) and Seurat version 4.1.3 (124), as described in the original articles (Article I–II). Co-expression of selected markers was visualized using the scCustomize (125) or Nebulosa packages (126). Dotplot heatmaps were created in Seurat. Differentially expressed genes and proteins for each cluster were determined using the Wilcoxon rank sum test (FindAllMarkers function). Genes and surface proteins were considered significantly differentially expressed when log2 fold change was ± 0.25, and Bonferroni adjusted p-value ≤ 0.05. Further details can be found at the original articles (I–II).

4.5.1 Pseudobulk analysis (II)

Pseudobulk analysis was carried out for CXCR5^{-}PD-1^{hi}, CXCR5^{+}PD-1^{hi}, CXCR5^{-}PD-1^{lo} and CXCR5^{+}PD-1^{lo} fractions that mirror the cell subsets analyzed in flow cytometry data. Pseudobulk samples were generated by aggregating the gene expression values from each of the four cell fractions in each individual. Pseudobulk analysis was performed using edgeR (127–130) version 3.40.0 according to the description in the original article (Article II). For differential expression testing, the generalized linear model quasi-likelihood pipeline was used (128) and the glmTreat test was performed to test for differential expression relative to FC >1.5. Genes with FDR value ≤0.05 were considered significantly differentially expressed. Heatmap was created using ComplexHeatmap package (131).

4.5.2 TCR CDR3 analysis (II)

Clones were defined according to identical TRAV, TRAJ, TRA CDR3, TRBV, TRBJ and TRB CDR3 sequences in given cells. Venn diagram was created using InteractiVenn (132).

4.6 QUANTERIX SIMOA (III)

Heparinized plasma samples were shipped on dry ice to Eli Lilly (Indianapolis, USA). Samples were thawed and aliquoted to batches. Plasma IL-21 levels were measured using the Quanterix SiMoA (Single Molecule Array) as previously reported (133). Plasma IL-17A, TNF-α and IL-6 were measured using the Cytokine 3 Plex B Quanterix SiMoA assay, according to manufacturer's instructions (Quanterix).

4.7 STATISTICAL TESTING (I–III)

Statistical analysis for flow cytometry (I–II) and plasma cytokine data (III) was carried out with GraphPad Prism (version 9.2.0). When two groups were compared either Wilcoxon signed-rank matched pairs test (II–III) or Mann-Whitney U test (I–III) were used as indicated in the original

publications. When >2 groups were compared, Kruskal-Wallis (I–III) or Friedmann test (II) with Dunn's multiple comparisons test were used. Spearman's correlation was used for correlation analyses (I, III). Simple linear regression was utilized for calculation of linear regression lines for the study groups. (I, III) $P<0.05$ was considered to indicate statistical significance, and $P<0.003$ where Bonferroni correction was applied as indicated in the original publications.

5 RESULTS

5.1 TEMPORAL ALTERATIONS IN CD8$^+$ T CELLS DURING THE PROGRESSION OF TYPE 1 DIABETES (I)

CD8$^+$ T cells likely play a major role in the T1D disease process as described in chapter 2.5.1. Certain autoreactive CD8$^+$ T-cell phenotypes have been linked with the risk of T1D progression (46,134) but characterization of global CD8$^+$ T-cell phenotypes in patients with T1D are only a few (80,135,136) and studies analyzing children before to the onset of T1D are lacking. Here, function and phenotype of blood polyclonal CD8$^+$ T cells were analyzed in a cross-sectional pediatric cohort consiting of 30 children with newly diagnosed T1D, 30 AAb$^+$ children and 60 age-matched healthy controls using multiparameter flow cytometry. (Article I, Table I, Suppl. Table. 1) In the following sections, the memory compartment is divided into CD27$^+$ (consisting of TCM and CD27$^+$ TEM cells) and CD27$^-$CD8$^+$ (consisting of CD27$^-$ TEM and TEMRA cells) T-cell subsets (Fig. 9A).

5.1.1 Polyfunctional proinflammatory CD27$^-$CD8$^+$ memory T cells are expanded in children with type 1 diabetes

First, the functionality of memory CD8$^+$ and CD4$^+$ T cells was assessed by examining the frequency of proinflammatory cytokine (IFN-γ, TNF-α, GM-CSF and IL-2) and degranulation marker CD107a expressing cells at different stages of T1D development. Cytokine signatures were studied upon stimulation with PMA and ionomycin.

The frequencies of CD27$^-$CD8$^+$ T cells expressing proinflammatory cytokines IFN-γ, TNF-α, GM-CSF, IL-2 and the degranulation marker CD107a were significantly higher in children with T1D compared to controls (Fig. 9B). Of note, when examining CD27$^-$ CD8$^+$ TEM (CD45RA$^-$CCR7$^-$CD27$^-$CD8$^+$) and CD27$^-$ CD8$^+$ TEMRA (CD45RA$^+$CCR7$^-$CD27$^-$CD8$^+$) subsets, similar cytokine expression patterns were seen in both of them indicating that the whole CD27$^-$CD8$^+$ T-cell compartment was altered rather than a particular memory CD8$^+$ T-cell subset (Article I, Suppl. Fig. 4), adding validity to our

chosen approach to analyze global $CD27^+$ and $CD27^-$ T-cell memory subsets. AAb^+ children, children before and after seroconversion and adults with established T1D showed comparable frequencies of proinflammatory cytokine expressing $CD27^-CD8^+$ T cells (Article I, Suppl. Fig. 4D, Fig. 3 and Suppl. Fig. 13). Of note, when $CD4^+$ T cells were studied, we observed a corresponding significant increase of IFN-γ^+, TNF-α^+, GM-CSF^+ and IL-2^+ $CD27^-CD4^+$ T cells in children with T1D. Again, we did not detect this signature in AAb^+ children or within less terminally differentiated $CD27^+CD4^+$ T-cell subsets (Article I, Suppl. Fig. 7). Of note, frequencies of $CD27^{+/-}$ T cells were not altered in the pediatric study groups (Article I, Suppl. Fig. 4).

Age and CMV serostatus have a major impact on the composition of $CD8^+$ T-cell compartment in children (19). CMV infection alters permanently $CD27^-CD8^+$ memory T cell compartment as described in chapter 2.2.4 (Fig. 4). Hence, we confirmed that we were able to detect the proinflammatory signature after stratifying for age and CMV serostatus. (Fig. 9C–D)

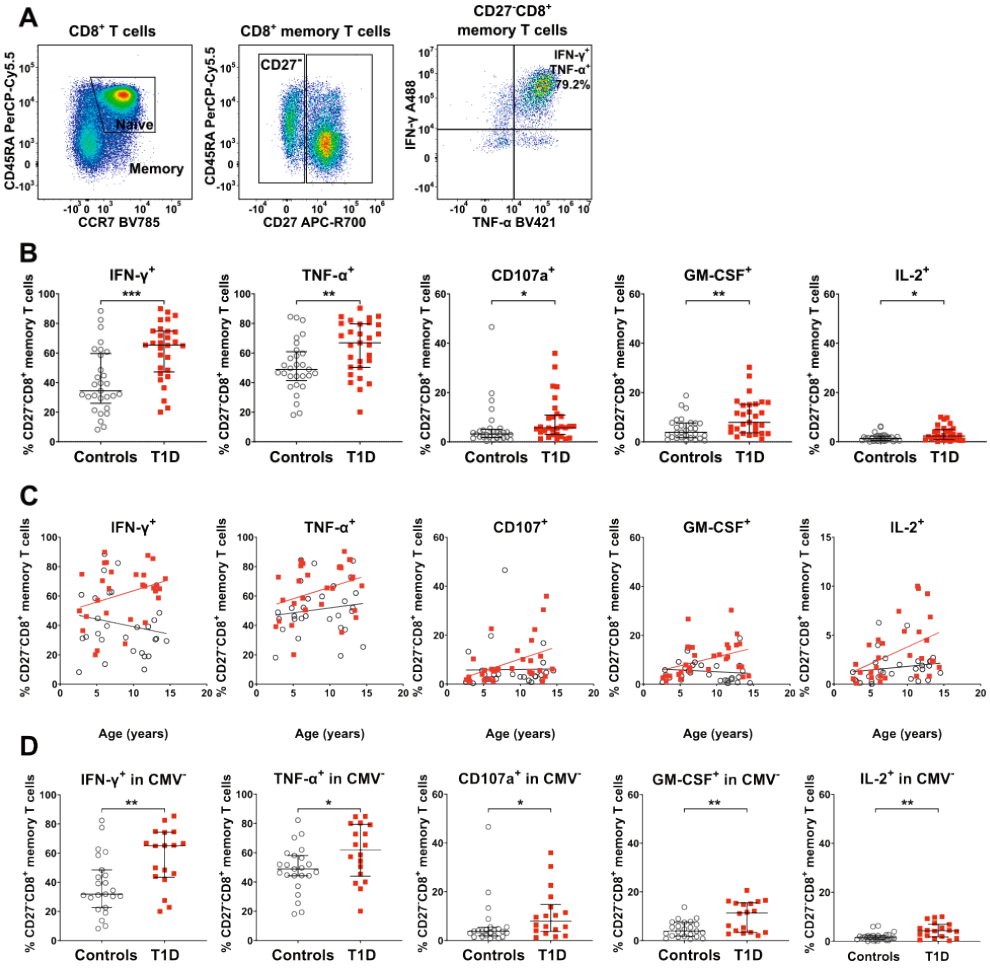

Figure 9. Frequencies of CD27$^-$CD8$^+$ memory T cells producing IFN-γ, TNF-α, CD107a, GM-CSF and IL-2 are increased in children with newly diagnosed T1D. (A) Representative gating strategy of CD27$^{+/-}$CD8$^+$ memory T cells. Naïve (CD45RA$^+$CCR7$^+$) CD8$^+$ T cells were excluded and only CD8$^+$ memory T cells were analyzed according to CD27 expression. Frequencies of IFN-γ$^+$, TNF-α$^+$, CD107a$^+$, GM-CSF$^+$, and IL-2$^+$ CD27$^-$CD8$^+$ memory T cells in children with T1D and controls (B). Linear regression analyses between age and the frequency of IFN-γ$^+$, TNF-α$^+$, CD107a$^+$, GM-CSF$^+$, and IL-2$^+$ CD27$^-$CD8$^+$ memory T cells in children with T1D (C). The elevations of the linear regression lines were significantly different between children with T1D and controls for IFN-γ$^+$ (p=0.0003), TNF-α$^+$ (p=0.0065) and IL-2$^+$ (p=0.0087) CD27$^-$CD8$^+$ memory T cells. Frequencies of CD107a and cytokines in in CD27$^-$CD8$^+$ memory T cells from CMV-seronegative (CMV-) children with T1D and healthy controls (D). Median and interquartile range (IQR) are shown in the figures. *P<0.05, **P<0.01, ***P<0.001.

As an increased frequency of $CD27^{-}CD8^{+}$ T cells expressing all the studied cytokines and CD107a was observed in children with T1D, we next investigated the polyfunctionality of the $CD27^{-}CD8^{+}$ T cells by analysing the frequency of cells co-expressing various combinations of proinflammatory cytokines and CD107a. A significantly higher frequency of IFN-γ^{+}TNF-α^{+} $CD27^{-}CD8^{+}$ T cells were observed alongside minor expansions of other combinations of cytokines and CD107a in children with T1D (Fig. 10A–B). AAb^{+} children, children before and after seroconversion or adults with established T1D did not show similar alterations (Fig. 10B). Next, polyfunctionality index (PI) (137), that represents the ability of T cells to co-produce multiple cytokines, was calculated. As expected, PI was significantly higher in children with T1D compared to controls. (Fig. 10C–D)

In summary, the signature consisting of highly differentiated proinflammatory $CD27^{-}CD8^{+}$ memory T cells was specific for newly diagnosed T1D disease stage in children. Corresponding alteration was also detected in $CD27^{-}CD4^{+}$ memory T cells indicating potentially a broader alteration in the memory T-cell compartment in children with newly diagnosed T1D.

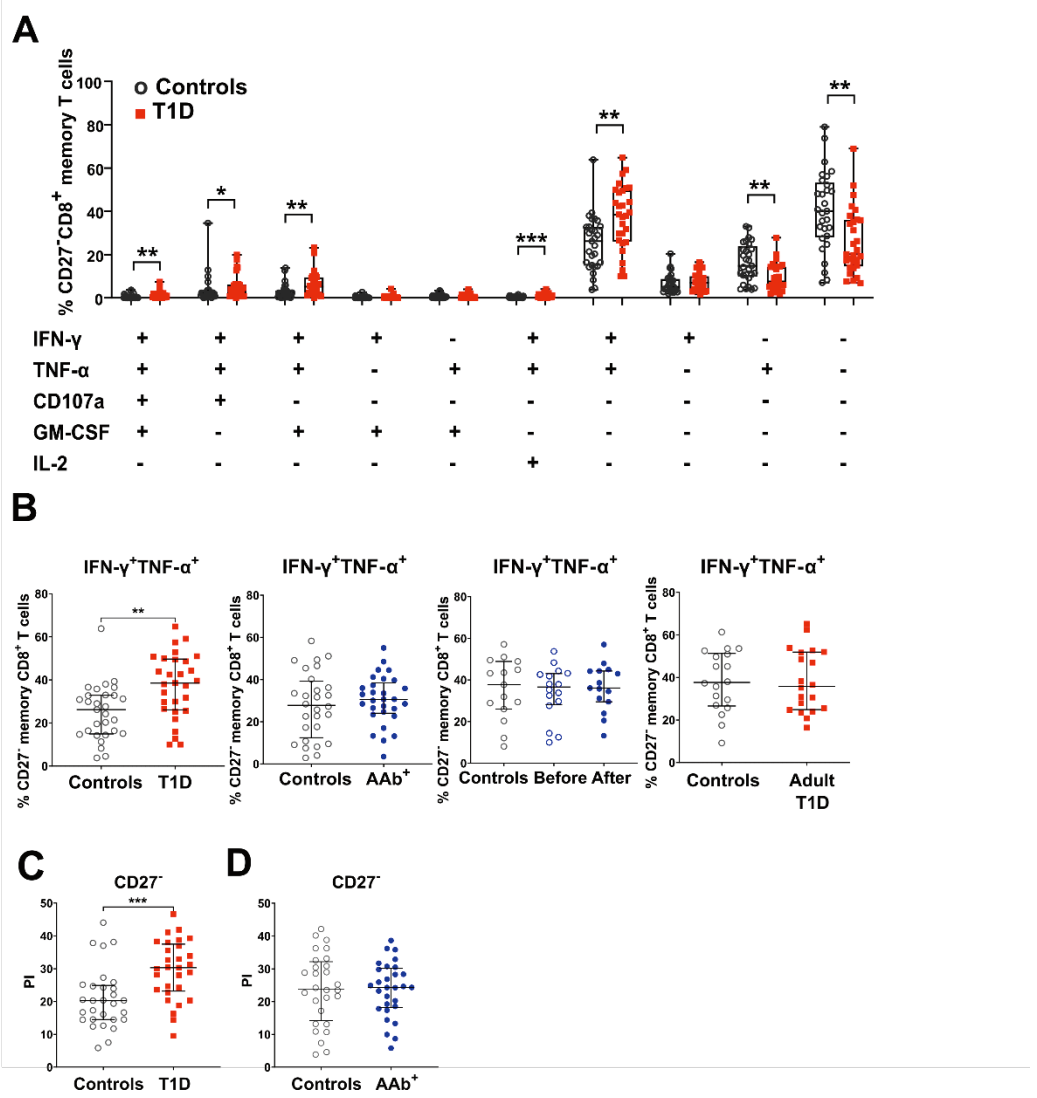

Figure 10. Polyfunctional IFN-γ$^+$TNF-α$^+$ CD27$^-$CD8$^+$ T cells are expanded in children with newly diagnosed T1D. Frequency of CD27$^-$CD8$^+$ memory T cells expressing various combinations of proinflammatory cytokines and CD107a (A). Frequency of IFN-γ$^+$TNF-α$^+$ CD27$^-$CD8$^+$ T cells in children with newly diagnosed T1D, AAb$^+$ children, children before and after seroconversion and adults with established T1D (B). Polyfunctionality index (PI) of CD27$^-$CD8$^+$ memory T cells in children with T1D and controls (C), and in AAb$^+$ children and controls (D). Median and interquartile range (IQR) are shown in the panels. *P<0.05, **P<0.01, ***P<0.001.

5.1.2 Expansion of CD27$^-$CD8$^+$ memory T cells co-expressing co-inhibitory receptors KLRG1 and TIGIT in autoantibody-positive children

Next, characterization of CD8$^+$ and CD4$^+$ memory T-cell phenotypes was conducted focusing on co-inhibitory receptor-expressing cells. KLRG1, TIGIT and PD-1 are co-inhibitory receptors that have been associated with immunotherapy treatment response and slower disease progression after disease onset T1D (2,46,95,97). Hence, we next studied expression of co-inhibitory surface receptors KLRG1, TIGIT, PD-1 and CD160 in the pediatric cohort (Article I, Suppl. Fig. 9). Additionally, the frequency of KLRG1$^+$TIGIT$^+$ CD8$^+$ T cells was examined as a highly similar phenotype has been associated both with slower progression of T1D after diagnosis as well as immunotherapy efficacies (2,46,95,97).

Naïve T cells were excluded from the analyses as before, and CD27$^+$ and CD27$^-$ memory T-cell subsets were subsequently analysed for the surface marker expression of the selected receptors (Fig. 11A). Interestingly, a significantly higher frequency of KLRG1$^+$TIGIT$^+$ CD27$^-$CD8$^+$ T cells was observed in AAb$^+$ children compared to controls. This alteration was not seen in children with T1D, in children before and after seroconversion or in adults with T1D. (Fig. 11B) After stratification with age and CVM serostatus in AAb$^+$ cohort, the results remained significant. (Fig. 11C–D)

Of note, the frequency of CD27$^-$CD8$^+$ and CD27$^-$CD4$^+$ memory T cells expressing co-inhibitory receptors (PD-1, CD160, KLRG1, TIGIT) were comparable (Article I, Suppl. Fig 10). Within the CD27$^+$ T-cell compartment, only higher frequency of PD-1$^+$ CD27$^+$CD8$^+$ and CD27$^+$CD4$^+$ were observed in children with AAb$^+$ compared to controls (Article I, Suppl. Fig. 12; II, Fig. 18A), a finding that is discussed more in chapter 5.2.6 that bridges the findings between projects I and II.

In conclusion, the signature consisting of co-inhibitory receptor expressing KLRG1$^+$TIGIT$^+$ CD27$^-$CD8$^+$ memory T cells was specific for AAb$^+$ children who later progressed to T1D.

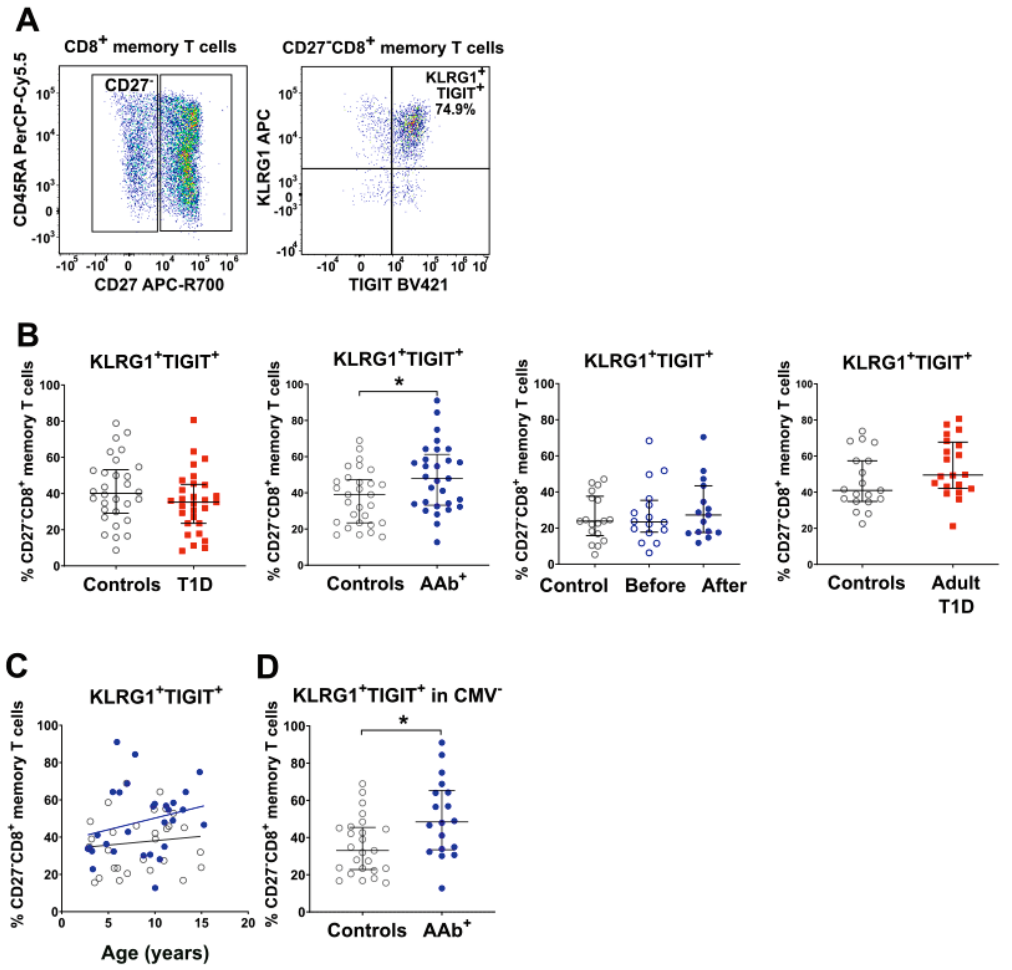

Figure 11. Frequency of CD27$^-$CD8$^+$ memory T cells co-expressing KLRG1 and TIGIT is increased in AAb$^+$ children that were later diagnosed with T1D. (A) Representative gating strategy of CD8$^+$ memory T cells. Naïve (CD45RA$^+$CCR7$^+$) CD8$^+$ T cells were excluded and only CD8$^+$ memory T cells were analyzed. Subsequently, memory T cells were gated according to CD27 expression. Frequencies of KLRG1$^+$TIGIT$^+$ CD27$^-$CD8$^+$ memory T cells in children with T1D, AAb$^+$ children, children before and after seroconversion, adults with T1D and controls (B). Linear regression analyses between age and KLRG1$^+$TIGIT$^+$ frequency within CD27$^-$CD8$^+$ T cells in AAb$^+$ children and controls (C). The elevations of the linear regression lines were significantly different between AAb$^+$ children and controls (p=0.0164). Frequencies of TIGIT$^+$KLRG1$^+$ CD27$^-$CD8$^+$ memory T cells in CMV-seronegative (CMV$^-$) AAb$^+$ children and controls (D). Median and IQR are shown in the panels B and D. Median and IQR are shown in the panels B and D. *P<0.05.

5.1.3 Proinflammatory cytokine expression in $CD27^-CD8^+$ memory T cells correlates with blood glucose levels at diagnosis in children with type 1 diabetes

Often co-inhibitory receptor expressing cells are considered to be less-functional than conventional memory T cells, or labeled even as exhausted. As we observed both proinflammatory and co-inhibitory signatures within the same rather rare subset of $CD27^-CD8^+$ T cells, it led us to ask whether there is any association between the signatures detected. Thus, a correlation analysis between the frequencies of proinflammatory cytokine and co-inhibitory receptor expressing $CD27^-CD8^+$ T cells was carried out.

