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

CD8$^+$ T-cell signatures during T1D progression

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Word count: 4369; 5 figures, 1 table

ABSTRACT

$CD8^+$ 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 co-inhibitory 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*$^+$ co-inhibitory 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.

Keywords: type 1 diabetes, autoimmunity, T cells, human, $CD8^+$ T cells, single-cell multiomics

ARTICLE HIGHLIGHTS

- Blood $CD8^+$ T-cell signatures have recently been associated with a slower progression of 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 co-inhibitory 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.

INTRODUCTION

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 (AAb) to islet autoantigens are almost invariably 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, stage 2 by the presence of multiple AAbs and dysglycemia, while 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. In both 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 (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 as well as "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 co-inhibitory 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 in both 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 utilizing 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 co-inhibitory 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^+ cases. 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 [IAA], insulinoma-associated-2 antibodies [IA-2A] and/or glutamic acid decarboxylase antibodies [GADA]). The adult cohort comprised 20 T1D patients and 19 healthy controls 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 controls (time between samples 3–13 months).

All participants and/or their legal guardians have given 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, and for the DIPP study by the ethics committee of the Hospital District of Northern Ostrobothnia.

PBMC and plasma sample preparation

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 x g, 10 min, and stored at -80°C until analyses.

CMV IgG-antibody measurements

Cytomegalovirus-specific (CMV) 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 i.e. no additional 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 controls 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 (Suppl. Table 4), BD Abseq oligonucleotide-conjugated monoclonal antibodies (BD Biosciences, Suppl. 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. Post-sort purity was determined after the sorting (Suppl. Fig. 1). Pooled $CD8^+$ T cells were captured either immediately after sorting using the BD Rhapsody Express System or subjected to 90 min stimulation with PMA (50 ng/ml, Sigma) 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 co-expression 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's multiple comparison test when comparing multiple groups. Correlation analyses were performed using Spearman's 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 at 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 datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. The single-cell multiomics data is 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 type 1 diabetes

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) (Suppl. Fig. 2). Using these definitions, we observed that Tscm $CD4^+$ T cells (p=0.008) were more frequent in children with T1D compared to controls, 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 controls. (Suppl. Fig. 2)

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

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. 1A, Suppl. 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 to controls (Suppl. 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 compared to controls. (Fig. 1B and Suppl. 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 (Suppl 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 (Suppl. 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 were comparable (Fig 1B and Suppl. 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 (Suppl. 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 co-expression 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. 1C.) 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 to controls (Fig.1D–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 (Suppl. Fig. 7), demonstrating that also highly differentiated $CD4^+$ T cells are 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. (Suppl. 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 co-inhibitory receptors KLRG1 and TIGIT

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

Again, the $CD27^+$ and $CD27^-$ memory T-cell subsets were studied separately (Fig. 2A, Suppl. Fig. 9). The frequency of $CD27^-CD8^+$ and $CD27^-CD4^+$ memory T cells expressing co-inhibitory receptors were comparable in children with T1D and AAb^+ children compared to controls (Fig. 2B and Suppl. Fig. 10), with the exception for a tendency for higher frequency of both $KLRG1^+$ (p=0.05) and $TIGIT^+$ (p=0.08) cells within $CD27^-CD8^+$ T cells (Suppl. Fig. 10). We also examined the frequency of $KLRG1^+TIGIT^+$ cells separately (Fig. 2C and D), as this marker combination has been associated both with slower progression of T1D after diagnosis as well as immunotherapy efficacy in recent studies (14–17). Interestingly, a higher frequency of $KLRG1^+TIGIT^+$ cells (p=0.02) was observed in AAb^+ children compared to controls within $CD27^-CD8^+$ memory T cells but not within $CD27^-CD4^+$

memory T cells (Fig. 2D, Suppl. Fig. 10). After taking age and CMV serostatus into account, the increase in KLRG1^{+}TIGIT^{+} CD27^{-}CD8^{+} memory T cells remained significant (Suppl. 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 to controls (p=0.007 and p=0.03, respectively, Suppl. Fig. 12).