Strong positive correlation was detected between the expression of proinflammatory cytokines and degranulation marker CD107a, and a separate positive correlation between the expression of different co-inhibitory receptors. Hierarchical clustering in the correlogram also clustered proinflammatory cells and co-inhibitory cells in separate areas in the correlogram. Of note, only a modest positive correlation was detected between the degranulation marker CD107a and $KLRG1^+TIGIT^+$ expressing cells. (Fig. 12A)

Finally, we also had clinical parameters (plasma glucose and β-hydroxybutyrate levels, with the latter indicative of the level of ketoacidosis, at diagnosis) available from 24 children with T1D. Plasma glucose levels at diagnosis (mmol/l) correlated positively with the frequency of IFN-γ^+, TNF-α^+ and GM-CSF^+ $CD27^-CD8^+$ memory T cells (Fig. 12B).

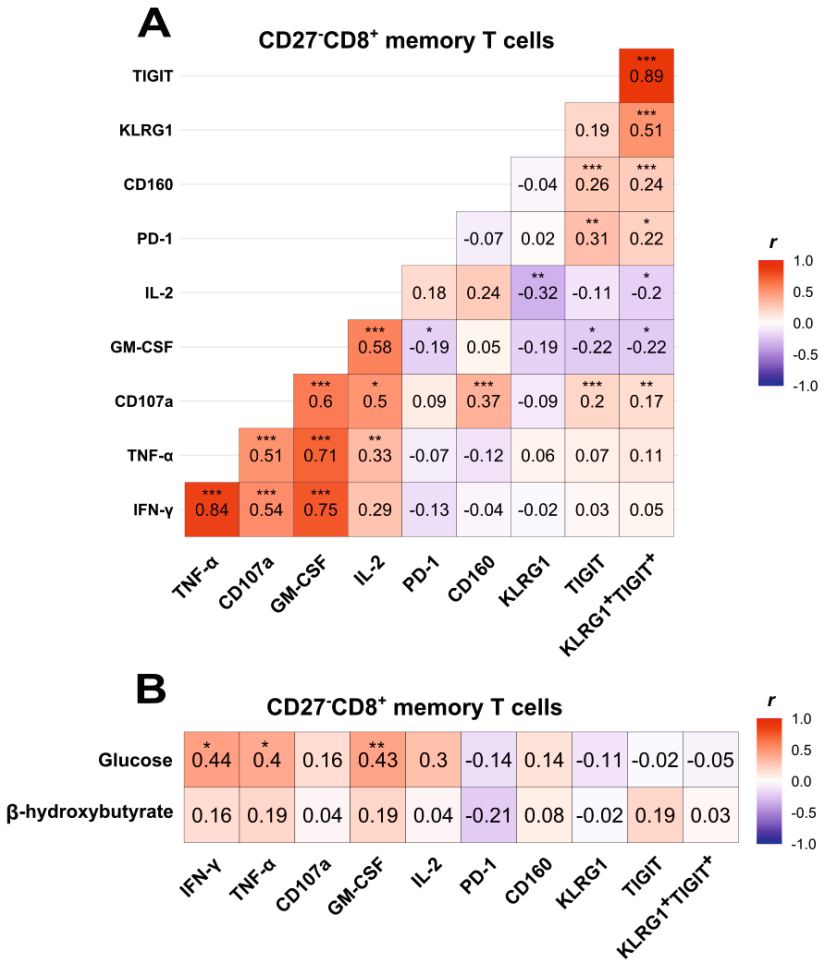

Figure 12. Plasma glucose levels at T1D diagnosis positively correlate with the frequency of CD27$^-$CD8$^+$ memory T cells expressing proinflammatory cytokines in children with T1D. Correlations of cytokine and co-inhibitory receptor expressing cells were examined using Spearman's correlation. Data from the whole pediatric cohort was pooled into the analysis (A). Correlation between plasma glucose levels (mmol/l) and plasma β-hydroxybutyrate (mmol/l) at T1D diagnosis and the frequency of CD27$^-$CD8$^+$ memory T cells was examined using Spearman's correlation in children with T1D (n=24) (B). Correlation analyses in A and B are depicted as correlograms where Spearman's r-values are presented within the squares, statistical significance between parameters is indicated as *P<0.05, **P<0.01, ***P<0.001 and color indicates positive (red hues) or negative (blue hues) correlation.

5.1.4 *IFNG, TNF, KLRG1* and *TIGIT* expression is co-localized with a subset of cytotoxic CD27$^-$CD8$^+$ memory T cells

The main findings in the flow cytometry data pointed at two signatures that consisted of proinflammatory cytokine expressing cells (Fig. 9) and co-inhibitory receptor expressing cells (Fig. 11) within the CD27$^-$CD8$^+$ T-cell subset. To further explore the molecular phenotypes of proinflammatory cytokine and co-inhibitory receptor expressing CD27$^-$CD8$^+$ T cells, a single-cell multiomics analysis was performed. For this purpose, CD27$^+$ and CD27$^-$ CD8$^+$ T cells were sorted and then shortly stimulated with PMA and ionomycin to induce mRNA expression of cytokines. Using this method, we profiled the mRNA expression of 475 immune genes together with 14 surface proteins utilizing the Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-Seq) approach.

Stimulated CD8$^+$ T cells clustered initially into six clusters. Four of the clusters consisted of CD27$^+$ naive and less differentiated memory CD8$^+$ T cells (clusters 1, 2, 3 and 4) and two cluster consisted of CD27$^-$ terminally-differentiated memory CD8$^+$ T cells (clusters 5 and 6) as indicated by the significant downregulation of CD27. (Fig. 13A–C)

As clusters 5 and 6 represented the CD27$^-$CD8$^+$ T cells of interest, the analysis was focused on on these clusters. Cluster 5 represented cytotoxic Teff CD8$^+$ T cells, as upregulation of *GZMB*, *GNLY* and *PRF1* (encoding cytolytic proteins granzyme B, granulysin and perforin, respectively) were observed. These cells also expressed *IFNG* and *TNF*. A smaller subset of cells within the cluster 5 also expressed *KLRG1*, *TIGIT* and *EOMES*. Cluster 6 represented TEMRA CD8$^+$ T cells, and they expressed CD45RA and CX3CR1. In accordance with generally acknowledged TEMRA phenotypes, the cells in the cluster 6 exhibited also a cytotoxic potential as upregulation of *GZMB* and *PRF1* were observed. *IFNG*, *TNF*, *KLRG1* or *TIGIT* were, however, not significantly upregulated within this cluster. (Fig. 13A–C)

Therefore, it appears that cluster 5 mirrors the phenotypes observed in the flow cytometry data on CD27$^-$CD8$^+$ T cells (Fig. 9 and Fig. 11). Next, cluster 5 was re-clustered for further examination of *IFNG*, *TNF*, *KLRG1* and *TIGIT* expression at a single-cell level. Five subclusters (clusters 5A–E) were

obtained using this approach. *IFNG* and *TNF* were co-expressed within cluster 5A, whereas *KLRG1* and *TIGIT* alongside transcripts for co-inhibitory receptors *CD160* and *KIR2DL1,* were observed within a separate cluster 5B. This observation could indicate that the proinflammatory and co-inhibitory signals originate from the same cluster of cells, but could potentially reflect distinct phenotypes, as *IFNG* and *TNF*, and *KLRG1* and *TIGIT* expression localized within different cells at the single-cell level. (Fig. 13D–E)

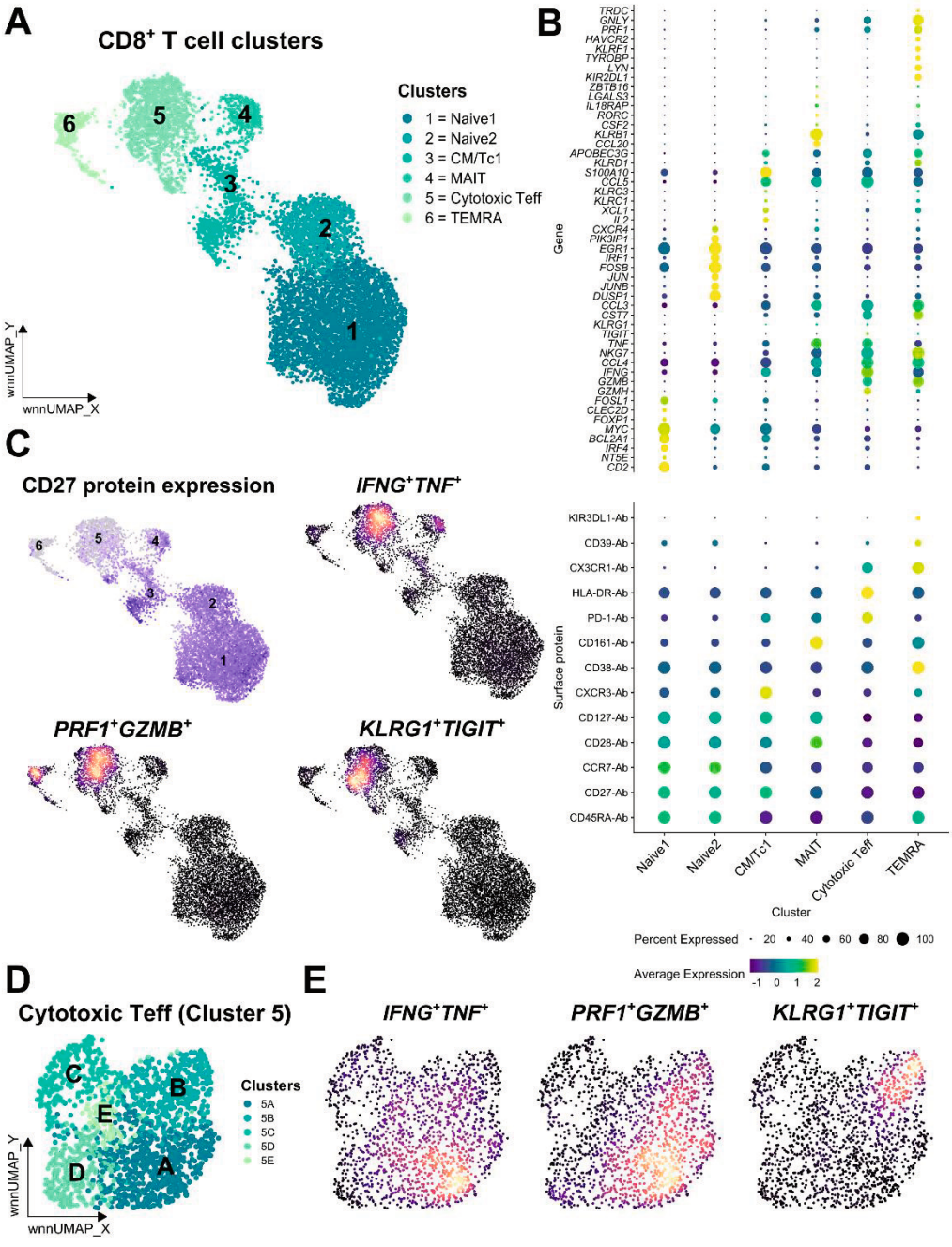
A
CD8⁺ T cell clusters
Clusters
1 = Naive1
2 = Naive2
3 = CM/Tc1
4 = MAIT
5 = Cytotoxic Teff
6 = TEMRA
wnnUMAP_Y
wnnUMAP_X
B
Gene
Surface protein
KIR3DL1-Ab
CD39-Ab
CX3CR1-Ab
HLA-DR-Ab
PD-1-Ab
CD161-Ab
CD38-Ab
CXCR3-Ab
CD127-Ab
CD28-Ab
CCR7-Ab
CD27-Ab
CD45RA-Ab
Naive1
Naive2
CM/Tc1
MAIT
Cytotoxic Teff
TEMRA
Cluster
Percent Expressed
Average Expression
C
CD27 protein expression
IFNG⁺TNF⁺
PRF1⁺GZMB⁺
KLRG1⁺TIGIT⁺
D
Cytotoxic Teff (Cluster 5)
Clusters
5A
5B
5C
5D
5E
E
IFNG⁺TNF⁺
PRF1⁺GZMB⁺
KLRG1⁺TIGIT⁺

Figure 13. Expression of *IFNG, TNF, KLRG1* and *TIGIT* is enriched within the $CD27^{-}CD8^{+}$ memory T-cell cluster exhibiting a cytotoxic signature. (A) WNN UMAP projection of $CD8^{+}$ T cells that clustered into six canonical $CD8^{+}$ T-cell subsets. Dot plot of expression of selected differentially expressed genes and 14 surface proteins (Ab) within the different $CD8^{+}$ T-cell clusters (B). CD27 protein (Ab) expression and co-expression of *IFNG* and *TNF*, *PRF1* and *GZMB*, and *KLRG1* and *TIGIT* within the clusters (C). WNN UMAP projection of reclustering of the cytotoxic Teff cluster (cluster 5) (D). Co-expression of *IFNG* and *TNF*, *PRF1* and *GZMB*, and *KLRG1* and *TIGIT* within the subclusters (E).

5.2 EXPANSION OF CIRCULATING FOLLICULAR AND PERIPHERAL T HELPER CELLS IS ASSOCIATED WITH DISEASE PROGRESSION RATHER THAN EARLY AUTOIMMUNITY IN TYPE 1 DIABETES (II)

5.2.1 Frequency of IL-21-producing $CD4^{+}$ T cells is increased in children with type 1 diabetes

Various $CD4^{+}$ T-cell subsets have been altered during the progression of T1D, and lot more focus have been placed upon studying $CD4^{+}$ T cells than $CD8^{+}$ T cells. The natural explanation for this is that $CD4^{+}$ T cells are required to orchestrate the responses of other T-cell subsets. In project I, the focus was mainly on $CD8^{+}$ T cells, but $CD4^{+}$ T cells were also studied in parallel. In the project I, IL-21-expressing TCM $CD4^{+}$ T cells were slightly expanded in children with T1D (Article I, Suppl. Fig. 8). This increase in IL-21-expressing cells likely originates from $CD4^{+}$ B-cell helper T-cells, cTfh and cTph cells, that we characterized in detail in project II.

5.2.2 $CXCR5^{-}PD\text{-}1^{hi}$ cTph cells are expanded in autoantibody-positive children and in children with type 1 diabetes

The importance of studying $CD4^{+}$ B-cell helper T cells is highlighted by the previous findings of increased frequencies of circulating Tfh and Tph cells in patients with T1D and AAb^{+} prediabetic individuals (81–83), but the exact

timing of the expansion is not well-defined. In this study characterization of the frequencies and phenotypes of CXCR5$^-$PD-1^{hi} and CXCR5$^+$PD-1^{hi} fractions consisting of cTph and cTfh cells, respectively, at different stages of T1D development was carried out, the relationship and heterogeneity of the cTph and PD-1^{hi} expressing cTfh phenotypes were investigated and the timing of the alterations in the frequencies of these cells were addressed.

First, the frequencies and phenotypes of CXCR5$^-$PD-1^{hi} cTph and CXCR5$^+$PD-1^{hi} cTfh cells (Fig. 14A) at the different stages of T1D were analyzed. 27 children with newly diagnosed T1D, 29 AAb$^+$ children who later developed T1D as well as 57 matched healthy controls were analysed. Both children with newly diagnosed T1D and AAb$^+$ children had a higher frequency of cTph cells compared to healthy, age-matched controls (Fig. 14B–C). In addition, CXCR5$^+$CD45RA$^-$PD-1$^+$ICOS$^+$ activated cTfh cells were increased in children with newly diagnosed T1D compared to healthy controls (Fig. 14D). These above-mentioned findings validate the previous findings by our group (81,83).

Next, the expression of 18 phenotypical surface markers by CXCR5$^-$PD-1^{hi} and CXCR5$^+$PD-1^{hi} fractions containing cTph and cTfh cells, respectively, as well as by their PD-1 low-expressing counterparts (CXCR5$^{-/+}$PD-1^{lo}) were studied in the pediatric study groups. In a principal component analysis (PCA), the cells clustered according to the cell subsets rather than the study groups (Fig. 14E). As already indicated by the PCA projection, no differences were detected in the phenotypes of CXCR5$^-$PD-1^{hi} cTph and CXCR5$^+$PD-1^{hi} cTfh between the study groups (Article II, Suppl. Fig. 6–9).

To understand the similarities and differences of CXCR5$^{+/-}$PD-1$^{hi/lo}$ cells, comparison of surface marker expression patterns were performed in these fractions. All study groups were pooled in to the analysis. The comparison between CXCR5$^-$PD-1^{hi} cTph and CXCR5$^+$PD-1^{hi} cTfh fractions showed significantly higher expression of CD226, CCR6, CD39, CD57, CCR5, HLA-DR and CCR2 in cTph compared cTfh fraction. Conversely, higher expression of CD27, CD84, CD38, β7 integrin, CCR9, CXCR3, TIGIT and CD200 were observed in cTfh cells compared to cTph cells. (Fig 14F; Article II, Suppl. Table 7) In addition, the expression of five surface receptors (CD84, CD38, TIGIT, CD200 and ICOS) were significantly higher, and CCR7

was significantly lower in both CXCR5^{-}PD-1^{hi} cTph and CXCR5^{+}PD-1^{hi} cTfh fractions compared to their PD-1^{lo} counterparts (Fig. 14F; Article II, Suppl. Table 5–6).

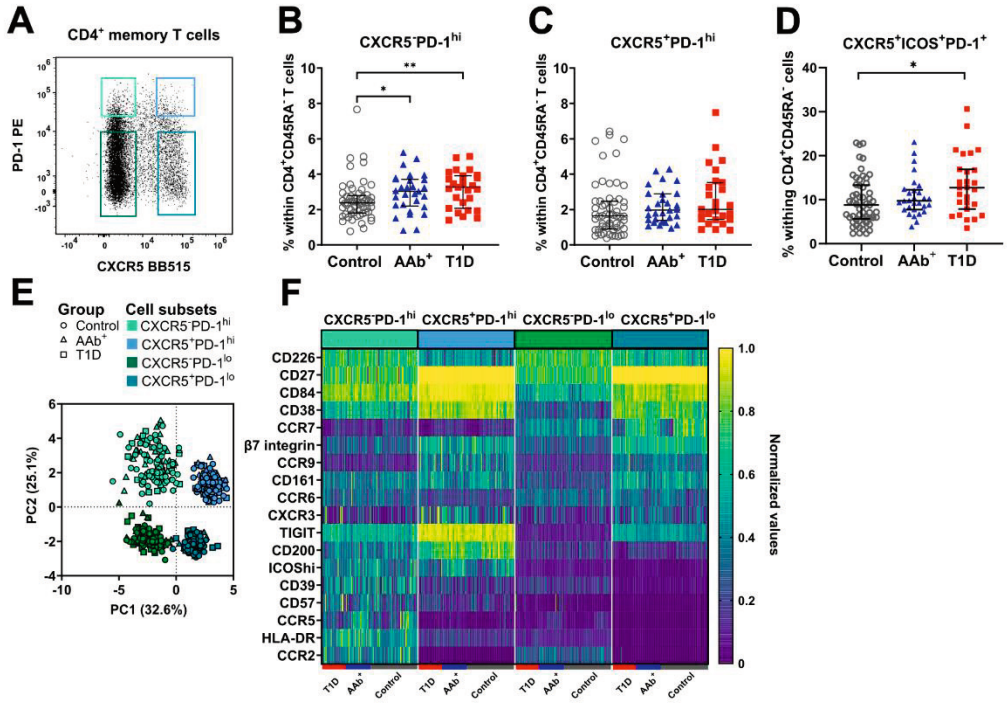

Figure 14. CXCR5^{-}PD-1^{hi} cTph cells are expanded in AAb^{+} children and children with T1D. (A) Representative gating of the flow cytometry data depicting CXCR5$^{-/+}$PD-1$^{hi/lo}$ fractions. Frequency of CXCR5^{-}PD-1^{hi} cTph (B), CXCR5^{+}PD-1^{hi} cTfh (C) and CXCR5^{+}PD-1^{+}ICOS^{+} activated cTfh in controls, AAb^{+} children and children with newly diagnosed T1D. PCA projection of phenotypical flow cytometry data (E). Heatmap of 18 surface marker expression in CXCR5$^{-/+}$PD-1$^{hi/lo}$ fractions (F). *P<0.05, **P<0.01.

5.2.3 CXCR5^{-}PD-1^{hi} and CXCR5^{+}PD-1^{hi} fractions contain cells expressing *IL21* and *CXCL13* consistent with B-cell help function

To further study the molecular phenotypes of cTph and cTfh fractions, targeted gene expression analysis in parallel with 14 surface protein and TCR repertoire analyses using BD Rhapsody Single-Cell Analysis system was

conducted. $CD4^+$ memory T cells were isolated from three children with newly diagnosed T1D and three age-matched healthy controls. The cells were stimulated for 90 min with PMA and ionomycin to allow the detection of cytokine transcripts. First, we studied $CXCR5^{-/+}PD\text{-}1^{hi/lo}$ fractions through a pseudobulk analysis (Fig. 15A–B) where the fractions mirror the ones presented in the flow cytometry analyses (Fig. 14A–B).

In the pseudobulk analyses, $CXCR5^{-}PD\text{-}1^{hi}$ cTph and $CXCR5^{+}PD\text{-}1^{hi}$ cTfh fractions were irst compared. $CXCR5^{-}PD\text{-}1^{hi}$ cTph fraction demonstrated an upregulated expression of *CCL4, CCL3, GZMA, IFNG, CCL20, CSF2, IL13, GNLY* and *IL22* compared to $CXCR5^{+}PD\text{-}1^{hi}$ fraction (Fig. 14; Article II, Suppl. Table 10). When comparing $CXCR5^{-}PD\text{-}1^{hi}$ cTph and $CXCR5^{+}PD\text{-}1^{hi}$ cTfh fractions to the corresponding $PD\text{-}1^{lo}$ fractions higher expression of *IL21, CXCL13, TIGIT, CCL4, CCL3, CD200, GZMK* and *EOMES* were seen in $CXCR5^{-}PD\text{-}1^{hi}$ cTph and $CXCR5^{+}PD\text{-}1^{hi}$ cTfh fractions (Fig. 14C; Article II, Suppl. Table 8–9). Of note, no major differences were observed between children with T1D and healthy controls in the pseudobulk analyses, with an exception of upregulated *IL5* expression within $CXCR5^{-}PD\text{-}1^{hi}$ cTph fraction of children with T1D (Fig. 14C; Article II, Suppl. Table 11). The comparison between children with T1D and controls, however, lacks statistical power due to the number of indviduals in the analyses (n=3 for both T1D and controls) so these findings have to be considered preliminary.

To examine clonal sharing between the $CXCR5^{-/+}PD\text{-}1^{hi/lo}$ fractions, TCR repertoire was analyzed in the single-cell multiomics dataset. 126 expanded clones (defined as ≥ 2 similar TCRs) were detected, all clonal expansions were private, i.e. observed within T cells from a single individual. (Article II, Suppl. Table 10) TCR sharing was the most commonly detected between $CXCR5^{-}PD\text{-}1^{hi}$ and $CXCR5^{-}PD\text{-}1^{lo}$ fractions (16 clones), and between $CXCR5^{-}PD\text{-}1^{hi}$ and $CXCR5^{+}PD\text{-}1^{hi}$ fractions (13 clones; Fig. 14D; Article II, Suppl. Table 12). This data suggests that $CXCR5^{-}PD\text{-}1^{hi}$ cTph cells may be clonally related both with $CXCR5^{+}PD\text{-}1^{hi}$ cTfh cells as well as with $CXCR5^{-}PD\text{-}1^{1o}$ cells.

To summarize, the pseudobulk analysis of the single-cell data demonstrated that both $CXCR5^{-}PD\text{-}1^{hi}$ and $CXCR5^{+}PD\text{-}1^{hi}$ fractions contain cells that have upregulated *CXCL13* and *IL21*, the hallmark chemokine and

cytokine produced by Tfh and Tph cells, consistent with a B-cell helper function. However, within CXCR5^{-}PD-1^{hi} cTph fraction, upregulation of proinflammatory chemokines and cytokines were also detected in comparison to the CXCR5^{+}PD-1^{hi} cTfh fraction. The findings in the pseudobulk approach (Fig. 15) corroborated our results with the flow cytometry approach (Fig. 14).

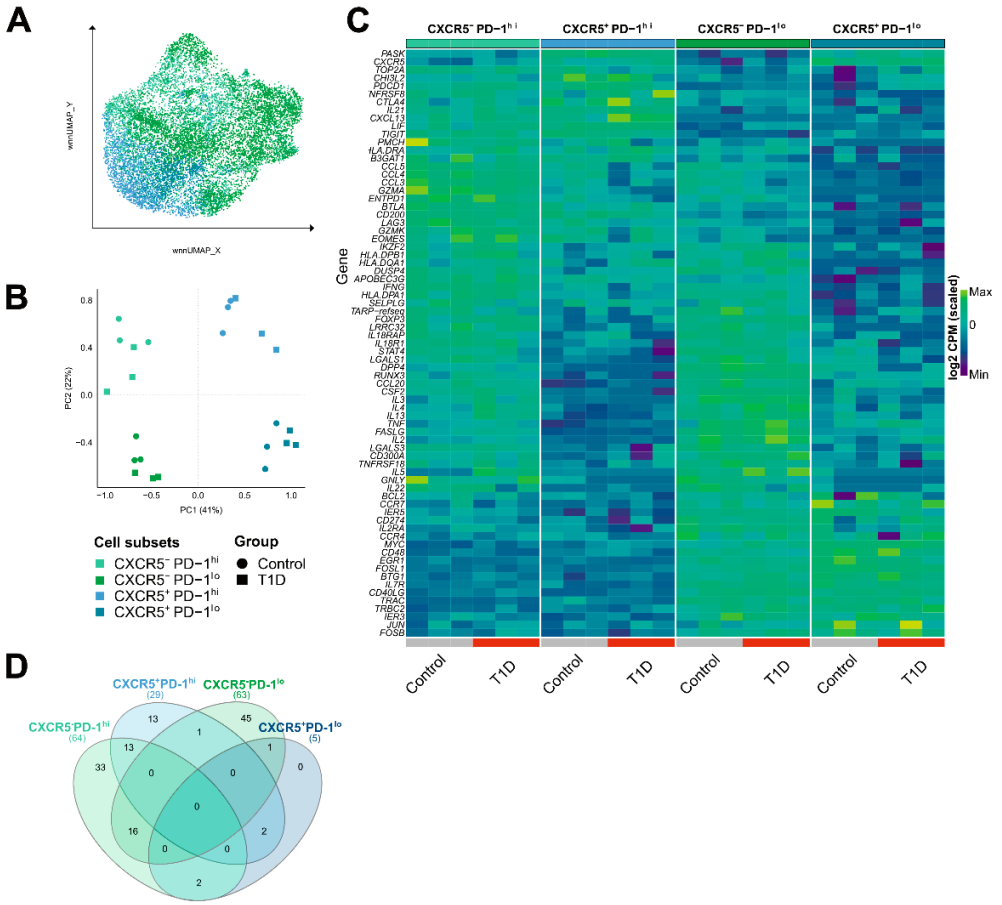

Figure 15. CXCR5^{-}PD-1^{hi} and CXCR5^{+}PD-1^{hi} fractions express genes associated with B-cell helper T cell function. (A) WNN UMAP projection with pseudobulk CXCR5$^{-/+}$PD-1$^{hi/lo}$ fractions overlaid in it. (B) PCA plot visualizing CXCR5$^{-/+}$PD-1$^{hi/lo}$ fractions and sample groups. (C) Heatmap of significantly differentially expressed genes in CXCR5$^{-/+}$PD-1$^{hi/lo}$ fractions. (D) TCR sharing is mostly observed between CXCR5^{-}PD-1^{hi} and CXCR5^{-}PD-1^{lo}, or CXCR5^{-}PD-1^{hi} and CXCR5^{+}PD-1^{hi} CD4^{+} T cells. The total number of expanded clones detected within each cell fraction is indicated in parentheses.

5.2.4 CXCR5^{-}PD-1^{hi} fraction exhibits substantial heterogeneity at the single- cell level

The single-cell analyses above present an analysis approach where we rely on known markers and marker combinations depicting cTph and cTfh cells. Next, an unsupervised clustering and differential expression analysis encompassing CXCR5$^{-/+}$PD-1$^{hi/lo}$ cells was performed. (Article II, Fig. 2A)

All major T-helper cell populations, such as Th1, Th2, Th17 cells and Tregs were identified with this approach (clusters 1–11 Fig. 16A; Article II, Suppl. Table 13–14). Cluster 1 appeared to represent cTfh cells, as indicated by the upregulation of CXCR5, *ICOS* and *IL21*, and cluster 3 cTph cells described in the literature (107), as indicated by the upregulation of *IL21*, *PDCD1*, *ICOS* and CD38 and downregulation of CXCR5. (Fig. 16B–D; Article II, Suppl. Table 13–14)

Next, the frequencies of the 11 clusters within the four CXCR5$^{+/-}$PD-1$^{hi/lo}$ fractions were analyzed. As one would expect, the majority of the cells within the CXCR5^{+}PD-1^{hi} fraction belonged to cluster 1 (cTfh). Surprisingly, in contrast, the composition of the CXCR5^{-}PD-1^{hi} fraction exhibited a much greater diversity as the CXCR5^{-}PD-1^{hi} fraction contained cells from several other clusters, especially enriching with proinflammatory and/or activated cell subsets in addition to the cluster 3 representing “classical” cTph phenotype. For example, Tregs (upregulation of *FOXP3,* cluster 6), Th1 cells (upregulation of *CXCR3*, *IFNG* and *GZMK,* cluster 7), and CD4^{+} CTLs cells (upregulation of *GZMB, NKG7* and *GNLY, c*luster 10) were enriched within the CXCR5^{-}PD-1^{hi} fraction. (Fig. 16E)

Taken together, the unsupervised clustering of the single-cell data demonstrates that cTph and cTfh characteristics localize, as expected, within the CXCR5^{-}PD-1^{hi} and CXCR5^{+}PD-1^{hi} fractions, respectively. However, while the CXCR5^{+}PD-1^{hi} fraction consists mainly of cTfh cells, the CXCR5^{-}PD-1^{hi} fraction, surprisingly, contains a variety of effector CD4^{+} T cell phenotypes in addition to the “classical” cTph cells.

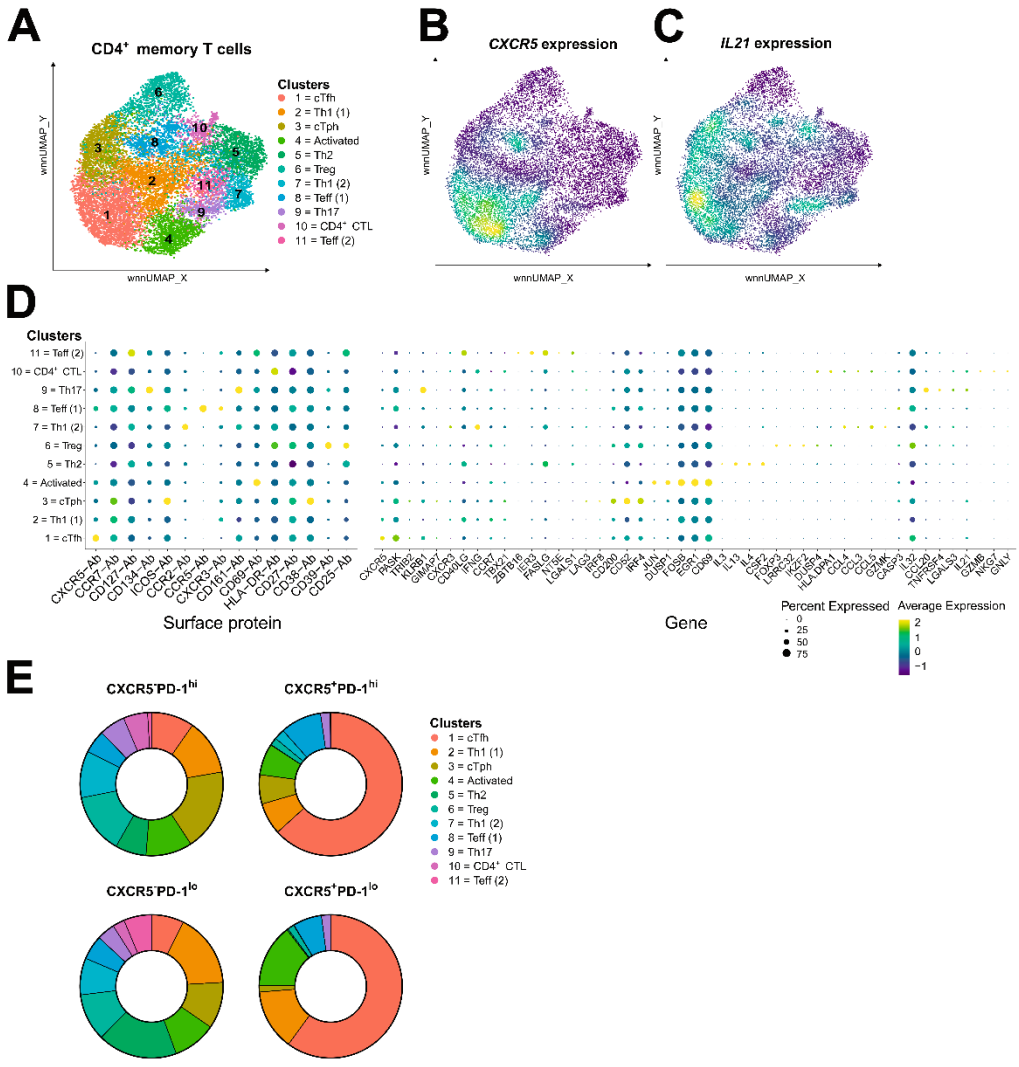

Figure 16. Heterogeneity of CXCR5^{-}PD-1^{hi} CD4^{+} T cells at the single-cell level. (A) 11 identified clusters overlaid in WNN UMAP projection. Expression of CXCR5 protein (Ab) (B) and *IL21* gene (C) within the WNN UMAP. (D) Dotplot of 15 surface proteins (Ab) expression and top 5 unique differentially expressed genes per clusters. (E) Distribution of cells from each cluster in the four CXCR5$^{+/-}$PD-1$^{hi/lo}$ cell fractions depicted as pie charts.

5.2.5 The expansions of cTph and cTfh cells are associated with later progression towards type 1 diabetes rather than seroconversion to autoantibody-positivity

Finally, to scrutinize the timing of the expansion of cTph and cTfh cells, the frequency of CXCR5$^-$PD-1^{hi} cTph, CXCR5$^+$PD-1^{hi} cTfh and CXCR5$^+$ICOS$^+$PD-1$^+$ activated cTfh cells were analysed in longitudinal samples collected from children before (n=17) and after (n=16) seroconversion to AAbs (collected 3–13 months apart), a time point believed to reflect the initiation of autoimmunity. However, no differences were observed between children before and after seroconversion compared to heatlhy children (Fig.17A–C), or in a pairwise-analysis of children before or after seroconversion who later developed T1D (progressors, 11 children) (Article II, Fig. 5).

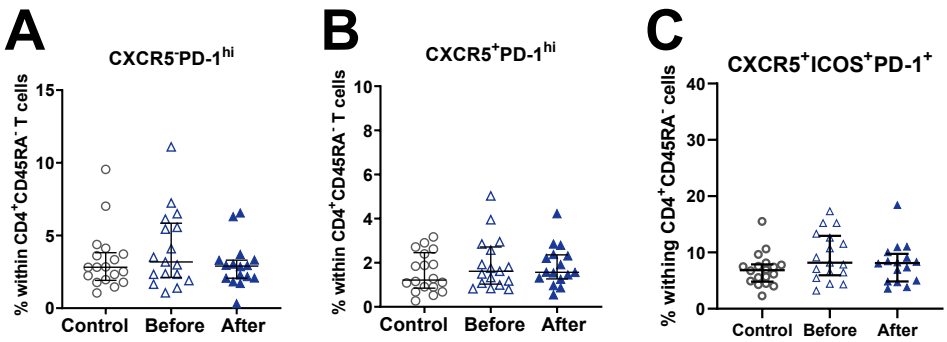

Figure 17. Comparable frequencies of CXCR5$^-$PD-1^{hi}, CXCR5$^+$PD-1^{hi}, activated cTfh CXCR5$^+$PD-1$^+$ICOS$^+$ T cells in children before and after seroconversion. Frequencies of CXCR5$^-$PD-1^{hi} (A), CXCR5$^+$PD-1^{hi} (B) and activated cTfh CXCR5$^+$PD-1$^+$ICOS$^+$ (C) cells in controls, and children before and after seroconversion.

5.2.6 PD-1-expressing CD27$^+$CD8$^+$ T cells are expanded in autoantibody-positive children who later progressed to type 1 diabetes

An interesting recurring theme between project I and II can be noted. When studying CD8$^+$ and CD4$^+$ T-cell subsets in project I, an increased frequency of PD-1 expressing CD27$^+$CD8$^+$ and CD27$^+$CD4$^+$ T cells were

detected in AAb^+ children compared to controls (Fig. 18A). It could be envisioned that the latter finding is explained by the increased frequencies of PD-1 expressing $CD4^+$ cTph and cTfh cells observed in project II. However, the finding that PD-1^+ $CD27^+CD8^+$ T cells were also increased would implicate the whole PD-1 expressing T-cell compartment being altered in AAb^+ children.

To examine potential associations indirectly between the cTph, cTfh and $CD8^+$ T cells, correlation analyses were performed between selected cell subsets by pooling the data from AAb^+ children and healthy controls examined in both projects I and II. cTph, but not cTfh, frequency (project II) signifcantly positively correlated with the PD-1^+ $CD27^+CD8^+$ frequency (project I). As $KLRG1^+TIGIT^+$ $CD27^-CD8^+$ cells were expanded in AAb^+ children in project I, the association between $KLRG1^+TIGIT^+$ expressing cells, cTph and cTfh cells was also examined. $KLRG1^+TIGIT^+$ expressing $CD8^+$ T cells did not show any correlation with cTph and cTfh cells. Conversely, $KLRG1^+TIGIT^+$ memory $CD4^+$ T cells positively correlated with cTph and cTfh frequencies. (Fig. 18B)

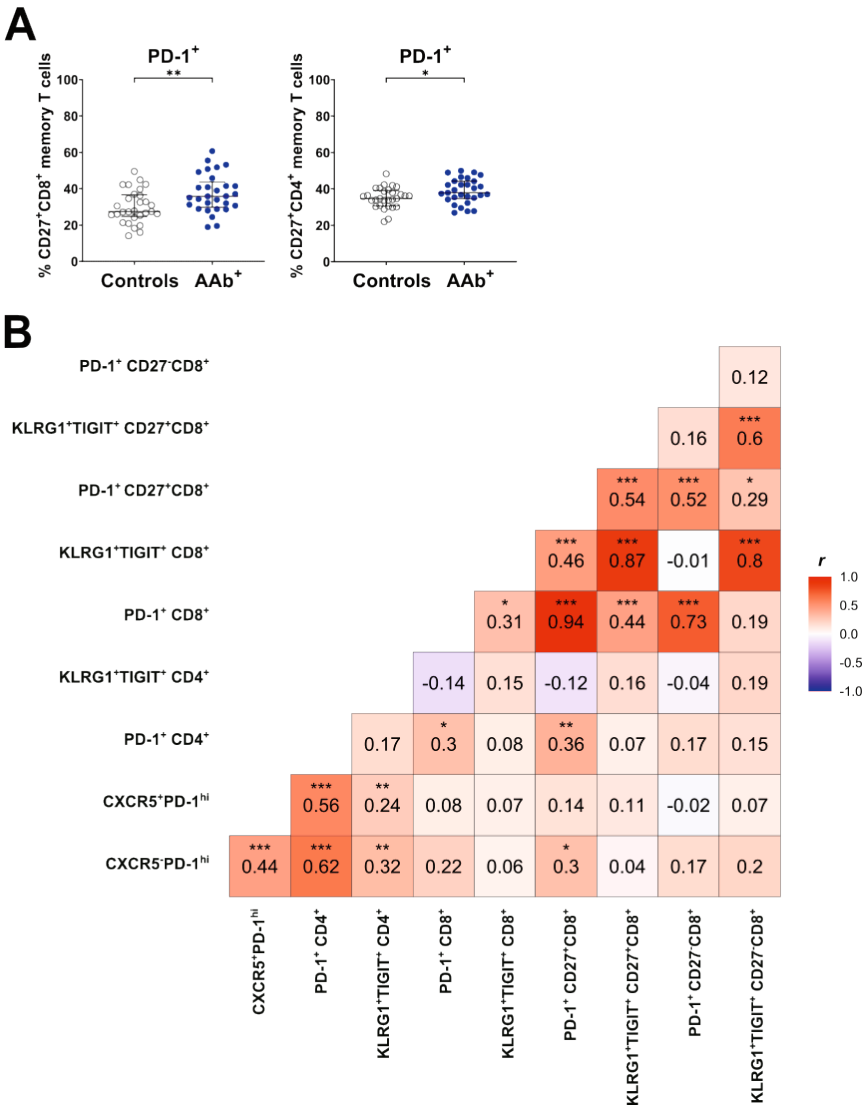

Figure 18. cTph, but not cTfh, frequency positively correlates with PD-1$^+$ CD27$^+$CD8$^+$ T cells. Frequency of PD-1$^+$ CD27$^+$CD8$^+$ and CD27$^+$CD4$^+$ memory T cells in AAb$^+$ children (A). (B) Spearman's correlation analyses were performed within AAb$^+$ cohort consisting of samples fro healthy controls (n=29) and AAb$^+$ children (n=28) on which both CD8 and cTph/cTfh stainings were performed. Correlogram with selected cell subsets is presented, red hues indicate positive correlation and blue hues negative correlation. Spearman's r-values are presented in squares. *P<0.05, **P<0.01, ***P<0.001. hclust function was used to assign the order of parameters in the correlogram.

5.3 EVALUATION OF PLASMA IL-21 AS A POTENTIAL BIOMARKER FOR TYPE 1 DIABETES PROGRESSION (III)

As indicated above, IL-21 producing $CD4^+$ T cells are more frequent in the circulation of AAb^+ children or children with T1D compared to healthy controls (II, Fig. 14). Hence, it was next investigated whether plasma IL-21 levels could serve as a biomarker for T1D progression. In order to evaluate the biomarker potential of plasma IL-21 levels during the development of T1D, we measured plasma levels of IL-21 alongside IL-17A, TNF-α and IL-6 levels in cross-sectional samples collected from individuals at different stages of T1D progression using an ultrasensitive SiMoA Quanterix assay.

5.3.1 Plasma IL-21 levels are not altered during the progression of pediatric type 1 diabetes

Plasma IL-21 levels were measured from 53 children with newly diagnosed T1D and 48 AAb^+ at-risk children, as well as from 123 healthy age-matched controls (Article III, Table 2). Plasma IL-21, as well as IL-17A, TNF-α and IL-6, levels were comparable between the study groups (Figure 19A; Article III, Fig. 2). Importantly, plasma IL-21 levels were ten-fold higher than in adults (median 0.26 pg/mL for the pooled pediatric cohort vs. median 0.03 pg/mL in adults). Accordingly, a negative correlation was observed between age and IL-21 levels in children. However, no differences were observed between the pediatric study groups even after stratification with age. (Fig. 19B)

In addition, to determine whether differences in IL-21 levels could be observed in a subset of study groups, AAb^+ at-risk children and children with T1D were assigned into two groups according to the number of autoantibodies detected (≤1 or ≥2). No differences were observed between the groups (Figure 19C–D). We retrospectively analyzed also whether progression to T1D in AAb^+ at-risk children had an effect on IL-21 levels. Plasma IL-21 levels were again comparable between AAb^+ at-risk children who were progressors (mean time to T1D after sampling 2.6 ± 1.4 years) or non-progressors to T1D (Fig. 12E). We observed no differences on IL-17A, TNF-α or IL-6 levels on either of the analyses (Article III, Suppl. Fig.2).

Finally, we retrospectively analysed correlation between plasma IL-21 levels and frequencies of IL-21 producing T cell subsets cTfh and Th17 cells, and also Tregs, since we had flow cytometry data originating from same sampling date as the plasma samples available (52,76, respectively) originating from same sampling date as the plasma samples. As expected, a significant positive correlation between activated cTfh cells ($CXCR5^+PD\text{-}1^+ICOS^+$ memory $CD4^+$ T cells) and plasma IL-21 levels were observed (Fig. 19A–B) indicating that possibly plasma IL-21 levels reflect Tfh activation *in vivo*. No correlation between Th17 frequencies ($CCR6^+CXCR3^-$ of memory $CD4^+$ T cells) and plasma IL-21 level was observed, and only a weak positive correlation was observed between Treg frequencies ($CD25^+CD127^{low}$ of $CD4^+$ T cells) and IL-21 levels. (Article III, Suppl. Fig. 6).

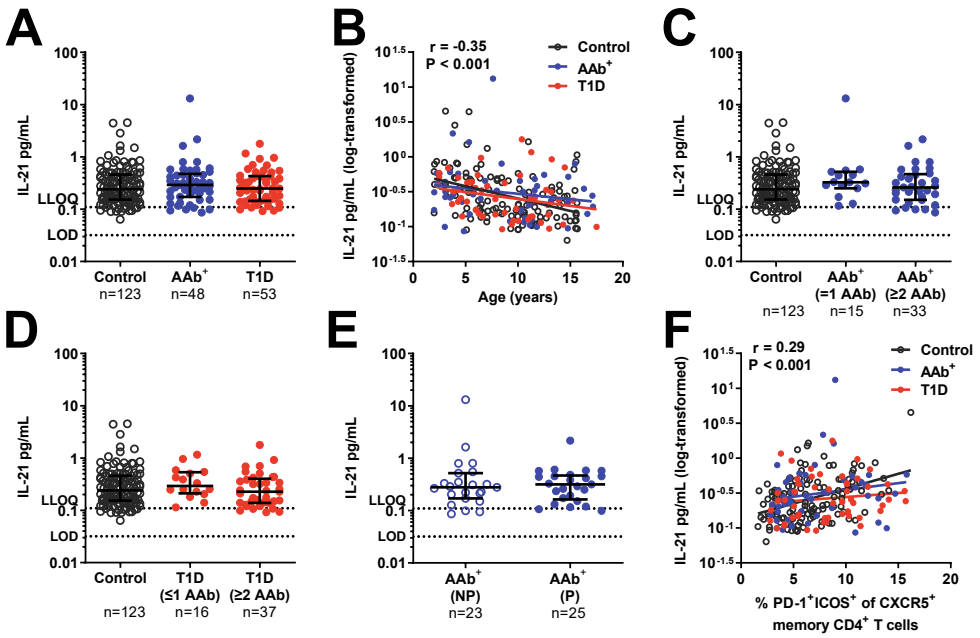

Figure 19. Plasma IL-21 levels in children with newly diagnosed T1D, AAb$^+$ at-risk children and healthy age-matched controls (A). (B) Spearman's correlation (r) was used to examine the correlation between age and plasma IL-21 levels. The data from all study groups were pooled into the analysis. r- and P-values from the correlation analysis are presented in the plot. AAb$^+$ at-risk children (C) and children with T1D (D) were assigned into two subgroups according on the number of AAbs detected at sampling (positive for ≤1 AAbs and ≥2 AAbs). (E) AAb$^+$ children were separated into non-progressor (NP) and progressor (P) subgroups depending on if the children had later progressed to T1D during follow-up (E). (F) Correlation between the % circulating activated cTfh (CXCR5$^+$PD-1$^+$ICOS$^+$ memory CD4$^+$ T cells) and plasma IL-21 levels was studied using Spearman's correlation (r). Data was pooled from all study groups. Medians with IQRs are shown, and Lower limit of detection (LOD) and Lower limit of quantification (LLOQ) are represented with dotted lines in the panels A, C–E.