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

Proinflammatory or co-inhibitory CD8^{+} T-cell signatures are not detectable in adults with established type 1 diabetes or in children during the seroconversion to autoantibody-positivity

Next, we wanted to determine whether either the proinflammatory or co-inhibitory 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 controls. However, the frequencies of cytokine and CD107a-producing, and co-inhibitory receptor expressing CD27^{-}CD8^{+} memory T cells were comparable between adults with T1D and controls. (Suppl. Fig. 13.)

We also analyzed rare longitudinal samples from 17 children collected 3 to 13 months apart before and after initial seroconversion to islet AAbs, alongside samples from healthy age-matched controls. 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 type 1 diabetes

Next, we examined potential associations between the expression of proinflammatory cytokines and co-inhibitory receptors within CD27$^-$CD8$^+$ memory T cells (Fig. 4A). 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 co-inhibitory receptors. However, no positive or negative correlations between the expression of the proinflammatory cytokines IFN-γ and TNF-α and co-inhibitory receptors were observed. (Fig. 4A)

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. 4B and C).

***IFNG, TNF, KLRG1* and *TIGIT* expression is co-localized 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 co-inhibitory receptors KLRG1 and TIGIT we performed a single-cell multiomics analysis. For this, we sorted from five children with T1D and five healthy controls 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* as well as 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. (Suppl. 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 naive and less differentiated memory cells, with features of CM and type 1 (Tc1) $CD8^+$ T cells as well as mucosal-associated invariant T (MAIT) cells, and two $CD27^-$ clusters (clusters 5 and 6) of more highly differentiated cells (Fig. 5A–C). Similar clustering was obtained both with the unstimulated and stimulated samples (Fig. 5A, Suppl. Fig. 14).

Of the two $CD27^-$ clusters, cluster 5 represented cytotoxic effector (Teff) $CD8^+$ 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 cytolytic potential. However, *IFNG* and *TNF,* or *KLRG1* and *TIGIT* were not significantly upregulated within this cluster. (Fig. 5A–C, Suppl. Table 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. 5D). Cells in cluster 5A displayed upregulated *IFNG* and *TNF* transcripts alongside *GZMB* and *PRF1* (Fig. 5D–E, Suppl. Fig. 15, Suppl. Table 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. 5D–E, Suppl. Fig. 15, Suppl. Table 9 and 10.). The subcluster 5B could therefore represent the $CD8^{+}CD27^{-}$ memory T cells with co-inhibitory 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 to the other clusters, suggesting a less differentiated phenotype (Supp. Fig. 15, Suppl. Table 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 controls (Suppl. Fig 15).

In conclusion, highly differentiated $CD27^{-}CD8^{+}$ memory T cells expressed genes consistent with cytolytic and proinflammatory function but also genes for co-inhibitory receptors. *IFNG* and *TNF* expression co-localized 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 co-inhibitory 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 co-inhibitory $KLRG1^+TIGIT^+$ 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 in adults with established T1D was comparable to age-matched controls.

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, i.e. 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), as well as 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 co-inhibitory 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 co-inhibitory 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 cell 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 TCR 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 to 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 both at the level of autoantigen-specific as well as 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 co-inhibitory $KLRG1^+TIGIT^+$ 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.

Acknowledgements

We 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 sc-multiomics analyses, and Laurène Pfajfer (University of Eastern Finland) for the help with flow sorting. Part of the work was carried out with the support of UEF Cell and Tissue Imaging Unit, University of Eastern Finland. NGS sequencing was performed in Finnish Functional Genomics Centre Facility supported by University of Turku, Åbo Akademi University and Biocenter Finland.

Author contributions

A-M.S., V.P., M-R.N., I.E. and D.S. performed the experiments. J.L., J.T., R.R. and J.P. 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.

Conflicts of interest

Authors declare that there are no conflicts of interest.

Funding

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

Prior Presentation

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

Abbreviations

AAb	Autoantibody
AAb+	Autoantibody-positive
CLR	Centered log ratio
CM	Central Memory
CMV	Cytomegalovirus
CX3CR1	CX3C Motif Chemokine Receptor 1
KIR	Killer Lectin Receptor
KLRG1	Killer Cell Lectin Like Receptor G1
PBMC	Peripheral blood mononuclear cell
PI	Polyfunctionality index
PD-1	Programmed Cell Death Protein 1
PMA	Phorbol myristic acid
Teff	T effector
TEM	T Effector Memory
TEMRA	RA-re-expressing T Effector Memory
TIGIT	T cell immunoreceptor with Ig and ITIM domains
Tscm	T stem cell memory
WNN	Weighted Nearest Neighbor

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TABLES

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 controls**	**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	26.70 ± 6.45	26.70 ± 6.45	3.58 ± 1.61	3.14 ± 1.50	3.76 ± 1.39
Female	42% (25/60)	47% (14/30)	40% (12/30)	47% (9/19)	50% (10/20)	67% (12/18)	41% (7/17)	41% (7/17)
Male	58% (35/60)	53% (16/30)	60% (18/30)	53% (10/19)	50% (10/20)	33% (6/18)	59% (10/17)	59% (10/17)