5.3.2 Plasma IL-21 level is elevated in adults with established type 1 diabetes

Plasma IL-21 levels were also analysed in 37 adults with established T1D and in 47 age-matched healthy controls (Article III, Table 1). Plasma IL-21 levels were significantly elevated in T1D patients compared to healthy controls (Fig. 20A). In addition, plasma IL-6 levels were also significantly higher in T1D patients (Fig. 20B). No differences were observed in the plasma IL-17A or TNF-α levels between the adult study groups (Article III, Suppl. Fig. 1).

When studying the relationship between plasma cytokine levels and selected clinical variables (age, hsCRP, C-peptide levels, HbA1c values, body-mass index (BMI), or disease duration) in adults with T1D, we observed that TNF-α and IL-6 positively correlated with hsCRP levels (Article III, Suppl. Fig. 1). IL-21 levels, however, did not correlate with age (Fig. 20C) as seen in the pediatric study groups, or with clinical parameters (Article III, Suppl. Fig. 1.).

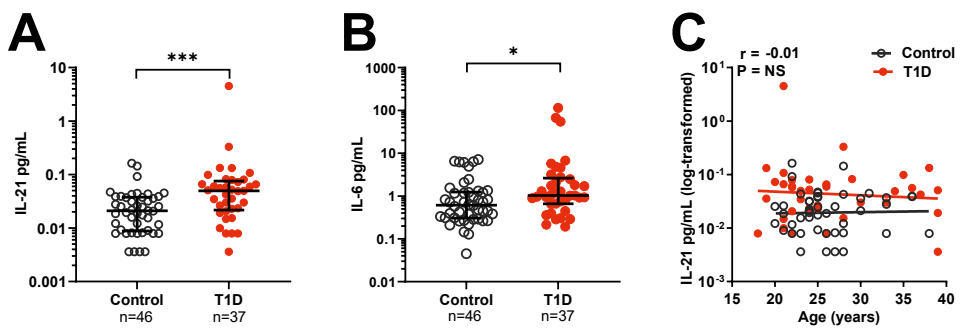

Figure 20. Elevated plasma IL-21 and IL-6 levels in adults with established T1D. Plasma IL-21 (A) and IL-6 (B) in adults with T1D and healthy controls. (C) Correlation between age and plasma IL-21 levels was examined using Spearman's correlation. The data from both study groups were pooled into the analysis. The elevations of the linear regression lines were significantly different between the groups (P = 0.001). Medians and IQRs are shown in the panels A–B.

6 DISCUSSION

6.1 POTENTIAL RELEVANCE OF CIRCULATING CD27$^-$CD8$^+$ T-CELL PHENOTYPES AT DIFFERENT STAGES OF T1D PROGRESSION

In project I, a comprehensive characterization of circulating CD8$^+$ T cells was carried out at different stages of T1D development in AAb$^+$ children, children with recent onset disease and in adults with established T1D, or during islet autoantibody seroconversion.

A proinflammatory signature consisting of polyfunctional IFN-γ$^+$TNF-α$^+$ producing CD27$^-$CD8$^+$ T cells was detected in children with newly diagnosed T1D and a co-inhibitory signature consisting of KLRG1$^+$TIGIT$^+$ expressing CD27$^-$CD8$^+$ T cells in AAb$^+$ children, who had later progressed to T1D. Both signatures were detected in a highly differentiated CD8$^+$ T-cell subset that lacked the expression of CD27. CD27$^+$ or CD27$^-$CD8$^+$ T-cell frequencies were, however, not altered indicating that phenotypical rather than quantitative differences explained the findings. Either of these signatures were not detected during early stages of autoimmunity at seroconversion or at later stages in the adults with established T1D. Of note, no major differences between CD4$^+$ and CD8$^+$ T-cell subsets were between the pediatric study groups compared to controls (Article I, Suppl. Fig. 2).

To consider the biological meaning of the findings, it could be envisioned that the expansion of proinflammatory T cells could be related to the metabolical distress during the clinical onset of T1D. To support this notion, the frequency of proinflammatory cytokine expressing cells positively correlated with the level of dysglycaemia at diagnosis in the children with newly diagnosed T1D. This may suggest a potential association of proinflammatory signature with dysglycaemia during the symptomatic onset of T1D, but the finding would have to be validated in the future in another cohort.

The biological background of the the co-inhibitory signature consisting of KLRG1$^+$TIGIT$^+$CD27$^-$CD8$^+$ T cells in AAb$^+$ children likely is associated with

a more complex phenomenon. KLRG1^{+}TIGIT^{+}CD8^{+} T cells is a phenotype of CD8^{+} T cells that was also observed in treatment responders in the teplizumab trials where a single 2-week teplizumab regimen in individuals with high-risk for T1D development delayd the onset of T1D for approximately an average 2 years (96). KLRG1^{+}TIGIT^{+} CD8^{+} T cells in the teplizumab study were suspected to represent partially exhausted T as their proliferative capacity had decreased but they still retained some functionality. It was speculated that the beneficial effect of teplizumab could be due to transient T-cell exhaustion that would reduce T-cell autoimmunity and therefore, support the preservation of β cells. (95) Intriguingly, a similar signature in CD8^{+} T cells have been observed in individuals with slower progression of T1D after diagnosis (46). Wiedeman et al. reported that individuals with slow progression of T1D, i.e. slower decline of endogeneous insulin production, exhibited a CD8^{+} T-cell phenotype consisting of elevated expression of EOMES, TIGIT, PD-1 and CD160 with a reduced functional and proliferative capacity (46). It has to be noted, that the characterization of the cells was done already during established T1D. Collectively, these studies suggest that the KLRG1^{+}TIGIT^{+} CD8^{+} T-cell signature is beneficially associated with suppression of autoimmunity. As the “exhausted” phenotype emerged in treatment responders after teplizumab treatment (2), a fair question is whether the cells are truly exhausted (=dysfunctional) or actually mediating some form of immune tolerance.

Labeling the KLRG1^{+}TIGIT^{+} CD8^{+} T cells as exhausted is a valid interpretation, and to some extent in line what should be labelled as exhausted (see chapter 2.2.6). The T-cell exhaustion storyline would explain the beneficial phenotype in such a manner, that as the KLRG1^{+}TIGIT^{+} CD8^{+} T cells would be exhausted indicating that they could not mediate the autoreactivity in pancreas, which in turn would lead to slowing down the β-cell decay. I would like to offer another explanation for the phenomenon. Inhibitory functions of co-inhibitory receptor-expressing CD8^{+} T cells could, at least to some extent, explain the beneficial association with decreased autoimmunity, whereas hypofunctionality would be then represented at a more advanced disease stage when the

immunological balance has tipped towards the clinical disease. The simplest explanation is likely the correct one, but probably understandably, it is difficult to assess whether T-cell exhaustion or tolerogenic function would be the simpler one in this case. Support for the idea of tolerogenic function of $CD8^+$ T cells expressing co-inibitory receptors comes from a recent publication (38) where it was shown that tolerogenic $CD8^+$ T cells could suppress autoimmunity development after viral infection. These tolerogenic $CD8^+$ T cells were distinguished by the expression of the KIR receptors KIR3DL1 or KIR3DL2, a higher expression of TIGIT, KLRG1, granzyme B and perforin, and lower expression of CD27, CD28 and CCR7 indicating a highly differentiated subset of $CD8^+$ memory T cells. In line with this, Jiang et al. (138) proposed already in 2010 that defects in tolerogenic $CD8^+$ T cells could potentially be linked with the development of T1D. The proposed mechanism would be that these tolerogenic $CD8^+$ T cells would directly kill the autoreactive $CD4^+$ T cells (38). Tolerogenic or suppressive function of $CD8^+$ T cells have been reported in other context as well, but it appears that the suppressive function is not yet well-understood (139). However, if we consider that $CD8^+$ T cells conventionally display their function in HLA class I restricted manner, would it be logical that tolerogenic $CD8^+$ T cells would also function in such a way? Regulatory action by killing the harmful target cell would likely be exterted in such a manner as indicated by the report by Li et al. (38). In addition, there are reports describing that suppressive $CD8^+$ T cells would be able to limit the action of $CD4^+$ T cells in a manner that does not result in killing of the $CD4^+$ T cells (140). This would indicate that the suppression would be extered through cytokines. These questions surely would need revisiting in the future.

Therefore, moving back to the results obtained in this study, it could be envisioned that $CD8^+$ T cells expressing co-inhibitory receptors could exert an inhibitory or tolerogenic functions to contain the ongoing autoimmunity before the symptomatic onset of T1D. Eventually, however, the chronic antigen stimulation leads to T-cell dysfunction (and/or T-cell exhaustion). Due to the dysfunction, autoimmunity is no longer contained, and the proinflammatory signature becomes dominant.

The functionality of the co-inhibitory receptor expressing $CD8^+$ T cells were not studied as a part of this project as intracellular cytokines and co-inhibitory receptors were regrettably examined using separate flow cytometry staining panels. Hence, we cannot determine whether the co-inhibitory receptor expressing cells were indeed showing signs of functional exhaustion. However, indirectly, we show no major negative correlations between the co-inhibitory receptor expressing $CD8^+$ T cells and the proinflammatory cytokine expressing $CD8^+$ T cells. Conversely, a positive correlation between $KLRG1^+TIGIT^+$ and degranulation marker CD107a expressing $CD27^-CD8^+$ memory T cells were observed in the flow cytometry dataset. Moreover, even though the single-cell multiomics analyses indicated that at the single-cell level *IFNG, TNF, KLRG1* and *TIGIT* transcripts are all expressed in cluster of cells with cytolytic capacity (as indicated by the expression of *GZMB* and *PRF1)*.

In the future, it would be important to determine the functionality of $KLRG1^+TIGIT^+$ $CD8^+$ T cells at different stages of T1D progression: are they exerting inhibitory or tolerogenic functions in stage 1–2 T1D, and, in turn, would these cells get dysfunctional or exhausted due to chronic antigen stimulation during the disease progression towards stage 3 T1D?

6.2 INCREASED FREQUENCY OF $CD4^+$ B-HELPER T CELLS DURING TYPE 1 DIABETES DEVELOPMENT IS ASSOCIATED WITH DISEASE PROGRESSION RATHER THAN EARLY AUTOIMMUNITY

One of the most prominent findings in the blood $CD4^+$ T-cell landscape in T1D is the expansion of B-cell helper T cells, cTfh and cTph cells, in AAb^+ children at-risk for T1D and in children with newly diagnosed T1D. Thus, a comprehensive characterization of cTph and cTfh frequencies, phenotypes and the timing of the expansion at different stages of T1D progression was carried out in project II.

The frequency of $CXCR5^-PD\text{-}1^{hi}$ cTph cells was increased in AAb^+ children and in children with newly diagnosed T1D, as also noted in previous study (83). In addition, $CXCR5^+PD\text{-}1^+ICOS^+$ activated cTfh cells were expanded in

children with T1D, and this finding was also supported by the data obtained in project I where we show that IL-21-producing $CD4^+$ TCM cells were slightly expanded in children with T1D.

Importantly, no differences in the frequencies of cTph and cTfh cells were detected during early autoimmunity, i.e. during seroconversion to islet autoantibodies were detected, further supporting the observations that the expansions seen in cTph and activated cTfh cells appear to be associated with later disease stages or higher disease activity in autoimmune diseases. Interestingly, we show that the cTph expansion is only seen in AAb^+ children who later progressed to T1D, while in our earlier study by Ekman et al. (83), AAb^+ non-progressor children did not exhibit the elevated frequency of cTph cells. This phenomenon was not observed at early autoimmunity as children who progressed later to T1D had no expanded cTph cells during seroconversion, suggesting that this expansion is specifically associated with the progression to T1D on a later point in time. Of note, as the seroconversion cohort is relatively small in project I and II, these analyses would need to be revisited in a larger cohort in the future even if obtaining the samples can be a difficult endeavour.

Similar to T1D, in RA, cTph, but not cTfh, expansion was reported in at-risk individuals before the clinical diagnosis (141). In addition, in RA, patients with moderate to high disease activity had a larger expansion of cTph cells (102). Additionally, in CeD, a higher frequency of cTph have been observed in untreated patients compared to treated patients (103). Collectively, these all could be an indication that cTph expansions are typically seen in stages of active autoimmune disease.

No phenotypical differences were detected in cTph or in cTfh cells between the study groups indicating that the detected expansions were quantitative and not qualitative in nature. However, in other autoimmune diseases, more distinct phenotypical differences in cTph cells have been observed between patients and controls. For example, in CeD, a higher frequency of $CD39^+CXCR3^+CD38^+PD\text{-}1^+ICOS^+CD161^+$ cTph-like cells were observed in the circulation of CeD patients (103). Also, in SLE and in RA, HLA class II-expressing cTph cells were expanded in patients (29,104). These phenotypical marker expression patterns would indicate that

activated cTph cells are enriched in individuals with active autoimmune diseases. The discrepancy between the phenotypic results presented here for T1D compared to other autoimmune diseases could be due to a variety of reasons, an obvious one being a less prominent $CD4^+$ B-cell helper T cell activation in patients with T1D *in vivo*, perhaps.

A major caveat of our study is that circulating T cells rather than the cells obtained from pancreas were examined, since the Tph signature llikely is stronger in T cells present in the inflamed tissue. Another obvious caveat in the current approach is that the polyclonal (or global) cTph and cTfh cell frequencies were studied. The phenotypes of autoreactive cTph or cTfh cells could reveal phenotypic alterations or specific phenotypes associated with different disease stages. However, due to the extremely low number of autoreactive T cells in circulation, the study of autoreactive cTph or cTfh cells is a difficult endeavour (142) beyond what could be achieved this project. In CeD, where gluten-specific $CD4^+$ T cells are more easily detected by HLA class II tetramers, it has been shown that the gluten-specific $CD4^+$ T cells have appeared as cTph-like cells (103). In addition, in autoimmune hepatitis, Tph-like signature has been reported in autoreactive $CD4^+$ T cells (143). This may indicate that the observed expansions could be, at least partially, related to the expansion of autoreactive T cells also in other autoimmune diseases.

Finally, to draw an interesting link between project I and II, we observed a higher frequency of $CD27^+PD\text{-}1^+$ $CD8^+$ and $CD4^+$ T cells in AAb^+ children compared to controls indicating that it is likely that also the $CD8^+$ $PD\text{-}1^+$ T-cell compartment has expanded in AAb^+ children who later progressed to T1D. It has to be also noted that there are some thematic similarities in the studied cell subsets as both $CD8^+$ and cTph $CD4^+$ T-cell subsets exhibited an activated and/or proinflammatory status. These observations warrant further investigations to study the potential phenotypical similarities and differences between $CD8^+$ and $CD4^+$ T-cell compartments especially in AAb^+ children who did or did not progress to T1D.

6.3 CIRCULATING TPH CELLS – INTERMIXED PHENOTYPES OF VARIOUS EFFECTOR CELLS?

The second part of project II consisted of general characterization of CXCR5^{-}PD-1^{hi} cTph and CXCR5^{+}PD-1^{hi} cTfh fractions, regardless of the study groups. cTph cells have been relatively recently discovered (102) and their exact phenotype or function is constantly being studied further. It was observed early on that the cTph cells had phenotypical similarities with cTfh cells, indicating the capacity to provide B-cell help (29). It has been envisioned that cTph cells would participate in B-cell help in TLOs i.e. outside SLOs, at the sites of autoimmune reaction, perhaps. (107) The phenotypes detected in our dataset supported these observations, as cTph cells expressed CCR2, CCR5 and HLA-DR, indicative of effector phenotype capable of migrating towards tissues. In addition, they also expressed *IL21*, *CXCL13*, TIGIT and ICOS, markers typically associated with cTfh cells and B-cell help.

However, single-cell multiomics analyses revealed a surprising heterogeneity within CXCR5^{-}PD-1^{hi} fraction containing cTph cells. This fraction contained cells with a "classical" Tph phenotype, but also many other cell types appeared to accumulate there. In particular, Th1, Treg and CTL CD4^{+} T cells. It could be ensivisoned that perhaps the CXCR5^{-}PD-1^{hi} fraction contains various activated effector types. Indeed, this heterogeneity has been demonstrated in previous studies, but surprisingly, the matter has gained quite modest attention even though it is quite likely that what we have labelled as "cTph cells" are not a single phenotype. Caielli et al. (144) report that in SLE circulating CXCR3^{+}CXCR5^{-}PD-1^{hi} cells express IL-10 but no IL-21 or CXCL13, and Goto et al. (145) reported CXCR3^{mid}CXCR5^{-}PD-1^{hi} cells expressing IL-21, CXCL13 and cytolytic molecules. Argyriou et al. (146) showed that cTph cells in blood possess CTL characteristics in patients with RA, whereas in synovial fluid Tph cells possess a more B-cell helper T-cell phenotype. It is possible that in the tissues Tph cells are a more uniform phenotype and in circulation there is more heterogeneity, and perhaps, enrichment of other activated cell types

within the CXCR5^{-}PD-1^{hi} fraction. I would envision that the cells we call "Tph cells" need more phenotypical refinement, at least in the circulation.

6.4 BIOMARKER POTENTIAL OF PLASMA IL-21 LEVELS IN TYPE 1 DIABETES

A higher frequency of IL-21-producing T-cell subsets have been observed in several studies (81–83) and these findings were corroborated in the current shown here (I, II). Thus, it was a logical next step to evaluate the biomarker potential of plasma IL-21 levels in the context of T1D. IL-21 in plasma could serve as an easy surrogate biomarker for activation of the IL-21 pathway, and potentially Tfh/Tph cells in T1D.

Plasma IL-21 levels in were measured in cross-sectional cohorts using ultrasensitive Quanterix SiMoA IL-21 detection method capable of capturing fg/mL concentrations, with markedly improved IL-21 detection sensitivity and specificity compared to older IL-21 detection methods (III).

Plasma IL-21 levels were not altered in children with T1D or children at-risk for T1D. Physiologically ten-fold higher plasma IL-21 levels in children (compared to adults) and a strong negative correlation with age was however observed. In contrast, increased plasma IL-21 levels were detected in adults, but these did not correlate with the clinical parameters analyzed in parallel. A larger cohort of individuals would be needed to validate this finding. Also, potentially a comparison of plasma IL-21 levels in adults with type 2 diabetes could reveal whether the increased IL-21 levels in adults with T1D are associated with autoimmunity or are, in fact, secondary to hyperglycemia and metabolic dysfunction.

In the few studies, that have analyzed IL-21 serum and/or plasma levels in T1D a slight elevation of IL-21 levels in serum or plasma in adults (147) and children with T1D (148), as well is AAb^{+} at-risk children (149) has been reported. Our findings are in line concerning the findings of adults, but we could not detect increased levels of IL-21 in the pediatric study groups. Of note, the earlier studies utilized older, likely inadequate IL-21 detection methodologies (133), and thus a new examination of the IL-21 levels in plasma was warranted.

As IL-21 producing T-cell subtypes have been reported to be increased in AAb^+ children and individuals with T1D, we would have expected that plasma IL-21 levels would have been increased in the pediatric study groups. As we observed only a modest increase of plasma IL-21 levels in adults with T1D, the potential alterations in the pediatric cohorts could be partially concealed by the higher background IL-21 levels in children. In addition, the plasma IL-21 levels do not entirely reflect the IL-21 produced by conventional T-cell subsets mentioned earlier, as also natural killer T (NKT) cells cells are shown to produce IL-21 (150). In the future, when potential disease endotypes of T1D are better established, the question of using plasma or serum IL-21 as a biomarker could be re-visited again. In addition, another potential viable approach would be to monitor the IL-21 levels in longitudinal samples. Novel biomarkers would be needed especially for at-risk children, since they would be the main target group for potential immunotherapy treatments to delay the onset of the disease.

6.5 POTENTIAL INTERPLAY BETWEEN TH1/TC1 T-CELL RESPONSES AND IL-21 PATHWAY IN TYPE 1 DIABETES

Various blood T-cell subsets have been found to be altered by us and others (Table 2, chapter 2.5) in T1D. It could be argued that blood may not be representative of the immune response in the tissues (pancreas in T1D). However, often blood represents only viable material for biomarker discovery purposes as it could be clinically contraindicated to routinely sample certain tissues. At least this is the case with pancreas in T1D. Often, it is considered, however, that the cell subsets found to be altered, especially the subsets that have increased, may reflect the immune response in the tissues. Biological significance of the alterations is often much more difficult to interpret as egress of certain cell types in the blood may be a form of bystander activation or perhaps a compensatory mechanism rather than indication of harmful cell type. Hence, in the future, more comparisons between blood and tissue T-cell phenotypes are needed. In this thesis, only blood T cells were studied. To bring the

findings, presented in the thesis into a broader perspective, I will discuss a potential interplay between the CD8$^+$ T cells, cTph cells, IL-21 pathway and Th1-like responses. The results of the thesis are summarized in Fig. 21.

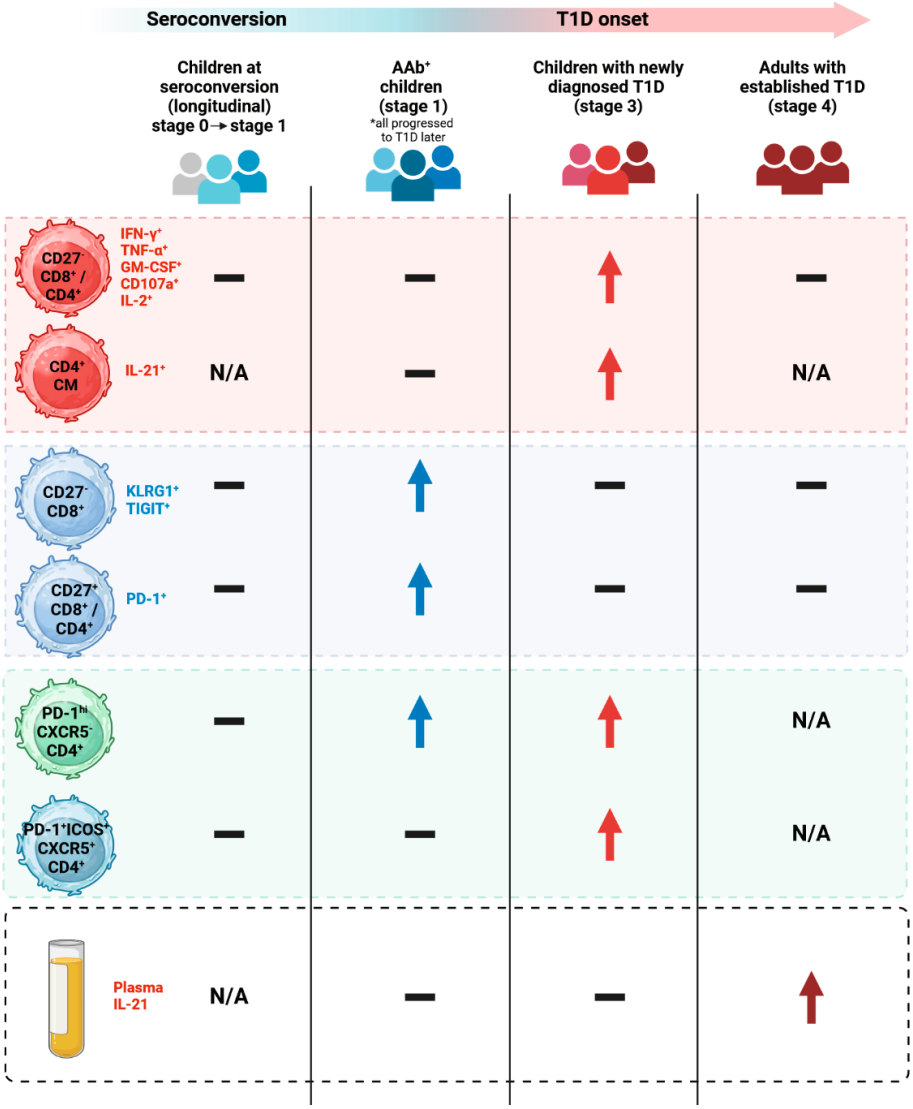

Figure 21. Summary of the results. Children at seroconversion did not exhibit alterations in the T-cell subsets studied. AAb$^+$ children who all progressed to T1D showed an increase of co-inhibitory receptor expressing CD8+ T cells and cTph cells whereas children with newly diagnosed T1D showed an elevation of proinflammatory CD8$^+$ T cells, IL-21 producing CD4$^+$ T cells, cTph and activated cTfh cells. Adults with T1D exhibited an elevated levels of IL-21 in plasma. N/A, not applicable. Created with Biorender.com.