FIGURES

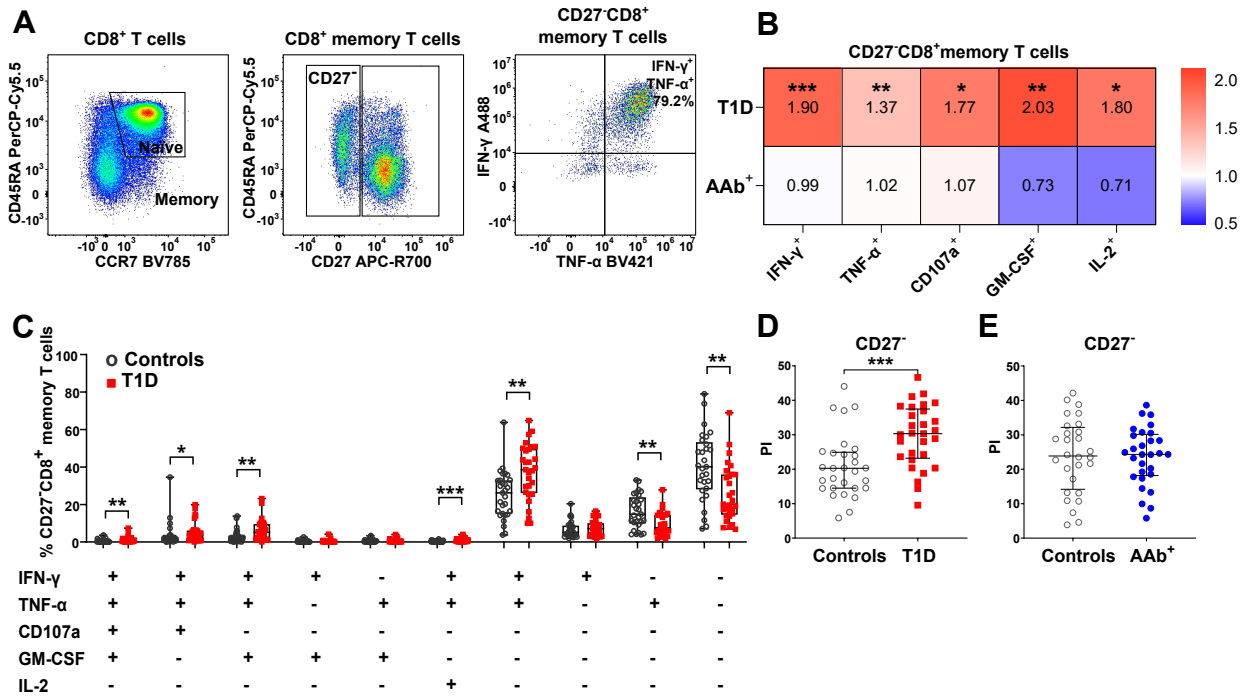

Figure 1. Frequencies of CD27$^-$CD8$^+$ memory T cells producing IFN-γ, TNF-α, CD107a, GM-CSF and IL-2 are increased in children with newly diagnosed T1D. (A) Representative gating strategy of CD8$^+$ memory T cells. Naïve (CD45RA$^+$CCR7$^+$) CD8+ T cells were excluded and only CD8$^+$ memory T cells were analyzed. Subsequently, memory T cells were gated according to CD27 expression into CD27$^+$ and CD27$^-$ subsets. **(B)** Heatmap 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 controls. **(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 controls **(D),** and in AAb$^+$ children and controls **(E)**. Mann-Whitney U test was used for statistical testing. Median and interquartile range (IQR) are shown in the C–E figures. *P<0.05, **P<0.01, ***P<0.001. Controls are depicted as open grey circles, children with newly diagnosed T1D as solid red squares and AAb$^+$ children as solid blue circles.

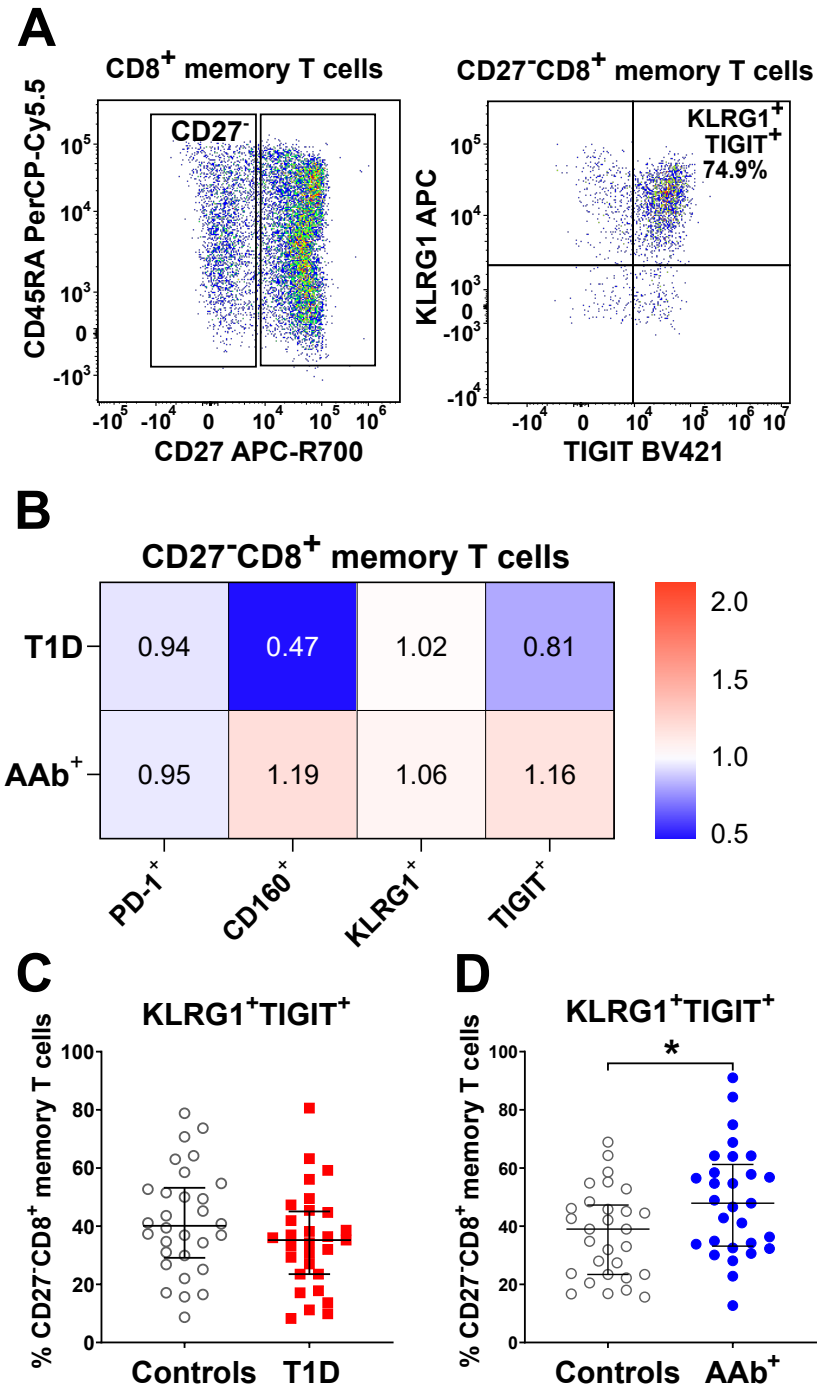

Figure 2. Frequency of CD27$^-$CD8$^+$ memory T cells co-expressing KLRG1 and TIGIT is increased in AAb$^+$ children. **(A)** Representative gating strategy of CD8$^+$ memory T cells. Naïve (CD45RA$^+$CCR7$^+$) CD8$^+$ T cells were excluded and only CD8$^+$ memory T cells were analyzed. Subsequently,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

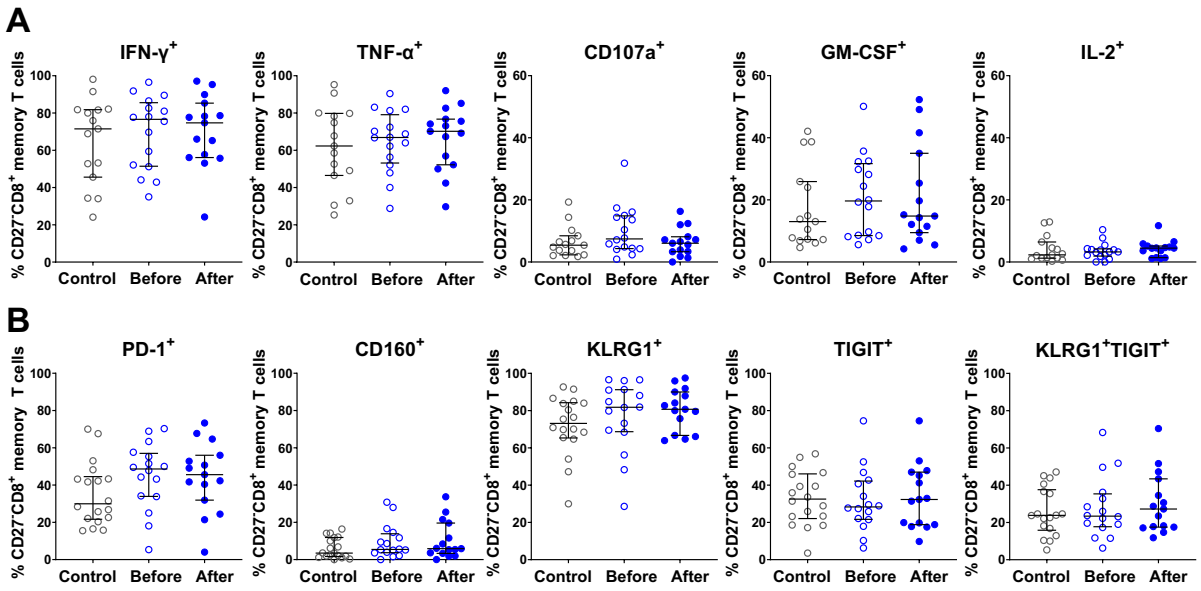

Figure 3. Frequencies of CD27$^-$CD8$^+$ memory T cells expressing cytokines, CD107a and co-inhibitory receptors in children before and after seroconversion to islet AAb positivity. Frequencies of IFN-γ$^+$, TNF-α$^+$, CD107a$^+$, GM-CSF$^+$ and IL-2$^+$ **(A)**, and PD-1$^+$, CD160$^+$, KLRG1$^+$, TIGIT$^+$ and TIGIT$^+$KLRG1$^+$ **(B)** CD27$^-$CD8$^+$ memory T cells in children before and after seroconversion to islet AAb positivity and controls. Kruskal-Wallis with Dunn's multiple comparison test was used for statistical testing in analysis of the pediatric samples. Median and IQR are shown in the figures. Controls are depicted as open grey circles and AAb$^+$ children as open (before seroconversion) or solid (after seroconversion) blue circles.