There must be an adequate immunological milieu within pancreas that enables the autoimmune process. In T1D, a Th1/Tc1-type, i.e. proinflammatory IFN-γ response, has been suggested to be responsible for driving the autoimmune destruction (27). Th1/Tc1-responses also fit to the story of viral infection as an environmental trigger of autoimmunity, as Th1/Tc1-responses are conventionally mediated against intracellular targets such as viruses (10). IFN-γ induces leukocyte migration into tissues, and it could be envisioned that IFN-γ expression within pancreas might aid migration of various T cells and other immune cell subsets to pancreas. In addition, IFN-γ expression enhances the expression of HLA molecules, which, in turn, might promote autoantigen presentation. IFN-γ signalling can also directly contribute to β-cell stress which makes them susceptible to cell death. (27) In the thesis project presented here, Th1/Tc1-type $CD4^+$ and $CD8^+$ T cells and the production of proinflammatory cytokines were shown to be expanded in blood of children with newly diagnosed T1D (I).

In addition to IFN-γ signaling pointing to a Th1/Tc1-type response, pancreatic β cells have been shown to express CXCL10 (151), a ligand for CXCR3 (a hallmark chemokine receptor of Th1/Tc1 T cells), which could aid the autoreactive Th1, Tph or Tc1 cells to migrate into pancreas. Indeed, it has been shown in a mouse model that CXCL10 expression at islets appears to attract $CD8^+$ T cells to islets (152). Furthermore, it has been shown in NOD mouse model that by blocking CXCL10, the infiltration of both $CD4^+$ and $CD8^+$ T cells were reduced (153). CXCR3-expressing autoreactive $CD8^+$ T cells have also been shown to have a potential to mediate β-cell killing (91). As increased frequency of polyclonal IFN-γ^+TNF-α^+CD8^+ T cells was observed in circulation of children with newly diagnosed T1D, a fair question is whether these cells were expanded due to metabolic distress or as a response to inflammatory milieu - as a form of bystander activation? Undoubtedly, not all of them represented autoreactive T cells as indicated by the low frequency of autoreactive $CD8^+$ T cells in circulation detected in various studies in T1D (79,93,154).

Building the story upon an inflammatory milieu sustaining the development of T1D, the activation of the IL-21 pathway can also be implicated as a mechanism of autoimmunity in T1D. IL-21-producing T-cell

subsets are increased in the circulation of individuals with newly diagnosed T1D (59–61, 63,64), and these findings were also confirmed here by demonstrating that IL-21-producing TCM $CD4^+$ T cells were increased in children with newly-diagnosed T1D (I). Moreover, PD-1^{hi}cTph and PD-1^+ICOS^+cTfh cells were expanded in children with T1D and/or AAb^+ children (II). In addition, adults with T1D had higher plasma IL-21 levels compared to healthy controls (III). IL-21 likely induces autoantibody production, which in turn, likely supports the autoimmune process. Importantly, IL-21 also supports cytolytic $CD8^+$ T-cell responses (155). $CD8^+$ T cells are the major component of immune cell infiltrates at pancreas in T1D, and they localize within insulitic islets (88), and autoreactivity of these cells has been confirmed (78,79). Could IL-21-producing $CD4^+$ T cells induce the cytolytic action of $CD8^+$ T cells within the pancreas? Recent confirmation of the existence of TLOs at pancreas of AAb^+ individuals and individuals with T1D (74) leads to an interesting scenario where TLOs could support the adequate immunological milieu for autoimmunity. IL-21 production could likely take place within TLOs and sustain autoimmunity in the pancreas, as discussed in chapter 6.2. In this study, the increase of cTph cells in circulation precedes the increase of blood proinflammatory $CD8^+$ T cells, whereas the increase of activated cTfh cells and the increase of proinflammatory $CD8^+$ T cells were observed in the children with newly diagnosed T1D (I–II).

The IL-21 pathway can be also linked to the dysfunction of the Tregs. Th1-type Tregs can likely migrate towards the CXCL10 gradient and potentially mediate suppression as indicated by a study conducted in NOD mice (156). However, perhaps the underlining immunological milieu does not enable the Tregs to function properly in autoimmunity. IL-21 appears to antagonize Treg function (157), so it could be envisioned that the increased IL-21 production by B-cell helper T cells could further enable autoreactive T cells to be released from the harness of peripheral tolerance mechanisms. In fact, Teff cells from patients with T1D have been shown to be resistant to Treg-mediated suppression (53). Moreover, it has been shown *in vitro*, that Teffs exposed to IL-6 and IL-21 are not effectively suppressed by Tregs (53,157). This could explain, at least partially, why the

autoreactive T cells can escape peripheral tolerance within the pancreas in T1D. Additionally, it can be speculated, that would it be possible that the development of non-suppressive "Treg" types are favored due to some polarizing conditions towards non-suppressive cell types. Fittingly, an increase of Tregs with low suppression capacity but enhanced Th17-like proinflammatory phenotype in children with T1D have been detected (86). However, the analysis of Treg frequencies in circulation of children with T1D has yielded various results; others show altered frequencies of Tregs in children with T1D (i.e. 84,85), others do not show differences between children with T1D and controls (i.e. 156). IL-6, for one, can also promote Th17-like responses. Indeed, higher IL-6 levels and altered IL-6 responsiveness have been observed in T1D (105). IL-21 and IL-6 could potentially create a milieu where Tregs would be inadequately capable to suppress autoimmunity (27). In addition to the expanded IL-21-producing $CD4^+$ T cells (I–II), IL-21 and IL-6 were found to be increased in plasma of individuals with T1D (III).

A final consideration of the immunological factors that shape the emergence of T1D could be T-cell exhaustion. In autoimmunity, the identification of self-antigens as non-self could be considered a chronic antigen exposure that, in the case of T1D, leads eventually to an eradication of insulin producing β cells. However, T-cell exhaustion is associated with slower progression of T1D after diagnosis (46) implicating it could be a favorable phenomenon. At least two potential explanations exist: either the autoreactive exhausted T cells can not mediate autoreactivity due to downmodulation of their effector functions or these exhausted cells expressing inhibitory receptors could alter the functionality of other, pathogenic cell types. PD-1 and TIGIT expression could lead to inhibitory signaling in T cells: PD-1 is known to alter CD28 co-stimulatory signaling and TIGIT signaling can lead to IL-10 production, further inhibiting the T-cell activation. Interestingly, IL-21 has also been suggested to be cytokine that would (partially) antagonize T-cell exhaustion (159), indicating that increased IL-21 production could suppress the potentially beneficial co-inhibitory receptor expressing phenotype, and induce the action of proinflammatory cell types.

The events described above create a potential link between IL-21-producing $CD4^+$ T-cell subsets, $CD8^+$ T-cell activation, exhaustion and Treg dysfunction leading eventually to the emergence of T1D. The findings in this project support the existence of Th1/Tc1 type immunopathology (I) and activation of the IL-21 pathway in T1D near the onset (II) and at established clinical disease (III).

7 CONCLUSIONS

The purpose of this thesis was to characterize the T-cell compartment during the development of T1D to shed light to T-cell alterations at different stages of T1D progression and to scrutinize the timing of observed T-cell expansions by us and others. Novel biomarkers are urgently needed for this heterogenous disease in order to better identify the individuals who might benefit from the immunotherapeutical treatments before the autoimmunity has destroyed the insulin-producing β cells.

The studies focused on the $CD8^+$ T cells, which are known to contribute to the demise of the β cells at pancreas, and on the $CD4^+$ T-cell subsets that produce IL-21 and give help to B cells, which are considered as some of the main players in T1D autoimmunity.

I) Two distinct, but temporal, $CD8^+$ T cell signatures were detected in pediatric study cohorts within circulating $CD8^+$ T cells: a proinflammatory signature associated with disease onset and a co-inhibitory signature in AAb^+ children, before the onset of T1D.

II) The timing of the known expansions in cTph and cTfh cells were scrutinized and the expansions were shown to be associated with the stages 1 to 3, close to the diagnosis. These signatures were not detected at early autoimmunity during seroconversion to AAbs. Substantial phenotypical heterogeneity was also detected within the $CXCR5^-PD\text{-}1^{hi}$ cTph subset, warranting further studies in the future.

III) Adults with established T1D exhibited higher levels of plasma IL-21 and IL-6 compared to controls. In contrast, no alteration was detected in the pediatric study groups. However, plasma IL-21 levels were 10-fold higher in children compared to adults, and this physiologically higher plasma IL-21 level could mask the potential differences between the pediatric study

groups. This analysis could be revisited in the future, when potential T1D disease subgroups are more established.

Taken together, using precious and unique clinical samples from the DIPP study, a comprehensive characterization of global $CD8^+$ and $CD4^+$ T-cell phenotypes at different stages of T1D progression are presented in this thesis. Specific $CD8^+$ and $CD4^+$ T-cell signatures that were temporal but associated with distinct stages of T1D development, close to the T1D diagnosis rather than at initial autoimmunity during AAb seroconversion, were discovered. A common theme for PD-1^+ expressing $CD8^+$ and $CD4^+$ cTph cells were that the expansions were mainly seen in AAb^+ progressor children, whereas the expansions of proinflammatory $CD8^+$ T cells and activated cTfh cells were detected in children with newly diagnosed T1D. The expansions of IL-21-producing cTph and cTfh in children did not translate into higher plasma IL-21 levels in children, whereas in adults with established disease higher plasma IL-21 level were detected.

The findings presented in this thesis reveal important insight of the alterations in the global T-cell landscape at different stages T1D progression, and the signatures could be utilized in further development of biomarkers for T1D progression.

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“We’re maintaining our position on the event horizon of chaos.”
Hannibal to Will on NBC’s Hannibal

”And this chaos, it defies imagination”

Panic Station by Muse

ORIGINAL PUBLICATIONS (I – III)

I

Temporal Alterations in CD8$^+$ T Cells During the Progression from Stage 1 to Stage 3 Type 1 Diabetes

Schroderus AM, Pitkänen V, Ekman I, Stevens D, Rytkönen-Nissinen M, Rintamäki R, Pihlajamäki J, Knip M, Veijola R, Toppari J, Ilonen J, Lempainen J, Kinnunen T

Diabetes 2024; db240159. https://doi.org/10.2337/db24-0159

diabetes
American Diabetes Association

Temporal Alterations in CD8$^+$ T Cells During the Progression From Stage 1 to Stage 3 Type 1 Diabetes

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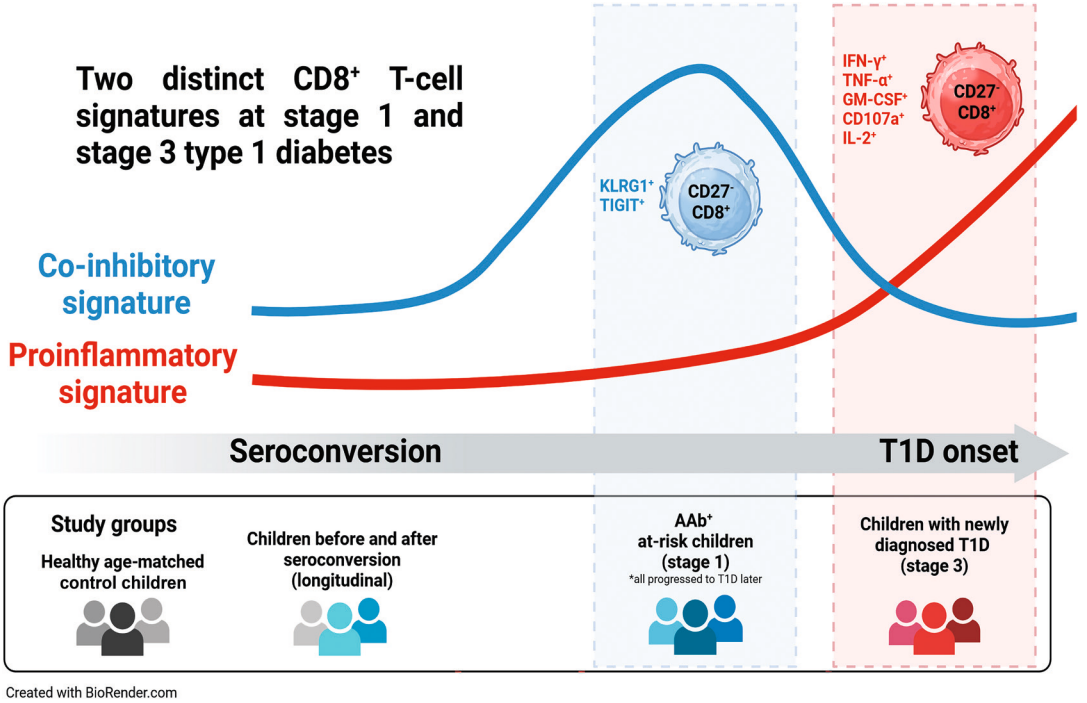
Diabetes 2024;73(10):1705–1715 | https://doi.org/10.2337/db24-0159



Temporal Alterations in CD8$^+$ T Cells During the Progression From Stage 1 to Stage 3 Type 1 Diabetes

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Diabetes 2024;73:1705–1715 | https://doi.org/10.2337/db24-0159

CD8$^+$ T cells are perceived to play a major role in the pathogenesis of type 1 diabetes (T1D). In this study, we characterized the function and phenotype of circulating CD8$^+$ memory T cells in samples from individuals at different stages of T1D progression using flow cytometry and single-cell multiomics. We observed two distinct CD8$^+$ T-cell signatures during progression of T1D within the highly differentiated CD27$^-$CD8$^+$ memory T-cell subset. A proinflammatory signature, with an increased frequency of IFN-γ$^+$TNF-α$^+$ CD27$^-$CD8$^+$ memory T cells, was observed in children with newly diagnosed T1D (stage 3) and correlated with the level of dysglycemia at diagnosis. In contrast, a coinhibitory signature, with an increased frequency of KLRG1$^+$TIGIT$^+$ CD27$^-$CD8$^+$ memory T cells, was observed in islet autoantibody-positive children who later progressed to T1D (stage 1). No alterations within CD27$^-$CD8$^+$ memory T cells were observed in adults with established T1D or in children during the initial seroconversion to islet autoantibody positivity. Single-cell multiomics analyses suggested that CD27$^-$CD8$^+$ T cells expressing the *IFNG*$^+$*TNF*$^+$ proinflammatory signature may be distinct from those expressing the *KLRG1*$^+$*TIGIT*$^+$ coinhibitory signature at the single-cell level. Collectively, our findings suggest that distinct blood CD8$^+$ T-cell signatures could be employed as potential biomarkers of T1D progression.

ARTICLE HIGHLIGHTS

- Blood CD8$^+$ T-cell signatures have recently been associated with a slower progression of type 1 diabetes (T1D) after diagnosis and with clinical response to immunotherapy.
- We investigated blood CD8$^+$ T-cell signatures in individuals at different stages of T1D progression.
- We observed two distinct CD8$^+$ T-cell signatures at different stages of T1D: a proinflammatory signature in children with newly diagnosed T1D (stage 3) and a coinhibitory signature in autoantibody-positive children who later progressed to T1D (stage 1).
- CD8$^+$ T-cell signatures could potentially be utilized as biomarkers for evaluating the risk of T1D progression.

Type 1 diabetes (T1D) is an autoimmune disease that leads to the destruction of the insulin-producing β-cells in the pancreas (1). The onset of T1D is preceded by a preclinical phase of variable length during which autoantibodies (AAbs) to islet autoantigens are almost invariably

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Received 27 February 2024 and accepted 6 June 2024

This article contains supplementary material online at https://doi.org/10.2337/figshare.26072404.

detected. AAb positivity can thus be used as a highly specific biomarker for future development of T1D in follow-up. (2). Currently, presymptomatic T1D can be classified into distinct stages, with stage 1 defined by the presence of multiple AAbs and stage 2 by the presence of multiple AAbs and dysglycemia, whereas stage 3 marks the onset of symptomatic disease (3).

$CD8^+$ T cells are implicated to play a major role in the T1D disease process by contributing to the direct killing of insulin-producing β-cells. Both in the NOD mouse model and in humans, $CD8^+$ T cells are a major component of the immune infiltrate in the islets (4–6). Islet antigen-reactive $CD8^+$ T cells can also be reproducibly detected in the blood and pancreas of patients with T1D, but also in healthy donors (7–9). This suggests that the simple presence of autoreactive $CD8^+$ T cells is not sufficient for autoimmunity to occur, but rather the quality of the $CD8^+$ T-cell response could differ in patients with T1D.

$CD8^+$ T cells exhibit a broad range of different developmental and functional states (10,11). Several studies have confirmed that circulating islet autoreactive $CD8^+$ T cells also have a wide phenotypic heterogeneity (reviewed in Wiedeman et al. [12]), and, interestingly, certain $CD8^+$ T-cell phenotypes have been associated with the risk of T1D progression (13,14). Both a higher frequency of terminally differentiated and "exhausted" $CD8^+$ autoreactive T cells have been correlated with slower C-peptide decline after T1D diagnosis (13,14). Interestingly, the exhausted signature, consisting of higher expression of coinhibitory markers PD-1, TIGIT, and CD160, was also observed to be increased at the level of total $CD8^+$ T cells in the latter study (14), suggesting a more global alteration of $CD8^+$ T cells. Importantly, a highly similar exhausted global $CD8^+$ T-cell signature has recently been also associated with treatment response in immunotherapy trials (15–17). An increased frequency of $KLRG1^+TIGIT^+$ $CD8^+$ T cells has been shown to correlate with a better clinical outcome in clinical trials of the anti-CD3 mAb teplizumab both in patients with recent-onset T1D and in at-risk subjects (15,16). More recently, a similar observation was also made in the alefacept (LFA-Ig) clinical trial (17).

Given these results, global changes in $CD8^+$ T-cell signatures could potentially be used as biomarkers of disease progression in T1D. However, apart from the above-mentioned studies, only a few studies have addressed $CD8^+$ T-cell phenotypes in patients with T1D, with variable results (18–20), and studies analyzing individuals prior to the onset of T1D are completely lacking.

Here, we have performed an extensive analysis of blood $CD8^+$ T-cell phenotypes using clinical cohorts of individuals at different stages of T1D progression. We observed distinct $CD8^+$ T-cell signatures at different stages of disease progression: a proinflammatory signature in children at the onset of T1D (stage 3) and a coinhibitory signature in AAb^+ children who later progressed to T1D (stage 1).

RESEARCH DESIGN AND METHODS

Study Subjects

The characteristics of the study cohorts are summarized in Table 1 and Supplementary Tables 1–3. The pediatric cohort consisted of 30 children with newly diagnosed T1D (0–7 days after clinical diagnosis), 30 AAb^+ children, who later progressed to clinical T1D (0.5–3.9 years after sampling), and 60 healthy autoantibody-negative control children that were age matched to T1D and AAb^+ case subjects. The AAb^+ children and healthy control children participated in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) follow-up study and carried HLA genotypes associated with increased risk for T1D. Autoantibody positivity was analyzed as previously described (21), and was defined based on positivity for one or more biochemical autoantibodies (insulin autoantibodies, insulinoma-associated-2 antibodies, and/or glutamic acid decarboxylase antibodies). The adult cohort comprised 20 T1D patients and 19 healthy control subjects that were enrolled in the T1D-IMMUNO study at Kuopio University Hospital and University of Eastern Finland, Kuopio. The seroconversion cohort consisted of longitudinal samples collected before and after islet AAb seroconversion in 17 children participating in the DIPP study and 18 age-matched healthy control children (time between samples 3–13 months).

All participants and/or their legal guardians gave written informed consent, as mandated by the Declaration of Helsinki. The study was approved by local ethics committees in the participating Turku and Kuopio University Hospitals (Finland) and, for the DIPP study, by the ethics committee of the Hospital District of Northern Ostrobothnia (Finland).

Peripheral Blood Mononuclear Cells and Plasma Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood samples using Ficoll gradient centrifugation and stored in liquid nitrogen until analyses. Plasma samples were collected from heparinized peripheral blood after centrifugation at 700*g*, 10 min, and stored at −80°C until analyses.

Cytomegalovirus-Specific IgG-Antibody Measurements

Cytomegalovirus (CMV)-specific IgG antibodies were measured from plasma samples stored at −80°C at the University of Turku with the Vidas CMV IgG assay (bioMérieux S.A.), according to manufacturer's instructions. The plasma sample originated from the same blood draw as the PBMCs.

Flow Cytometry

Flow cytometry analyses were performed essentially as previously described (22,23). Polyfunctionality index (PI) (24) was calculated using CD107a, IFN-γ, TNF-α, GM-CSF, and IL-2 expressing cells, q = 1; that is, no additional

Table 1—Summary of the study cohorts

	Pediatric cohort			Adult cohort		Pediatric seroconversion cohort		
	Healthy children	Autoantibody-positive children	Children with newly diagnosed T1D	Adult control subjects	Adults with established T1D	Healthy children	Before seroconversion	After seroconversion
n	60	30	30	19	20	18	17	17
Age (years) on sampling, mean ± SD	8.61 ± 3.65	8.49 ± 3.61	8.63 ± 3.66	25.37 ± 4.21	26.70 ± 6.45	3.58 ± 1.61	3.14 ± 1.50	3.76 ± 1.39
Female, % (ratio)	42 (25 of 60)	47 (14 of 30)	40 (12 of 30)	47 (9 of 19)	50 (10 of 20)	67 (12 of 18)	41 (7 of 17)	41 (7 of 17)
Male, % (ratio)	58 (35 of 60)	53 (16 of 30)	60 (18 of 30)	53 (10 of 19)	50 (10 of 20)	33 (6 of 18)	59 (10 of 17)	59 (10 of 17)

weighting was added. For additional details, see the Supplementary Material.

Single-Cell Multiomics Analyses

Single-cell multiomics analyses were conducted using the BD Rhapsody Single-Cell Analysis (BD Biosciences) platform. First, PBMCs from five children with newly diagnosed T1D and five age-matched healthy control children were thawed. Next, $CD8^+$ T cells were enriched using the human $CD8^+$ T-cell isolation kit (Miltenyi). The cells were labeled with fluorochrome-labeled monoclonal antibodies (Supplementary Table 4), BD Abseq oligonucleotide-conjugated monoclonal antibodies (BD Biosciences, Supplementary Table 4), and the Single Cell Sample Multiplexing Kit (BD Biosciences), according to the manufacturer's instructions. Finally, viability staining was performed using 7-AAD (Biolegend), according to manufacturer's instructions.

Next, viable $CD27^+$ and $CD27^-CD8^+$ T cells were sorted from each donor using the Sony MA-900 cell sorter (Sony Biotechnology) and pooled after sorting. Postsort purity was determined after the sorting (Supplementary Fig. 1). Pooled $CD8^+$ T cells either were captured immediately after sorting using the BD Rhapsody Express System or subjected to 90 min of stimulation with phorbol mystic acid (PMA) (50 ng/mL, Sigma) and ionomycin (1 μg/mL, Sigma) before single-cell capture. Data analysis was carried out in R (25) using Seurat version 4.3.0 (26). Seurat (26) or scCustomize (27) packages were used for visualization of dot plots and coexpression of selected markers. For additional details on library preparation, sequencing, and data analysis, see the Supplementary Material.

Statistical Testing

Statistical testing for the flow cytometry data was performed using GraphPad Prism (GraphPad, version 9.2.0). Mann-Whitney *U* test was used when comparing two groups, and Kruskal-Wallis test with Dunn multiple comparison test was used when comparing multiple groups. Correlation analyses were performed using Spearman correlation in R (25) (package stats version 4.2.2). Correlograms in Fig. 4 were prepared in R (25) using ggcorrplot (28). Simple linear regression was used to calculate regression lines for the study groups. Slopes and intercepts of the regression lines were compared, and two-tailed *P* values were calculated for the comparisons with Prism. P values < 0.05 were considered to indicate statistical significance. The study was exploratory in nature, with a limited number of parameters analyzed, and, therefore, correction for multiple comparisons was not performed for the flow cytometry data. Sex was registered for each study subject, but it was not considered as a factor in the statistical analysis.

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. The single-cell multiomics

data are accessible via ImmPort (https://www.immport.org, accession number SDY2666).

No applicable resources were generated or analyzed during the current study.

RESULTS

Minor Alterations in CD4$^+$ and CD8$^+$ Memory T-Cell Subset Frequencies in Children With Newly Diagnosed T1D

Circulating CD8$^+$ and CD4$^+$ T cells were analyzed with flow cytometry in a cross-sectional pediatric cohort consisting of 30 children with newly diagnosed T1D (stage 3), 30 AAb$^+$ children who later progressed to T1D (stage 1), and 60 age-matched healthy children. T cells are classically differentiated into naïve (CD45RA$^+$CCR7$^+$CD28$^+$CD27$^+$CD95^{lo}), T stem cell memory (Tscm) (CD45RA$^+$CCR7$^+$CD28$^+$CD27$^+$CD95^{hi}), central memory (CM) (CD45RA$^-$CCR7$^+$CD28$^+$CD27$^+$), T effector memory (TEM) (CD45RA$^-$CCR7$^-$CD27$^{+/-}$), and TEM re-expressing CD45RA (TEMRA) (CD45RA$^+$CCR7$^-$CD27$^-$) subsets (10,29) (Supplementary Fig. 2). Using these definitions, we observed that Tscm CD4$^+$ T cells (P = 0.008) were more frequent in children with T1D compared with control children, whereas CD4$^+$ CM (P = 0.02) and CD27$^+$ TEM CD8$^+$ (P = 0.01) T cells were less frequent in children with T1D. T-cell subset frequencies were comparable between AAb$^+$ children and control children (Supplementary Fig. 2).

Polyfunctional Proinflammatory CD27$^-$CD8$^+$ Memory T Cells Are Expanded in Children With Newly Diagnosed T1D

Next, we examined the functionality of CD8$^+$ memory T cells by analyzing the production of proinflammatory cytokines (IFN-γ, TNF-α, GM-CSF, and IL-2), as well as the expression of the cytotoxic degranulation marker CD107a upon PMA and ionomycin stimulation. For the analysis, CD8$^+$ memory T cells were subdivided into CD27$^+$ (consisting of less differentiated CM and CD27$^+$ TEM subsets) and CD27$^-$ fractions (consisting of more highly differentiated CD27$^-$ TEM and TEMRA subsets) (Fig. 1*A* and Supplementary Fig. 3). CD27$^-$CD8$^+$ T cells represented a median 15.10% of total CD8$^+$ memory T cells in pediatric samples, and their frequency was similar in children with T1D or AAb$^+$ children compared with control children (Supplementary Fig. 4).

Interestingly, the frequencies of CD27$^-$CD8$^+$ memory T cells producing IFN-γ, TNF-α, GM-CSF, and IL-2 as well as those expressing CD107a were higher in children with T1D

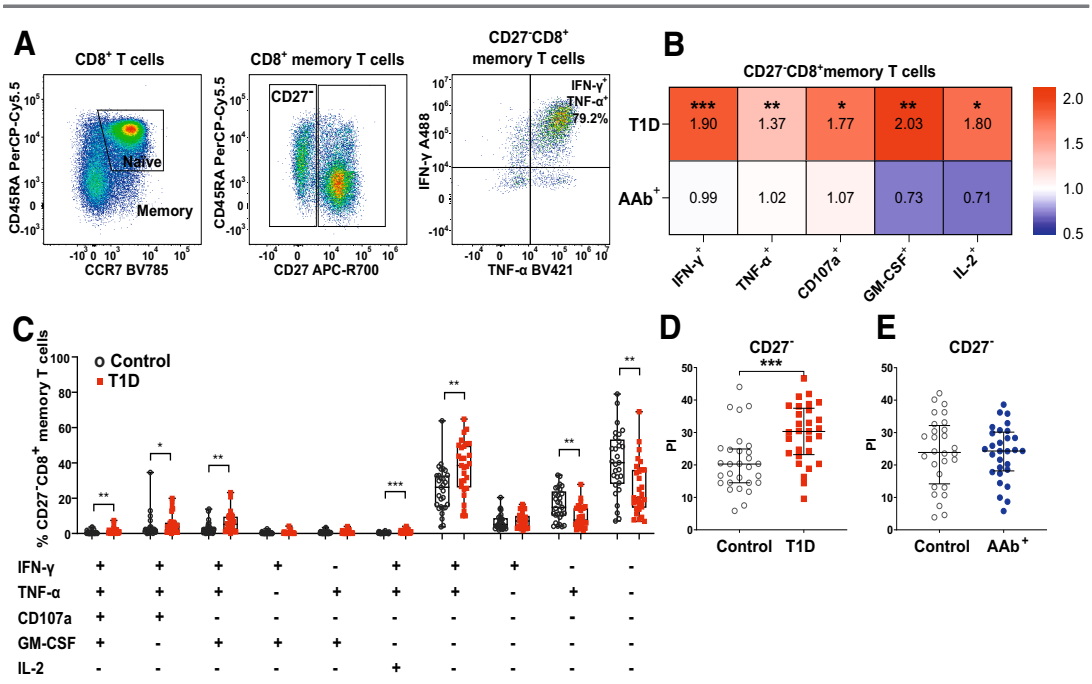

Figure 1—Frequencies of CD27$^-$CD8$^+$ memory T cells producing IFN-γ, TNF-α, CD107a, GM-CSF, and IL-2 are increased in children with newly diagnosed T1D. *A*: Representative gating strategy of CD8$^+$ memory T cells. Naïve (CD45RA$^+$CCR7$^+$) CD8$^+$ T cells were excluded, and only CD8$^+$ memory T cells were analyzed. Subsequently, memory T cells were gated according to CD27 expression into CD27$^+$ and CD27$^-$ subsets. *B*: Heat map of median fold differences of IFN-γ$^+$, TNF-α$^+$, CD107a$^+$, GM-CSF$^+$, and IL-2$^+$ CD27$^-$CD8$^+$ memory T-cell frequencies between T1D or AAb$^+$ children and matched control children. *C*: Frequency of CD27$^-$CD8$^+$ memory T cells expressing various combinations of CD107a and cytokines. Polyfunctionality index (PI) of CD27$^-$CD8$^+$ memory T cells in children with T1D and control children (*D*), and in AAb$^+$ children and control children (*E*). Mann-Whitney *U* test was used for statistical testing. Median and interquartile range are shown in *C*–*E*. *P < 0.05, **P < 0.01, ***P < 0.001. Control children are depicted as open gray circles, children with newly diagnosed T1D as solid red squares, and AAb$^+$ children as solid blue circles.

compared with control children (Fig. 1*B* and Supplementary Fig. 4) (P = 0.0003, P = 0.008, P = 0.003, P = 0.03 and P = 0.01, respectively). This increase was detected within both $CD27^-CD45RA^-$ TEM and $CD27^-CD45RA^+$ TEMRA subsets (Supplementary Fig. 4). However, within the less differentiated $CD27^+CD8^+$ memory T cells, no such increase in cytokine production or CD107a expression was detected in children with T1D (Supplementary Fig. 5). Moreover, the production of cytokines and expression of CD107a within both $CD27^-$ and $CD27^+CD8^+$ memory T cells between AAb^+ and control children was comparable (Fig. 1*B* and Supplementary Figs. 4 and 5).

Both age and CMV infection have a profound impact on the composition of the $CD8^+$ T-cell compartment in children (30). Importantly, the increased production of proinflammatory cytokines within $CD27^-CD8^+$ memory T cells in children with T1D remained significant even after taking age and CMV serostatus into account (Supplementary Fig. 6).

To investigate the polyfunctionality of $CD27^-CD8^+$ memory T cells in detail, we first used a Boolean gating strategy to determine different combinations of coexpression of different cytokines and CD107a. Significantly increased frequencies of IFN-γ^+TNF-α^+ (P = 0.001) and the minor populations of IFN-γ^+TNF-α^+GM-CSF$^{+/-}$ CD107a$^{+/-}$ cells were observed in children with T1D (Fig. 1*C*). The capacity of T cells to produce multiple cytokines can also be quantified through a calculated polyfunctionality index (PI) (24). PI of $CD27^-CD8^+$ memory T cells was increased in children with T1D (P = 0.001) but not in AAb^+ children compared with control children (Fig. 1*D* and *E*).

Interestingly, a similar increase in IFN-γ^+, TNF-α^+, GM-CSF$^+$, and IL-2$^+$ proinflammatory T cells (P = 0.02, P = 0.02, P = 0.01, and P = 0.04, respectively) was observed within $CD4^+CD27^-$ memory T cells (gated as $CD8^-CD27^-$) in children with T1D (Supplementary Fig. 7), demonstrating that highly differentiated $CD4^+$ T cells are also affected. Of note, using a separate staining panel containing additional cytokines produced by $CD4^+$ T cells (IL-4, IL-17A, and IL-21), we confirmed the increased production of IFN-γ (P = 0.008) within $CD45RA^-CCR7^-CD4^+$ TEM cells in children with T1D. Moreover, in line with previous studies (31,32), an increased production of IL-21 within $CD45RA^-CCR7^+CD4^+$CM cells (P = 0.04) was also noted in children with T1D (Supplementary Fig. 8).

In conclusion, the highly differentiated $CD27^-CD8^+$ memory T-cell subset in children with newly diagnosed T1D contains an increased frequency of polyfunctional cells producing multiple proinflammatory cytokines and expressing the degranulation marker CD107a. A similar phenomenon was also observed within the $CD27^-CD4^+$ memory T-cell subset.

AAb^+ Children Have a Higher Frequency of $CD27^-CD8^+$ Memory T Cells Expressing Coinhibitory Receptors KLRG1 and TIGIT

We next studied the expression of the coinhibitory surface receptors KLRG1, TIGIT, PD-1, and CD160 on $CD8^+$ and $CD4^+$ T cells in the pediatric cohort (Fig. 2).

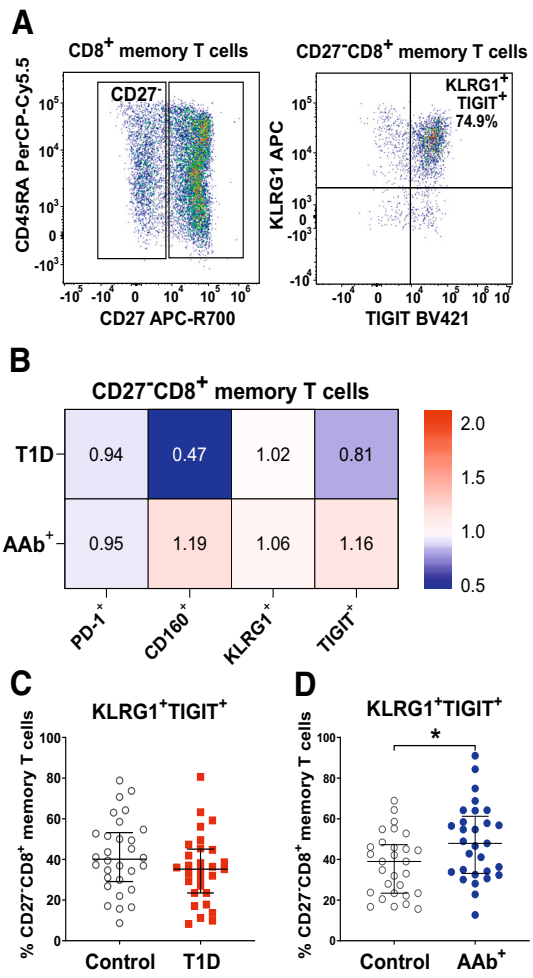

Figure 2—Frequency of $CD27^-CD8^+$ memory T cells coexpressing KLRG1 and TIGIT is increased in AAb^+ children. *A*: Representative gating strategy of $CD8^+$ memory T cells. Naïve ($CD45RA^+CCR7^+$) $CD8^+$ T cells were excluded, and only $CD8^+$ memory T cells were analyzed. Subsequently, memory T cells were gated according to CD27 expression into $CD27^+$ and $CD27^-$ subsets. *B*: Heat map of median fold differences of PD-1$^+$, CD160$^+$, KLRG1$^+$, and TIGIT$^+$ $CD27^-CD8^+$ memory T-cell frequencies between T1D or AAb^+ children and matched control children. Frequencies of $KLRG1^+TIGIT^+$ $CD27^-CD8^+$ memory T cells in children with T1D and control children (*C*) and in AAb^+ children and control children (*D*). Mann-Whitney *U* test was used for statistical testing. Median and interquartile range are shown. *$P < 0.05$. Control children are depicted as open gray circles, children with newly diagnosed T1D as solid red squares, and AAb^+ children as solid blue circles.

Again, the $CD27^+$ and $CD27^-$ memory T-cell subsets were studied separately (Fig. 2*A* and Supplementary Fig. 9). The frequency of $CD27^-CD8^+$ and $CD27^-CD4^+$ memory T cells expressing coinhibitory receptors were comparable in children with T1D and AAb^+ children compared with control children (Fig. 2*B* and Supplementary Fig. 10), with the exception of a tendency for higher frequency of both $KLRG1^+$ (P = 0.05) and $TIGIT^+$ (P = 0.08) cells within

CD27$^-$CD8$^+$ T cells (Supplementary Fig. 10). We also examined the frequency of KLRG1$^+$TIGIT$^+$ cells separately (Fig. 2*C* and *D*), as this marker combination has been associated both with slower progression of T1D after diagnosis and immunotherapy efficacy in recent studies (14–17). Interestingly, a higher frequency of KLRG1$^+$TIGIT$^+$ cells (P = 0.02) was observed in AAb$^+$ children compared with control children within CD27$^-$CD8$^+$ memory T cells but not within CD27$^-$CD4$^+$ memory T cells (Fig. 2*D* and Supplementary Fig. 10). After taking age and CMV serostatus into account, the increase in KLRG1$^+$TIGIT$^+$ CD27$^-$CD8$^+$ memory T cells remained significant (Supplementary Fig. 11). Of note, we also observed a higher frequency of PD-1$^+$ CD27$^+$CD8$^+$ and CD27$^+$CD4$^+$ memory T cells in AAb$^+$ children compared with control children (P = 0.007 and P = 0.03, respectively) (Supplementary Fig. 12).

In conclusion, AAb$^+$ children but not children with newly diagnosed T1D exhibited a signature consisting of higher expression of coinhibitory receptors KLRG1 and TIGIT within highly differentiated CD27$^-$CD8$^+$ memory T cells.

Proinflammatory or Coinhibitory CD8$^+$ T-Cell Signatures Are Not Detectable in Adults With Established T1D or in Children During the Seroconversion to Autoantibody Positivity

Next, we wanted to determine whether either the proinflammatory or coinhibitory signatures identified in children with T1D or AAb$^+$ children could be detected also at other stages of T1D. For this, we first analyzed a cohort of 20 adults with established T1D (mean time after diagnosis 11.2 ± 8.1 years) and 19 age-matched healthy control subjects. However, the frequencies of cytokine- and CD107a-producing and coinhibitory receptor–expressing CD27$^-$CD8$^+$ memory T cells were comparable between adults with T1D and control subjects (Supplementary Fig. 13).

We also analyzed rare longitudinal samples from 17 children collected 3–13 months apart before and after initial seroconversion to islet AAbs, alongside samples from healthy age-matched control children. However, no differences in the frequencies of cells producing proinflammatory cytokines or expressing coinhibitory receptors within the CD27$^-$CD8$^+$ memory T-cell subset were observed (Fig. 3).

In conclusion, the CD8$^+$ T-cell signature changes observed in children with T1D and AAb$^+$ children were not detected in samples from adults with established T1D or in samples collected during seroconversion to AAb positivity (representing the beginning of stage 1), suggesting that these signatures are associated with disease progression close to the diagnosis of T1D rather than early autoimmunity or a more established disease stage.

Proinflammatory Cytokine Expression in CD27$^-$CD8$^+$ Memory T Cells Correlates With Blood Glucose Levels in Children With Newly Diagnosed T1D

Next, we examined potential associations between the expression of proinflammatory cytokines and coinhibitory

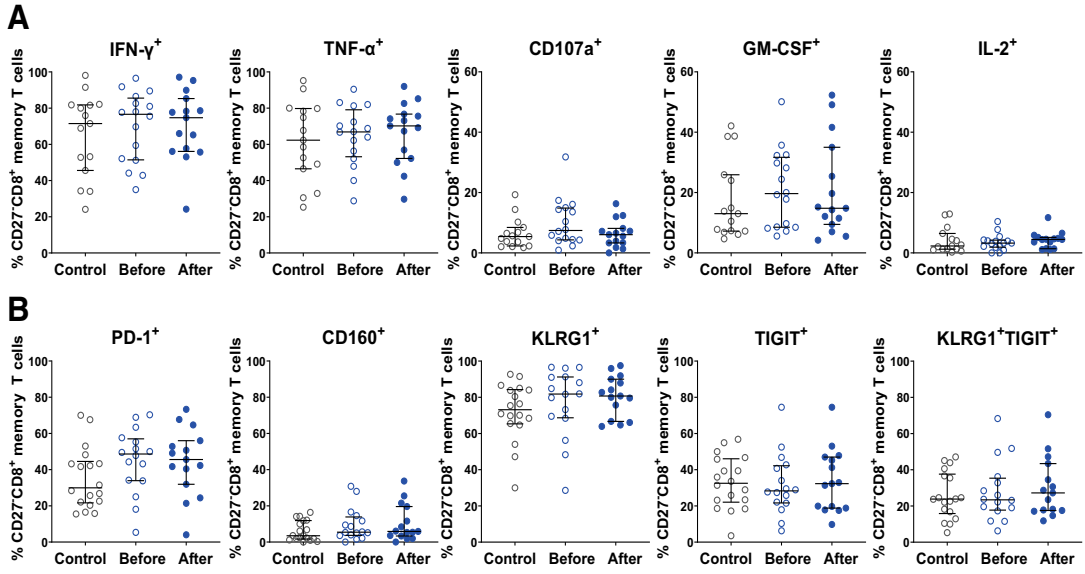

Figure 3—Frequencies of CD27$^-$CD8$^+$ memory T cells expressing cytokines, CD107a, and coinhibitory receptors in children before and after seroconversion to islet AAb positivity. Frequencies of IFN-γ$^+$, TNF-α$^+$, CD107a$^+$, GM-CSF$^+$, and IL-2$^+$ (*A*), and PD-1$^+$, CD160$^+$, KLRG1$^+$, TIGIT$^+$, and TIGIT$^+$KLRG1$^+$ (*B*) CD27$^-$CD8$^+$ memory T cells in children before and after seroconversion to islet AAb positivity and in control children. Kruskal-Wallis with Dunn multiple comparison test was used for statistical testing. Median and interquartile range are shown. Control children are depicted as open gray circles, and AAb$^+$ children as open (before seroconversion) or solid (after seroconversion) blue circles.

receptors within CD27$^-$CD8$^+$ memory T cells (Fig. 4*A*). Strong positive correlations were observed between the expression of different proinflammatory cytokines and the degranulation marker CD107a, and, separately, also between the expression of different coinhibitory receptors. However, no positive or negative correlations between the expression of the proinflammatory cytokines IFN-γ and TNF-α and coinhibitory receptors were observed (Fig. 4*A*).

In addition, we had clinical parameters (plasma glucose and plasma β-hydroxybutyrate levels at diagnosis) available from 24 children with T1D. Interestingly, plasma glucose levels at diagnosis correlated positively with the frequency of IFN-γ$^+$, TNF-α$^+$, and GM-CSF$^+$CD27$^-$CD8$^+$ memory T cells (Fig. 4*B* and *C*).

IFNG, *TNF*, *KLRG1*, and *TIGIT* Expression Is Colocalized Within a Subset of Cytotoxic CD27$^-$CD8$^+$ Memory T Cells

Finally, to further explore the phenotype of CD27$^-$CD8$^+$ memory T cells expressing proinflammatory cytokines and the coinhibitory receptors KLRG1 and TIGIT, we performed a single-cell multiomics analysis. For this, we sorted, from five children with T1D and five healthy control children, CD27$^-$ and CD27$^+$CD8$^+$ T cells and pooled them at a ratio of 1:1 to improve the analysis of the rare CD27$^-$ memory T cells at the single-cell level. We analyzed the cells both directly ex vivo and after a short stimulation with PMA and ionomycin, which enables a more sensitive detection of cytokine transcripts (33). We profiled the expression of 475 immune genes at the mRNA level together with the expression of 14 surface protein targets using the BD Rhapsody Single-Cell Analysis system (Supplementary Fig. 14). Stimulated CD8$^+$ T cells were initially clustered into six clusters that consisted of four CD27$^+$ clusters (clusters 1, 2, 3, and 4) representing naïve and less differentiated memory cells, with features of CM and type 1 CD8$^+$ T cells as well as mucosal-associated invariant T cells, and two CD27$^-$ clusters (clusters 5 and 6) of more highly differentiated cells (Fig. 5*A*–*C*). Similar clustering was obtained with both the unstimulated and stimulated samples (Fig. 5*A* and Supplementary Fig. 14).