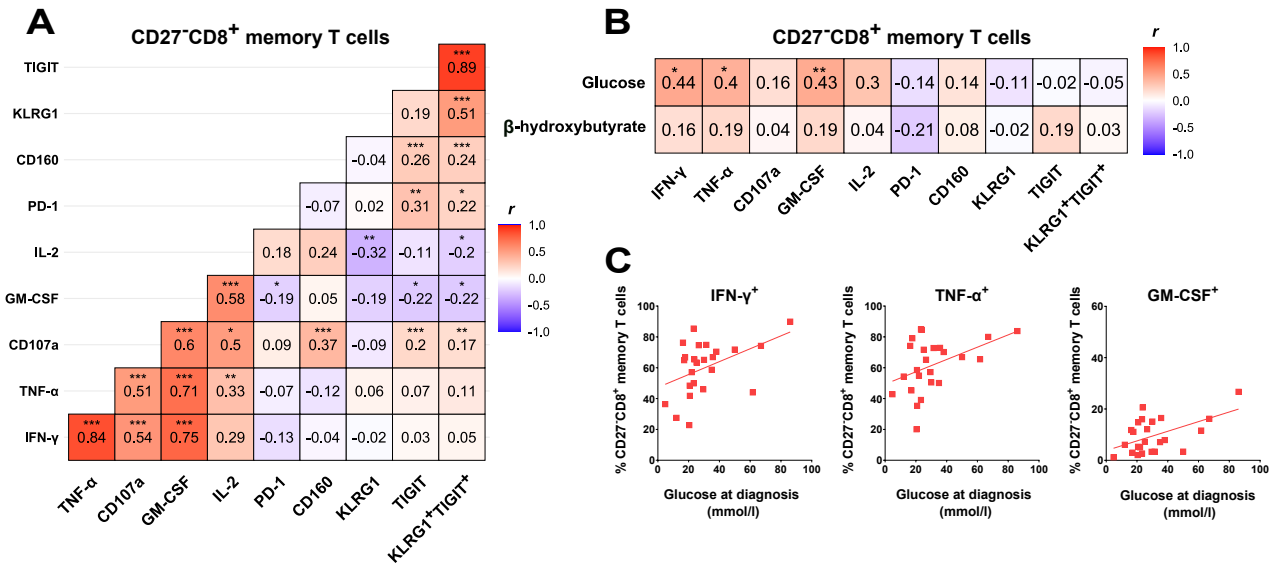

Figure 4. Plasma glucose levels at T1D diagnosis positively correlate with the frequency of CD27$^-$CD8$^+$ memory T cells expressing proinflammatory cytokines. (A) Correlations between the frequencies of cytokine and co-inhibitory receptor expressing CD27$^-$CD8$^+$ memory T cells was examined using Spearman's 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 co-inhibitory receptor expressing CD27$^-$CD8$^+$ memory T cells was examined using Spearman's 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's r-values are presented within the squares, statistical significance is indicated as *P<0.05, **P<0.01, ***P<0.001.