Of the two CD27$^-$ clusters, cluster 5 represented cytotoxic effector CD8$^+$ T cells, with upregulated CX3CR1 surface protein as well as *NKG7*, *GNLY* (encoding granulysin), *GZMB* (encoding Granzyme B), and *PRF1* (encoding perforin) gene expression, all markers associated with cytolytic function. These cells also expressed *IFNG* and *TNF*. A smaller proportion of cells within this cluster also expressed *KLRG1*, *TIGIT*, and *EOMES*. Cluster 6 represented Killer Lectin Receptor (KIR) expressing TEMRA cells, as they had significantly upregulated KIR3DL1, CD39, and CX3CR1 surface protein expression as well as *KIR3DL1*, *KIR2DL1*, *KLRC1* (encoding NKG2A), and *HAVCR2* (encoding TIM-3) gene expression. In addition to expressing transcripts for KIR receptors, the cells in this cluster displayed upregulated *GZMB* and *PRF1* expression indicative of

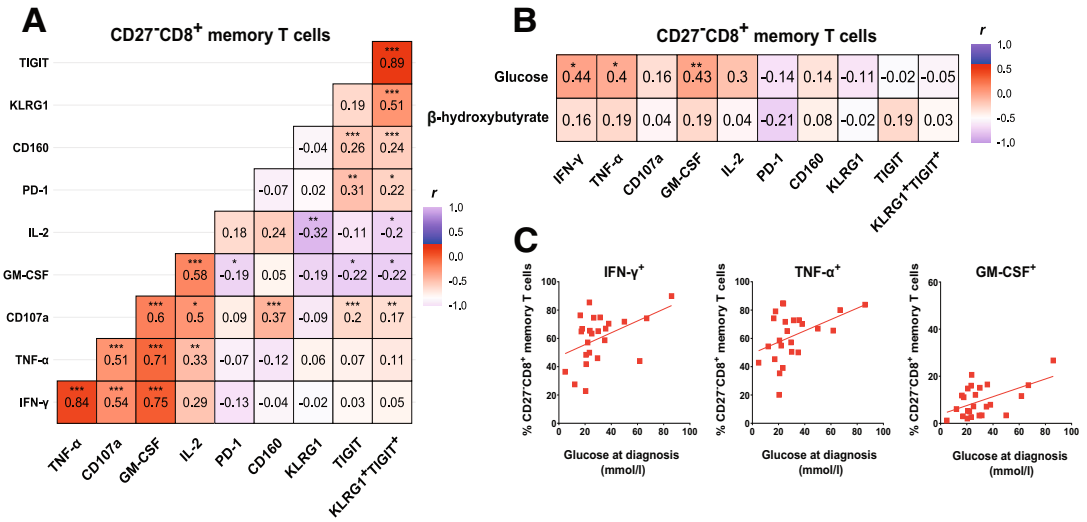

Figure 4—Plasma glucose levels at T1D diagnosis positively correlate with the frequency of CD27$^-$CD8$^+$ memory T cells expressing proinflammatory cytokines. *A*: Correlations between the frequencies of cytokine and coinhibitory receptor expressing CD27$^-$CD8$^+$ memory T cells was examined using Spearman correlation by pooling the data from the whole pediatric cohort into the analysis. *B*: Correlation between plasma glucose and β-hydroxybutyrate levels (mmol/L) at T1D diagnosis and the frequency of cytokine and/or coinhibitory receptor expressing CD27$^-$CD8$^+$ memory T cells was examined using Spearman correlation in children with T1D with data available (n = 24). *C*: Linear regression lines of frequencies of IFN-γ$^+$, TNF-α$^+$, and GM-CSF$^+$ CD27$^-$CD8$^+$ memory T cells vs. plasma glucose levels (mmol/L) in children with T1D. Correlation analyses in *A* and *B* are depicted as correlograms where Spearman R values are presented within the squares; statistical significance is indicated as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

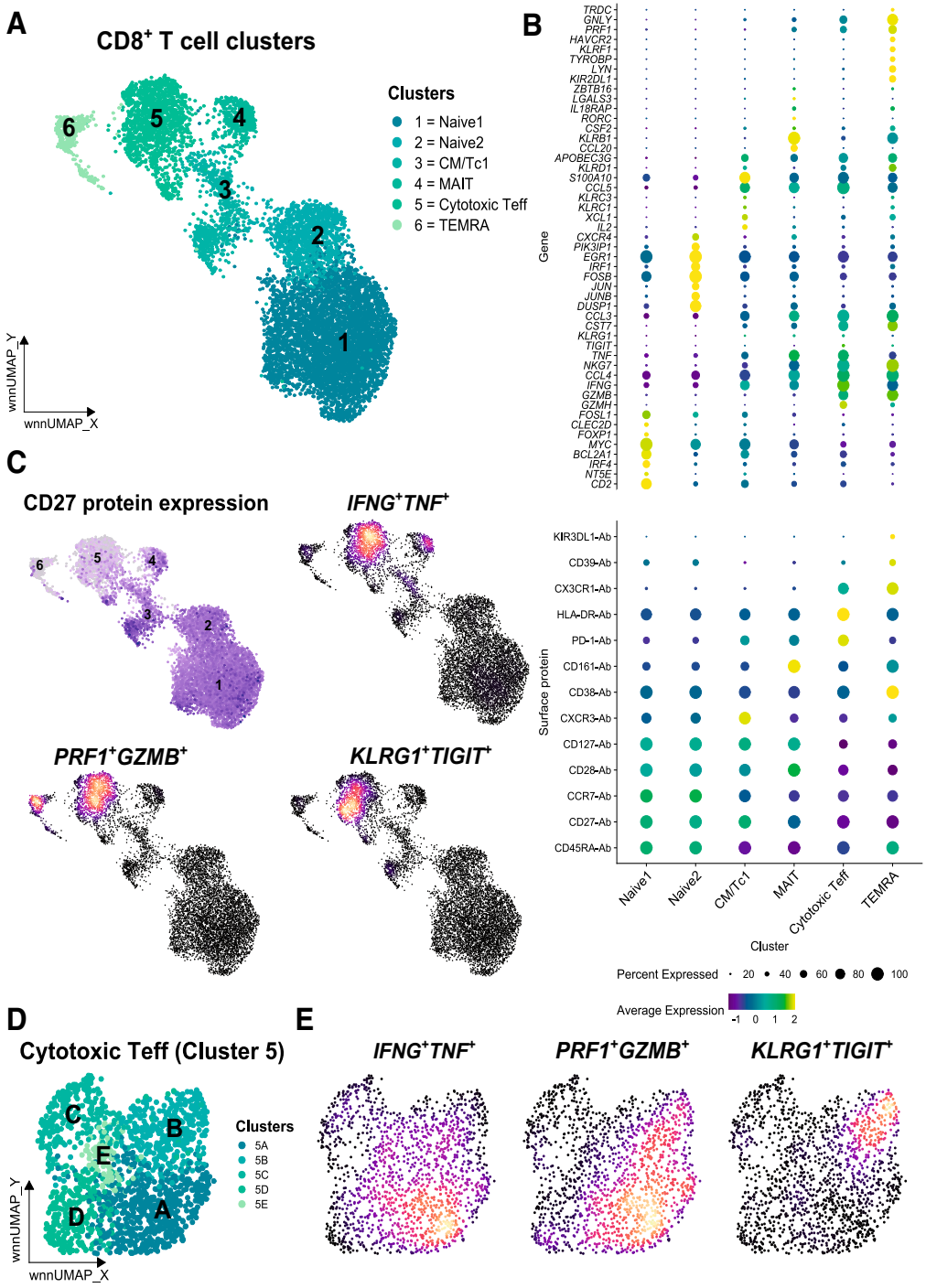

Figure 5—*IFNG*, *TNF*, *KLRG1*, and *TIGIT* expression is enriched within the cytotoxic CD27^{-}CD8^{+} memory T-cell cluster in single-cell multiomics analysis. *A*: CD8^{+} T cells were clustered into six clusters that represent canonical CD8^{+} T-cell subsets in a weighted nearest neighbor (WNN) uniform manifold approximation and projection (UMAP). *B*: Heat map of gene and surface protein expression within the various CD8^{+} T-cell clusters. *C*: Visualization of CD27 protein expression and coexpression of *IFNG* and *TNF*, *PRF1* and *GZMB*, and *KLRG1* and *TIGIT* within the various clusters. *D*: Reclustering of the cytotoxic T effector cluster (cluster 5) results in the identification of five subclusters. *E*: Coexpression of *IFNG* and *TNF*, *PRF1* and *GZMB*, and *KLRG1* and *TIGIT* within the subclusters. Wilcoxon rank sum test was used for statistical testing in the Seurat weighted nearest neighbor pipeline to identify significantly differently expressed genes within clusters.

cytolytic potential. However, *IFNG* and *TNF*, or *KLRG1* and *TIGIT*, were not significantly upregulated within this cluster (Fig. 5*A–C* and Supplementary Tables 7 and 8).

Since cluster 5 appeared to represent the molecular phenotype corresponding to our flow cytometry findings in $CD8^+CD27^-$ memory T cells, we reclustered these cells into five subclusters (Fig. 5*D*). Cells in cluster 5A displayed upregulated *IFNG* and *TNF* transcripts alongside *GZMB* and *PRF1* (Fig. 5*D* and *E*, Supplementary Fig. 15, and Supplementary Tables 9 and 10), possibly corresponding to the proinflammatory phenotype observed in $CD8^+CD27^-$ memory T cells in the flow cytometry data. Cells in cluster 5B, in contrast, displayed significantly upregulated *CD160*, *KIR2DL1*, *TIGIT*, and *KLRG1* expression (Fig. 5*D* and *E*, Supplementary Fig. 15, and Supplementary Tables 9 and 10). The subcluster 5B could therefore represent the $CD8^+$ $CD27^-$ memory T cells with coinhibitory signature in our flow cytometry data.

Cells in cluster 5C expressed *IL2*, *CCR7*, and *IL21* and had downregulated *GZMB*. In cluster 5D, upregulation of *NKG7* and *GZMH* (encoding Granzyme H) was detected alongside downregulation of *IFNG*. Finally, cells in cluster 5E expressed *IFNG* and had downregulated *GZMB*. CD27 protein expression was also higher in clusters 5C and 5E compared with the other clusters, suggesting a less differentiated phenotype (Supplementary Fig. 15 and Supplementary Tables 9 and 10). Due to the small number of samples analyzed, we did not observe any statistical differences in the frequencies of cells within the subclusters between children with T1D and control children (Supplementary Fig. 15).

In conclusion, highly differentiated $CD27^-CD8^+$ memory T cells expressed genes consistent with cytolytic and proinflammatory function but also genes for coinhibitory receptors. *IFNG* and *TNF* expression colocalized with *GZMB* and *PRF1* expression within the cytotoxic cluster, and expression of *KLRG1* and *TIGIT* was also detected within the same cluster. However, in more detailed analysis of this cluster, the proinflammatory signature and coinhibitory signature appeared to localize in different cells at the single-cell level.

DISCUSSION

In this study, we analyzed circulating $CD8^+$ T cells at different stages of T1D progression. We observed two distinct $CD8^+$ T-cell signatures within highly differentiated $CD27^-CD8^+$ memory T cells: a proinflammatory signature in children with newly diagnosed T1D (stage 3) and a coinhibitory $KLRG1^+TIGIT^+$ signature in AAb^+ children who later progressed to T1D (stage 1).

The proinflammatory signature in children with T1D consisted of a higher frequency of polyfunctional $CD27^-$ $CD8^+$ memory T cells expressing the proinflammatory cytokines IFN-γ and TNF-α, and, to a lesser extent, also GM-CSF and IL-2 as well as the degranulation marker CD107a. We observed a minor, albeit significant, positive correlation between the frequency of IFN-γ^+, TNF-α^+, and GM-CSF^+ $CD27^-CD8^+$ T cells and plasma glucose levels, suggesting a possible association of the proinflammatory signature with the level of dysglycemia at the onset of T1D. The proinflammatory signature appears likely to disappear with time, as the frequency of cytokine-producing $CD27^-CD8^+$ T cells in adults with established T1D was comparable to age-matched control subjects.

The second signature was represented by a higher frequency of $KLRG1^+TIGIT^+$ expressing $CD27^-CD8^+$ memory T cells in AAb^+ children who later progressed to T1D. This phenomenon appeared to be associated with later stages of autoimmunity, since these cells were not expanded during the time of initial seroconversion, that is, the earliest identifiable stage of autoimmunity before stage 1. Interestingly, a highly similar $CD8^+$ T-cell phenotype has been recently observed both in T1D patients with a slower disease progression after diagnosis (14) and in patients and at-risk individuals showing a better treatment response in clinical trials with the teplizumab and alefacept mAbs (15–17). These $KLRG1^+TIGIT^+$ $CD8^+$ T cells have been suggested to be partially exhausted since they exhibit a reduced proliferative capacity while still retaining some functionality (14,15). Of note, we also observed an increased expression of another coinhibitory receptor, PD-1, within the less differentiated $CD27^+CD8^+$ memory T cells in AAb^+ children, which could reflect the same phenomenon as the increase of $KLRG1^+TIGIT^+$ within the highly differentiated $CD27^-CD8^+$ T cells.

An exhausted $CD8^+$ T-cell signature in blood could be beneficial for autoimmunity in general, since, in a previous study, it has been associated with a better outcome for multiple autoimmune or inflammatory diseases but, conversely, a worse outcome for infectious diseases (34). Given the beneficial association observed for the $KLRG1^+$ $TIGIT^+$ $CD8^+$ T-cell signature in other studies, it can be speculated that, in AAb^+ children, the increase of coinhibitory receptor expression on $CD8^+$ T cells could represent an effort to contain the ongoing autoimmunity before the symptomatic onset of T1D. Then, at the onset of T1D, a proinflammatory, potentially pathogenic $CD8^+$ T-cell signature becomes dominant. Interestingly, a study in the NOD mouse model demonstrated that most $CD8^+$ T cells that infiltrate the islets are non–autoantigen specific and their presence in the islets actually suppresses autoimmunity (35). It can thus be speculated that the polyclonal expansion of $KLRG1^+TIGIT^+$ $CD8^+$ T cells in circulation could also, in humans, reflect a similar, protective phenomenon at the level of inflamed islets during the preclinical stages of the disease.

An important outstanding question is whether the IFN-γ^+TNF-α^+ proinflammatory and $KLRG1^+TIGIT^+$ $CD27^-CD8^+$ T cells represent a phenotypic shift within the same $CD8^+$ T-cell population or whether they represent different developmental lineages. Here, we could not demonstrate any correlation between the frequencies of these $CD8^+$ T-cell subsets in blood. Moreover, using a

single-cell multiomics approach, we demonstrated that *IFNG*, *TNF*, *KLRG1*, and *TIGIT* transcripts are expressed within a cluster of $CD27^-CD8^+$ T cells that also exhibit cytotoxic capacity (*GZMB*, *PRF1*). However, upon closer analysis, it appears that the cells expressing *IFNG*, *TNF*, *GZMB*, and *PRF1* may be distinct from those expressing *KLRG1* and *TIGIT* at the single-cell level. In the future, analysis of longitudinal blood samples together with T-cell receptor profiling would be needed to conclusively address how the phenotype of individual $CD27^-CD8^+$ T cells shifts during progression from preclinical to clinical T1D.

Our study has a few caveats that need to be considered. First, the study was exploratory in nature, and the results need confirmation in additional cohorts. Second, we used frozen PBMCs in our analyses, which potentially could impact $CD8^+$ T-cell phenotypes as compared with analysis of fresh blood samples. Potentially, the genetic background of the individuals could also have an effect on $CD8^+$ T-cell phenotypes, which should be addressed in the future. Third, it is important to emphasize that the $CD8^+$ T-cell phenotypes were observed at the level of total $CD8^+$ T cells, and additional studies would be needed to validate whether autoreactive $CD8^+$ T cells also exhibit similar changes. However, an exhausted $CD8^+$ T-cell phenotype has been observed at the level of both autoantigen-specific and total $CD8^+$ T cells in T1D patients with a slower disease progression after diagnosis (14), which supports the idea that global $CD8^+$ T-cell changes may also reflect changes in autoantigen-specific $CD8^+$ T cells. Finally, a detailed analysis of $CD8^+$ T-cell phenotypes at the level of inflamed islets at different stages of T1D progression would provide important additional insight for the role of distinct $CD8^+$ T-cell phenotypes in T1D pathogenesis.

In addition to $CD8^+$ T cells, we also analyzed the $CD4^+$ T-cell compartment in parallel. Importantly, we noted that proinflammatory signature observed within $CD27^-CD8^+$ memory T cells in children with T1D was also detected within $CD27^-CD4^+$ memory T cells, suggesting a broader functional alteration of highly differentiated T cells at T1D onset. However, the $KLRG1^+TIGIT^+$ signature detected in AAb^+ children appears to be unique to $CD8^+$ T cells, since no increase in $KLRG1^+TIGIT^+$ $CD27^-CD4^+$ T cells was observed. Within $CD4^+$ memory T cells, we also observed an increased expression of both PD-1 and IL-21, potentially explained by the increased follicular helper T-cell signature previously reported by us and others in individuals with T1D and AAb^+ at-risk children (22,23,31,32).

Taken together, we present here a comprehensive characterization of $CD8^+$ T cells at different stages of T1D progression. A coinhibitory $KLRG1^+TIGIT^+$ signature within highly differentiated $CD27^-CD8^+$ T cells was increased in AAb^+ children (stage 1), who later progressed to clinical T1D. In contrast, a proinflammatory signature was observed in children with newly diagnosed T1D (stage 3). We were not able to detect these signatures at the earliest definable stage of autoimmunity, during seroconversion to autoantibody positivity, or in adults with established disease. Collectively, our results imply that these $CD8^+$ T-cell signatures are transient in nature and are associated with distinct disease stages close to the clinical diagnosis. Taken together, our results support the concept that $CD8^+$ T-cell signatures could have potential as biomarkers of disease progression, in a similar way as they appear to reflect a clinical response to immunotherapy.

Acknowledgments. The authors thank the skillful technical assistance of Hanna Eskelinen (University of Eastern Finland), Anne Suominen (University of Turku), and Matti Laitinen (Kuopio University Hospital). We also thank Andrea Hanel (University of Eastern Finland), Merja Heinäniemi (University of Eastern Finland), and Yujuan Gui (BD Biosciences) for help with the single-cell multiomics analyses, and Laurène Pfajfer (University of Eastern Finland) for help with flow sorting. Part of the work was carried out with the support of University of Eastern Finland Cell and Tissue Imaging Unit, University of Eastern Finland. Next generation sequencing was performed in Finnish Functional Genomics Centre Facility supported by University of Turku, Åbo Akademi University and Biocenter Finland.

Funding. This work was supported by the Academy of Finland (decision no. 331282) and the Sigrid Jusélius Foundation.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. A.-M.S., V.P., I.E., D.S., and M.R.-N. performed the experiments. R.R., J.P., J.T., and J.L. provided the clinical samples. M.K. and R.V. were responsible for the islet autoantibody analyses of the pediatric cohort. J.I. and J.L. were responsible for the HLA screening of the pediatric study subjects. A.-M.S., V.P., M.R.-N., and T.K. analyzed the data. T.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented at the 19th Congress of the Immunology of Diabetes Society 2023, Paris, France, 25 May 2023, and at the 49th Annual Meeting and Summer School of the Scandinavian Society for Immunology, Turku, Finland, 6 June 2023.

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II

The frequency but not the phenotype of circulating peripheral T helper cells is increased at later stages of progression to type 1 diabetes

Schroderus AM, Hanel A, Vandamme C, Pitkänen V, Rytkönen-Nissinen M, Heinäniemi M, Knip M, Veijola R, Toppari J, Ilonen J, Lempainen J, Kinnunen T

Submitted

III

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Frontiers in Immunology: 14: 1157265, 2023,
https://doi.org/10.3389/fimmu.2023.1157265

frontiers | Frontiers in Immunology

TYPE Brief Research Report
PUBLISHED 21 June 2023
DOI 10.3389/fimmu.2023.1157265

OPEN ACCESS

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RECEIVED 02 February 2023
ACCEPTED 05 June 2023
PUBLISHED 21 June 2023

CITATION
Schroderus AM, Poorbaugh J, McElyea S, Beasley S, Zhang L, Näntö-Salonen K, Rintamäki R, Pihlajamäki J, Knip M, Veijola R, Toppari J, Ilonen J, Benschop RJ and Kinnunen T (2023) Evaluation of plasma IL-21 as a potential biomarker for type 1 diabetes progression. *Front. Immunol.* 14:1157265. doi: 10.3389/fimmu.2023.1157265

Evaluation of plasma IL-21 as a potential biomarker for type 1 diabetes progression

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IL-21 is a multifunctional cytokine linked with the pathophysiology of several autoimmune diseases, including type 1 diabetes. In this study, our aim was to examine plasma IL-21 levels in individuals at different stages of type 1 diabetes progression. We measured plasma IL-21 levels, as well as levels of other key pro-inflammatory cytokines (IL-17A, TNF-α and IL-6), from 37 adults with established type 1 diabetes and 46 healthy age-matched adult controls, as well as from 53 children with newly diagnosed type 1 diabetes, 48 at-risk children positive for type 1 diabetes-associated autoantibodies and 123 healthy age-matched pediatric controls using the ultrasensitive Quanterix SiMoA technology. Adults with established type 1 diabetes had higher plasma IL-21 levels compared to healthy controls. However, the plasma IL-21 levels showed no statistically significant correlation with clinical variables, such as BMI, C-peptide, HbA1c, or hsCRP levels, evaluated in parallel. In children, plasma IL-21 levels were almost ten times higher than in adults. However, no significant differences in plasma IL-21 levels were detected between healthy children, autoantibody-positive at-risk children, and children with newly diagnosed type 1 diabetes. In conclusion, plasma IL-21 levels in adults with established type 1 diabetes were increased, which may be associated with autoimmunity. The physiologically high plasma IL-21 levels in children may, however, reduce the potential of IL-21 as a biomarker for autoimmunity in pediatric subjects.

KEYWORDS

autoimmunity, autoimmune diseases, interleukin-21, IL-21, human, type 1 diabetes

1 Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease, in which autoreactive T cells mediate the progressive destruction of insulin-producing beta cells in the pancreas. T1D is preceded by a preclinical phase of variable length, during which circulating autoantibodies (AAb) to islet autoantigens are almost invariably detected (1). It is well-established that T cells play a central role in the T1D disease process (2). However, the exact T-cell phenotypes or immune pathways involved remain largely elusive. Recently, the interleukin-21 (IL-21) pathway has gained attention, as it has been associated with the development of T1D both in murine models (3) as well as in human studies (4–6).

IL-21 is a pleiotropic cytokine with multiple functions. It is a hallmark cytokine of $CD4^+CXCR5^+$ T follicular helper (Tfh) cells (7, 8). In addition, it is produced by Th17 cells (7) and a recently identified population, coined T peripheral helper (Tph) cells (9, 10). IL-21 has a fundamental role in B-cell helper functions. In order to activate, expand and produce antibodies, B cells are largely dependent on the help provided by IL-21-producing Tfh cells in lymph nodes (7, 8), and possibly also by Tph cells within inflamed tissues (10). Importantly, IL-21 is also known to support the effector functions and cytotoxicity of $CD8^+$ T cells (7). IL-21 produced by $CD4^+$ T cells may therefore contribute to the T1D disease process also by activating autoreactive $CD8^+$ T cells in the pancreas (11–13).

Interestingly, an expansion of circulating Tfh and Tph cells and increased IL-21 production by T cells has been observed both in children with newly diagnosed T1D (6, 14), as well as in adults with established T1D (4, 5). Moreover, Th17 cells, another potential source of IL-21, appear to be increased both in the blood and pancreatic lymph nodes of patients with T1D (15, 16). These findings implicate the IL-21 pathway to be an appealing candidate for immunotherapy of T1D. Promisingly, in a recently completed phase 2 trial, an anti-IL-21 antibody combined with liraglutide was shown to preserve beta-cell function in patients with recent-onset T1D (17).