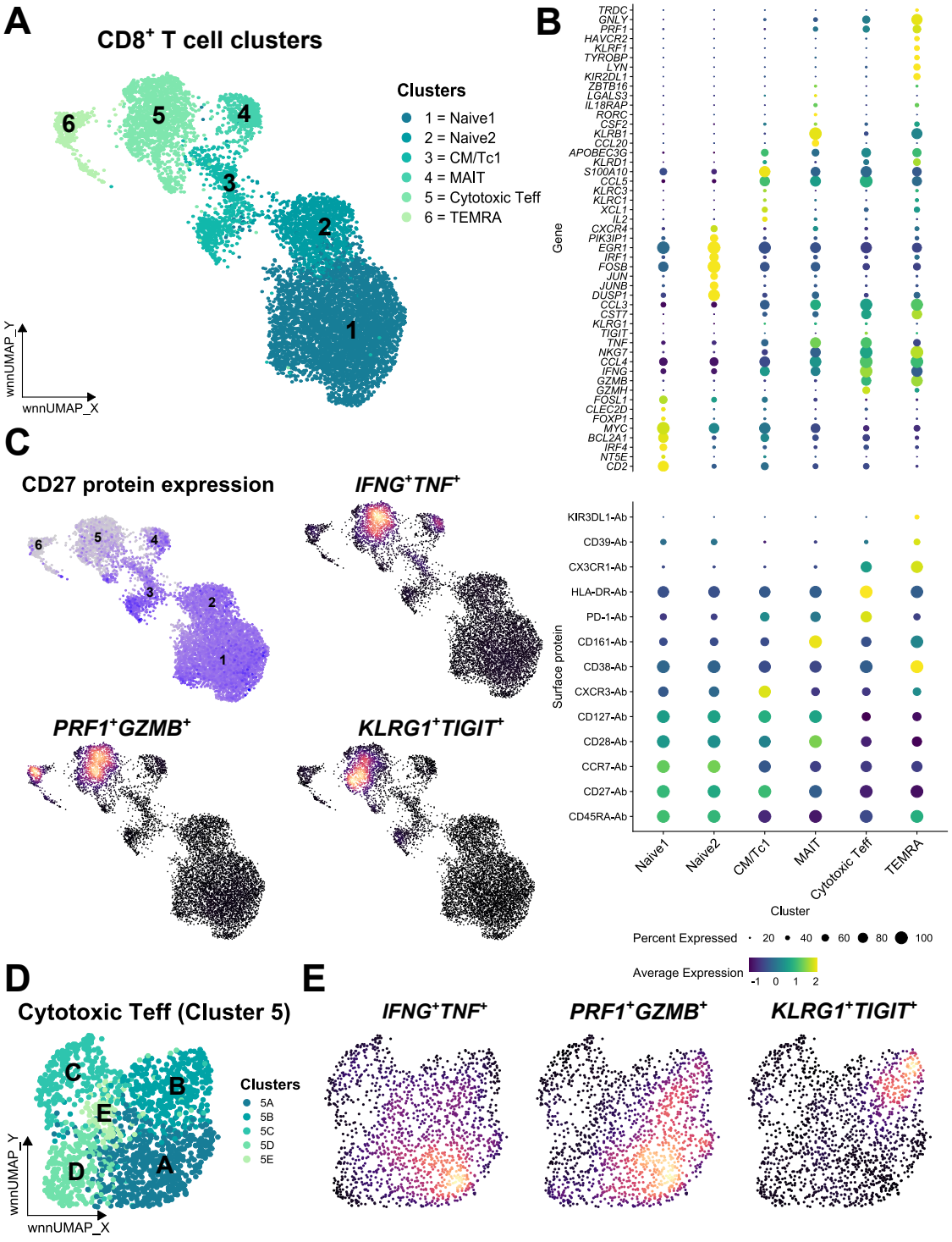

Figure 5. *IFNG, TNF, KLRG1* and *TIGIT* expression is enriched within the cytotoxic CD27$^-$ CD8$^+$ memory T-cell cluster in single-cell multiomics analysis. (A) CD8$^+$ T cells were clustered into six clusters that represent canonical CD8$^+$ T-cell subsets in a WNN UMAP projection. Heatmap of gene and surface protein expression within the different CD8$^+$ T-cell clusters **(B)**. Visualization of CD27 protein expression and co-expression of *IFNG* and *TNF*, *PRF1* and *GZMB*, and *KLRG1* and *TIGIT* within the different clusters **(C)**. Reclustering of the cytotoxic Teff cluster (cluster 5) results in the identification of five subclusters **(D)**. Co-expression of *IFNG* and *TNF*, *PRF1* and *GZMB*, and *KLRG1* and *TIGIT* within the subclusters **(E)**. Wilcoxon rank sum test was used for statistical testing in the Seurat WNN pipeline to identify significantly differently expressed genes within clusters.