Given the importance of IL-21 for autoimmunity, blood IL-21 levels could serve as a potential biomarker of disease activity in autoimmune disorders. Indeed, in some autoimmune diseases, such as Sjögren's syndrome (18) and systemic lupus erythematosus (SLE) (19), plasma and/or serum IL-21 levels are elevated in patients. Recently, limitations in the specificity and sensitivity of commercially available human IL-21 ELISA kits have been identified, and as a result, a new ultrasensitive assay for detecting IL-21 based on the Quanterix SiMoA (Single Molecule Array) technology was developed (20). With this assay, it was demonstrated that IL-21 concentrations in plasma and serum are considerably lower than previously published data using less sensitive and less specific assays suggested. Importantly, higher plasma IL-21 levels in patients with Sjögren's syndrome and SLE compared to healthy individuals were also validated with this ultrasensitive assay (20).

To our knowledge, plasma IL-21 levels have previously been investigated in a limited number of studies related to T1D, all of which have suggested that patients with T1D (21, 22) and AAb^+ at-risk children (23) have higher IL-21 levels in blood than controls. In this study, we analyzed plasma IL-21, as well as levels of other key pro-inflammatory cytokines (IL-17A, TNF-α and IL-6) with the ultrasensitive Quanterix SiMoA technology at different stages of T1D development, utilizing samples from cross-sectional cohorts of adults with established T1D, children with newly diagnosed T1D, and AAb^+ at-risk children together with age-matched controls.

2 Materials and methods

2.1 Study subjects

The adult cohort comprised 37 patients with established T1D and 46 healthy age-matched controls (Table 1). The pediatric cohort consisted of 53 children with newly diagnosed T1D (0 to 7 days after clinical diagnosis), 48 autoantibody-positive (AAb^+) at-risk children, and 123 healthy control children who were autoantibody-negative and age-matched with the T1D and AAb^+ cases (Table 2). The AAb^+ and healthy control children participated in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) follow-up study and carried HLA genotypes associated with increased risk for T1D. Autoantibody-positivity was analyzed, as previously described (24). Autoantibody-positivity was defined based on the presence of one or more biochemical autoantibodies (insulin autoantibodies [IAA], insulinoma-associated-2 antibodies [IA-2A] and glutamic acid decarboxylase antibodies [GADA]).

Plasma samples were collected between May 2013 and January 2016 for the pediatric cohort at the DIPP study center in Turku, Finland, and between February 2012 and March 2020 for the adult cohort at the University of Eastern Finland, Kuopio, Finland. Plasma was collected from heparinized peripheral blood samples after centrifugation at 700 x g for 10 min and stored at -80°C until analysis.

All participants and/or their legal guardians provided written informed consent, as mandated by the Declaration of Helsinki. The study was approved by local ethics committees of the University Hospitals of Turku and Kuopio, and for the DIPP study by the ethics committee of the Hospital District of Northern Ostrobothnia.

2.2 Quanterix SiMoA assays

The heparin plasma samples were randomized for plating, then thawed and aliquoted in batches to reduce freeze-thaw cycles. For all samples, assessment of IL-21 levels was performed using the Quanterix SiMoA assay as previously reported (20). Assessment of IL-17A, IL-6 and TNF-α levels was performed using the Cytokine 3 Plex B Quanterix SiMoA assay, according to manufacturer's instructions.

The spike recoveries were determined by comparing the spike samples of the observed back-calculated concentration from calibration curve against known spiked concentration. Limits of detection (LODs) were determined by the selection criteria of 75%–125% of the calibration recovery (i.e., comparing the calibration samples of the calculated concentration to expected concentration). Limits of Quantitation (LOQs) were determined by the selection criteria of 75%–125% of the spike recovery (i.e., comparing the spike samples of the calculated concentration to expected

concentration). The analyses for the adult cohort were performed in two batches, with slightly different LOD and Lower Limit of Quantitation (LLOQ) values, and batch correction was calculated using linear model in R (25).

2.3 C-peptide and hsCRP measurements

C-peptide was measured from heparinized plasma with electrochemiluminescence immunoassay (detection limit 0.007 nmol/L, Cobas, Roche Diagnostics) and high-sensitivity C-reactive protein (hsCRP) from heparinized plasma with particle enhanced immunoturbidimetric assay (detection limit 0.15 mg/L, Cobas, Roche Diagnostics).

2.4 Statistical analyses

Graphpad Prism version 9.1.0 (GraphPad Software, San Diego, California USA) was used for statistical analyses. Kruskal-Wallis test followed by Dunn's multiple comparison test was applied when comparing more than two groups. Mann-Whitney test or Wilcoxon matched-pairs signed-rank test was used when comparing two groups. No power calculations were made as the study was exploratory in nature with very limited previous data to support the calculations, and sample sizes were determined in part by feasibility. Spearman's correlation was calculated when relationships between plasma cytokine concentrations and different variables were assessed. Simple linear regression was used to calculate regression lines for each group. Slopes and intercepts of the regression lines were compared, and two-tailed P-values were calculated for them in Prism. P-values <0.05 were considered to indicate statistical significance.

3 Results

3.1 Plasma IL-21 levels are higher in adults with established type 1 diabetes than in healthy controls

Plasma IL-21, as well as IL-17A, TNF-α, and IL-6 levels were analyzed in 37 adults with established T1D and in 47 age-matched healthy controls. Plasma IL-21 levels were 2.5-fold higher in T1D patients (median 0.05 pg/mL) compared to healthy controls (median 0.02 pg/mL, $P < 0.001$, Figure 1A). Plasma IL-6 levels were also elevated in T1D patients compared to controls (median 1.03 pg/mL vs. 0.61 pg/mL, $P < 0.05$), but plasma IL-17A and TNF-α levels were similar between the study groups (Supplementary Figure 1).

Next, we examined whether selected clinical variables were associated with plasma IL-21 levels. No correlations were observed between age, hsCRP, C-peptide levels, HbA1c values, body-mass index (BMI), or disease duration with plasma IL-21 levels in the T1D patients (Figure 1B, Supplementary Figure 1). However, plasma IL-21 levels correlated positively with both TNF-α ($P < 0.001$, r=0.40) and IL-6 ($P < 0.05$, r=0.27) levels. Moreover, IL-17A levels correlated with TNF-α ($P < 0.001$, r=0.57) and IL-6 ($P < 0.001$, r=0.60) levels, while TNF-α levels correlated with hsCRP ($P < 0.05$, r=0.26) (Supplementary Figure 1).

3.2 Plasma IL-21 levels are similar in children with newly diagnosed type 1 diabetes, children at-risk for type 1 diabetes and healthy controls

Next, we analyzed the plasma cytokine levels in samples from 53 children with newly diagnosed T1D and 48 AAb^+ at-risk children, as well as from 123 healthy age-matched controls. In the pediatric

TABLE 1 Characteristics of the adult cohort.

Study group	Healthy controls	T1D patients
n	46	37
Age (years), mean ± SD	26.1 ± 4.3	26.6 ± 6.4
Age range (years)	20–38	18–39
Disease duration (years), mean ± SD	N/A*	11.7 ± 8.1
Disease duration range (years)	N/A	0–36
Male	40% (20/46)	57% (21/37)
Female	60% (26/46)	43% (16/37)
Clinical variables:		
HbA1c (mmol/mol), mean ± SD	ND†	64.6 ± 13.7 (n=32)
C-peptide (nmol/L), mean ± SD	ND	0.11± 0.17
BMI (kg/m^2), mean ± SD	ND	26.6 ± 5.1 (n=29)
hsCRP (mg/L), mean ± SD	1.31± 1.96	3.15 ± 5.14

*not applicable, †not determined.

cohort, plasma IL-21 levels (Figure 2A), as well as IL-17A, TNF-α, and IL-6 levels Supplementary Figure 2) were similar between children with T1D, AAb^+ at-risk children, and healthy controls. Of note, plasma IL-21 levels in children (median 0.26 pg/mL for the whole pediatric cohort) were around ten-fold higher compared to those observed in the adult cohort (Figures 1A, 2A). Accordingly, a strong negative correlation between age and IL-21 levels ($P < 0.001$, r=-0.35), as well as to a lesser extent between age and IL-17A ($P < 0.01$, r=-0.20) and TNF-α ($P < 0.001$, r=-0.24) levels, was observed in the pediatric cohort (Figure 2B, Supplementary Figure 2). However, even after stratification with age, no differences in plasma IL-21 levels between the pediatric study groups were detected (Figure 2B, Supplementary Figure 2). Finally, these results were further corroborated by a stringent pairwise analysis of a subset of samples from T1D and AAb^+ children that were drawn and processed in parallel with a sample from an age-matched healthy control child on the same day (Supplementary Figure 3). In addition, we analyzed correlation between selected clinical variables and plasma cytokine levels in children with newly diagnosed T1D. We did not observe correlations between cytokine levels and clinical variables at diagnosis (plasma glucose, HbA1c, blood pH, or beta-hydroxybutyrate levels) (Supplementary Figure 2). Of note, similar to the adult cohort, IL-17A levels correlated with TNF-α ($P < 0.001$, r=0.66) and IL-6 ($P < 0.001$, r=0.48) levels also in the pediatric cohort (Supplementary Figure 2).

We have previously observed that the frequency of both circulating Tfh (6) and Tph (14) cells was increased in children with newly diagnosed T1D positive for at least two AAbs but not in those positive for one AAb or none. Hence, we next stratified both AAb^+ children and children with T1D into two subgroups based on the number of autoantibodies detected (≤1 or ≥2) in these children. However, plasma IL-21, as well as IL-17A, TNF-α, and IL-6 levels were again similar in AAb^+ children and children with T1D positive for either ≤1 or ≥2 autoantibodies (Figures 2C, D, Supplementary Figure 4). In addition, we analyzed whether plasma cytokine levels differed between those AAb^+ at-risk children who had progressed to T1D (mean time to T1D after sampling 2.6 ± 1.4 years) and those who had not. However, again, no differences were detected between the groups (Figure 2E, Supplementary Figure 5).

Finally, flow cytometry data were available on selected T-cell subset frequencies for the pediatric samples (6, 26). Hence, we examined the correlation between plasma IL-21 levels and selected T-cell subset frequencies. The frequencies of activated Tfh cells ($PD\text{-}1^+ICOS^+$ of $CXCR5^+$ memory $CD4^+$ T cells) (6) positively correlated with plasma IL-21 levels ($P < 0.001$, r=0.29, Figure 2F). A weaker positive correlation was also observed with the frequency of

TABLE 2 Characteristics of the pediatric cohort.

Study group	Healthy children	AAb^+ at-risk children	Newly diagnosed T1D patients
n	123	48	53
Age (years), mean ± SD	8.9 ± 4.0	8.6 ± 4.7	8.5 ± 3.8
Age range (years)	2.0–15.7	2.0–17.2	2.4–17.5
Male	63% (77/123)	54% (26/48)	51% (27/53)
Female	37% (46/123)	46% (22/48)	49% (26/53)
Progressors*	N/A†	52% (25/48)	N/A
Time (years) from sampling to clinical T1D, mean ± SD	N/A	2.6 ± 1.4	N/A
Time (years) from birth to clinical T1D, mean ± SD	N/A	10.0 ± 4.1	N/A
Autoantibodies:			
GADA	N/A	63% (30/48)	68% (36/53)
IA-2A	N/A	67% (32/48)	75% (40/53)
IAA	N/A	48% (23/48)	25% (13/53)
Number of autoantibodies:			
≤1 AAb	N/A	31% (15/48)	30% (16/53)
≥2 AAb	N/A	69% (33/48)	70% (37/53)
Clinical variables at diagnosis:			
Plasma glucose (mmol/L), mean ± SD	N/A	N/A	24.3 ± 10.7 (n=43)
HbA1c (mmol/mol), mean ± SD	N/A	N/A	79.0 ± 30.1 (n=31)
Blood pH, mean ± SD	N/A	N/A	7.34 ± 0.11 (n=42)
Beta-hydroxybutyrate (mmol/L), mean ± SD	N/A	N/A	2.83 ± 2.79 (n=44)

*individuals, who have progressed from AAb^+ stage to clinical T1D during follow-up, †not applicable.

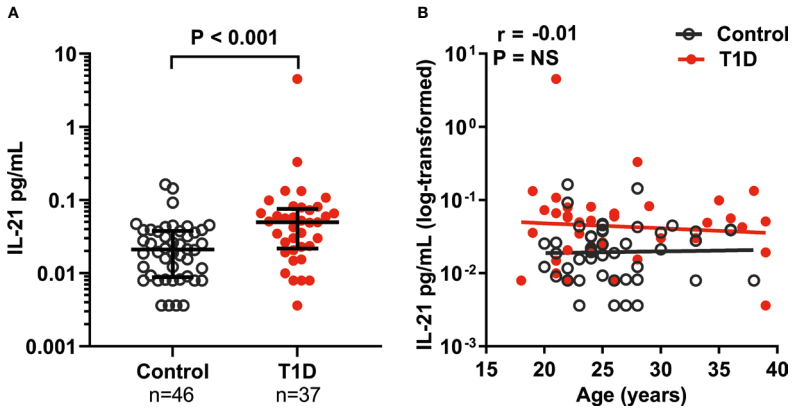

FIGURE 1
Plasma IL-21 levels are elevated in adults with T1D. **(A)** Plasma IL-21 levels in adults with established T1D and healthy controls. Mann-Whitney U-test was used for statistical analysis. **(B)** Correlation between age and $\log_{10}$-transformed plasma IL-21 levels was examined using Spearman's correlation by pooling the data from both study groups and is expressed together with the P value on the plot. The elevations of the linear regression lines were significantly different between the study groups (P = 0.001). Medians and IQRs are shown in the **(A)**.

regulatory T cells (Treg; $CD25^{+}CD127^{low}$ of $CD4^{+}$ T cells) (26) and plasma IL-21 levels ($P < 0.05$, r=0.17, Supplementary Figure 6). However, no correlation was observed between the frequency of Th17 cells ($CCR6^{+}CXCR3^{-}$ of memory $CD4^{+}$ T cells) (26) and plasma IL-21 levels (Supplementary Figure 6).

4 Discussion

In this study, we demonstrated that adults with established T1D had significantly elevated plasma IL-21 and IL-6 levels compared to age-matched controls. IL-21 levels did not correlate with the time from diagnosis or C-peptide levels, a marker of residual beta-cell function. Moreover, no correlation between plasma IL-21 levels and HbA1c levels, a marker related to glycemic control, or hsCRP, a marker related to inflammation, was observed. Therefore, the elevated plasma IL-21 levels do not appear to be associated with the extent of beta-cell destruction, hyperglycemia itself, or with underlying inflammation in T1D patients in the cohort studied.

In the pediatric cohort, we did not observe differences in plasma IL-21 levels between patients with newly diagnosed T1D, AAb^{+} at-risk children, and healthy age-matched children. Similar to adults, no correlation between plasma IL-21 levels and clinical variables, such as level of hyperglycemia (blood glucose and HbA1c levels at diagnosis) or markers of ketoacidosis (blood pH and beta-hydroxybutyrate levels) were found in children with T1D. Moreover, no associations between plasma IL-21 levels and autoantibody status or the risk of progression to clinical T1D in AAb^{+} at-risk children were observed.

An important observation in our study was that plasma IL-21 levels appear to be physiologically around ten-fold higher in children than in adults. Importantly, for the other cytokines, IL-17A, TNF-α, and IL-6, assessed in this study, no such phenomenon was observed. This observation could potentially explain the discrepancy that higher plasma IL-21 levels were observed only in adult T1D patients but not in children with T1D. As the plasma IL-21 levels were only modestly elevated in adult T1D patients compared to controls, a corresponding small change would be masked by the considerably higher physiological background levels in children. Another interesting finding in the pediatric cohort was that plasma IL-21 levels correlated with the frequency of activated Tfh cells in blood, potentially implicating Tfh cells as a major source of plasma IL-21.

Three previous studies have reported slightly elevated plasma IL-21 levels in both adult and pediatric patients with T1D (21, 22), as well as in AAb^{+} at-risk children (23). However, in these studies either ELISA or Luminex xMAP technologies were used, and the IL-21 levels reported were >100-fold higher than in our study using the ultrasensitive Quanterix SiMoA technology. Previous validation results strongly suggest that these conspicuously higher IL-21 levels reported in the previous studies may not accurately reflect physiological cytokine levels, as these older methods lack both the sensitivity and specificity required to detect endogenous IL-21 levels reliably (20).

In addition to using an ultrasensitive analysis method, the major strengths of our study are the large cohort sizes and the stringent matching with HLA background, age, and sampling date in the pediatric cohort, which all considerably strengthen the validity of our results. One caveat of our study is that we were only able to analyze cross-sectional cohorts. In the future, longitudinal analyses could potentially better detect subtle intraindividual alterations in cytokine levels occurring during T1D progression. Moreover, the analysis of patients with type 2 diabetes could help confirm whether the increased plasma IL-21 levels in adults with T1D are truly associated with autoimmunity and are not secondary to hyperglycemia and/or exogenous insulin use.

In conclusion, for the first time, IL-21 was quantified using an ultrasensitive and specific method and found to be elevated in adults with established T1D, supporting a potential role of IL-21 in the T1D disease process. In contrast, in the pediatric cohort, comprising children with newly diagnosed T1D, AAb^{+} children at-risk for T1D,

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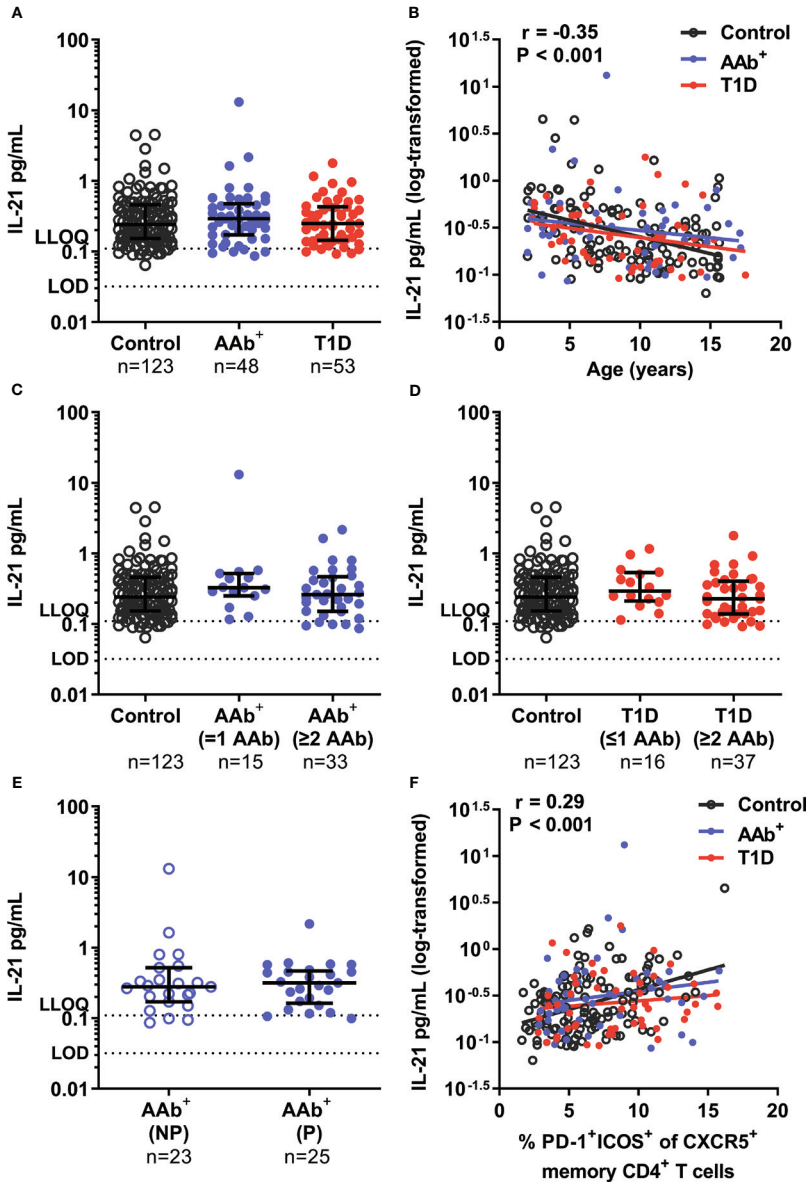

FIGURE 2
Similar plasma IL-21 levels in children with newly diagnosed T1D, AAb+ at-risk children and healthy children. **(A)** Plasma IL-21 levels in children with newly diagnosed T1D, AAb$^+$ at-risk children and control children. **(B)** Correlation between age and $\log_{10}$-transformed plasma IL-21 levels was examined using Spearman's correlation by pooling the data from all study groups and is presented together with the P value on the plot. The elevations of the linear regression lines were comparable between the study groups. AAb$^+$ at-risk children **(C)** and children with newly diagnosed T1D **(D)** were stratified into two subgroups based on the number of persistent autoantibodies detected at sampling (positive for ≤1 autoantibodies and ≥2 autoantibodies). **(E)** AAb$^+$ at-risk children were divided into non-progressors (NP) and progressors (P) depending on whether they had later progressed to T1D during follow-up. Kruskal-Wallis with Dunn's multiple comparisons test or Mann-Whitney U-test was used for statistical analyses. Medians with IQRs are shown, and limit of detection (LOD) and lower limit of quantification (LLOQ) are represented with dotted lines in A, C–E. **(F)** Correlation between the frequency of circulating activated Tfh (PD-1$^+$ICOS$^+$ of CXCR5$^+$ memory CD4$^+$ T cells) and $\log_{10}$-transformed plasma IL-21 levels was examined using Spearman's correlation by pooling the data from all study groups, and is expressed together with the P value on the plot. The elevations of the linear regression lines were comparable between the study groups.

and age-matched healthy controls, we did not observe differences in plasma IL-21 levels between the study groups. The physiologically higher plasma IL-21 levels in children may, however, mask potential changes caused by T1D autoimmunity. Taken together, the direct detection of plasma IL-21 levels, even by the ultrasensitive method capable of detecting fg/mL concentrations of cytokines employed here, may have limited potential as a biomarker of T1D progression, particularly in children.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by local ethics committees of the University Hospitals of Turku and Kuopio and the ethics committee of the Hospital District of Northern Ostrobothnia. Written informed consent to participate in this study was provided by the participants or participants' legal guardian/next of kin.

Author contributions

JPo, SM and SB measured the cytokines. RR, JPi, KN-S and JT provided the clinical research samples. MK and RV were responsible for the islet autoantibody analyses of the pediatric cohort. JI was responsible for the HLA screening of the pediatric study subjects. A-MS, JPo, SM, SB, LZ, RJB and TK analyzed the data and drafted the manuscript. TK is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Academy of Finland (decision no. 331282) and the Sigrid Jusélius Foundation.

Acknowledgments

We thank the skillful technical assistance of Hanna Eskelinen (University of Eastern Finland), Anne Suominen (University of Turku), Matti Laitinen (Kuopio University Hospital) and Satu Miettinen (Eastern Finland Laboratory Centre).

Conflict of interest

JPo, SM, SB, LZ, and RB are employees of Eli Lilly & Company and may own stock.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1157265/full#supplementary-material

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ANNA-MARI SCHRODERUS

Type 1 diabetes (T1D) is an autoimmune disease where T cells mediate the progressive destruction of insulin-producing β cells in the pancreas. $CD8^+$ T cells and $CD4^+$ B-cell helper T cells are implicated as major cell subsets associated with T1D disease process but their phenotypes during the disease progression remain elusive. This dissertation investigated $CD8^+$ T-cell and $CD4^+$ B-cell helper T-cell signatures during the progression of T1D.

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ISBN 978-952-61-5422-0
ISSN 1798-5706