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ORIGINAL ARTICLE

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Children With Celiac Disease Have a Higher Degree of Activated or Exhausted CD4⁺ and CD8⁺ T Cells Compared to Healthy References

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ABSTRACT

Type 1 diabetes (T1D) and celiac disease (CeD) are two chronic autoimmune disorders commonly diagnosed during childhood. In this exploratory study we performed flow cytometric immunophenotyping of various immune cell populations in peripheral blood from children with T1D, CeD, a T1D and CeD comorbidity, and from age-matched healthy references (controls). With extensive flow cytometry panels covering subpopulations of both CD4⁺ and CD8⁺ T cells, as well as monocytes and NK cells, our main finding is a tendency towards a higher degree of activated/exhausted T cells in children with a sole diagnosis of CeD compared with healthy references. This was seen through a higher fraction of CD4⁺ T cells positive for PD-1, CCR5 and CCR10, as well as a higher fraction of CD8⁺ T cells expressing PD-1 and CD39. In contrast, children with CeD showed a lower percentage of naïve CD8⁺ T cells compared with healthy references. Other important findings are a skewed CD4⁺/CD8⁺ ratio for children with a comorbidity compared with references, increased fractions of T regulatory cells (Tregs, CD4⁺CD25⁺CD127^{low}) for all three diagnosis groups compared with references, and a higher percentage of CD56^{dim}CD16⁺ NK cells with a corresponding lower percentage in CD56^{dim}CD16⁻ NK cells in CeD compared to the T1D and CeD comorbidity. Ultimately, analysis of the peripheral immunological milieu might lead to the development of more efficient tools for diagnosis and monitoring, and better treatment options, for children with T1D, CeD, and the rare combination of T1D and CeD.

1 | Introduction

Type 1 diabetes (T1D) and celiac disease (CeD) are two incurable chronic autoimmune disorders commonly diagnosed during childhood [1–4]. In T1D there is a T cell-driven destruction of the pancreatic β -cells, subsequently leading to hyperglycemia due to insufficient insulin production [1, 2]. In CeD the ingestion of gluten in genetically predisposed individuals triggers an autoimmune attack on primarily the small intestine, causing,

for example, villous atrophy and gastrointestinal, as well as non-gastrointestinal, issues [3, 4].

In addition to both being autoimmune diseases, T1D and CeD share the same genetic risk factors, that is, the human leukocyte antigen (HLA) genotypes coding for HLA-DQ2 and HLA-DQ8. For both diseases, viral infections have been implicated to be causative agents, and studies have shown that the composition of the gut microbiota is important for the

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pathogenesis of both disorders [5–7]. Thus, T1D sometimes co-occurs together with CeD. The prevalence of confirmed CeD in children that are diagnosed with T1D in Sweden, country of origin for this study, has been shown to be between 9% and 10% [8–10].

It is known that T cells are important players in the pathogenesis of both T1D and CeD, as extensively reviewed [11–16]. Regulatory T cells (Tregs), in this study defined as either CD4⁺CD25⁺CD127^{low} or CD4⁺CD25⁺CD127^{low}FoxP3⁺, are a subset of CD4⁺ T cells having immunoregulatory properties used to maintain peripheral tolerance with implications in the development of, for example, autoimmune disorders and cancer [17, 18]. Tregs have shown in animal models to delay or totally protect against diabetes development, and they are reported to be functionally altered in patients with T1D [19–21]. There are also indications of Tregs being altered in both duodenal mucosa and in peripheral blood in patients with CeD [22].

In addition to Tregs, we also investigated the expression of T cell markers associated with activation and exhaustion on CD4⁺ and CD8⁺ T cells since T cell autoreactivity is a key driver in T1D and CeD. This includes CD26, CD39, and PD-1. CD26, also known as dipeptidyl peptidase IV, is a type II transmembrane glycoprotein present on activated T cells that facilitates, for example, T cell proliferation and cytokine production [23, 24]. CD39 is a cell surface enzyme which, together with CD73, converts extracellular ATP into immunosuppressive adenosine [25], and it can be used for the identification of exhausted CD8⁺ T cells [26, 27]. PD-1, programmed cell death protein 1, is an inhibitory receptor expressed on activated and exhausted T cells which functions as an immune checkpoint, negatively regulating, for example, proliferation and survival upon binding to its ligands PD-L1 and PD-L2 [28, 29].

Other analysed markers include CD122, CCR5 and CCR10. CD122 is a subunit of the receptors for IL-2 and IL-15, which are two cytokines implied in the proliferation and differentiation of lymphocytes [30]. It has been shown in mice that CD8⁺CD122⁺ cells might constitute a CD8⁺ regulatory T cell population [31] which can prevent disease onset in a T1D murine model [32]. CCR5 and CCR10 are chemokine receptors found on, for example, T cells. CCR5 binds to CCL3 (MIP-1 α), CCL4 (MIP-1 β) and CCL5 (RANTES) and is associated with a type 1 CD4⁺ T helper cell (Th1) profile, while CCR10 is often seen as a skin-homing receptor for T cells and binds CCL27 (CTACK) and CCL28 (MEC) [33, 34]. We have previously shown that children with CeD and children with a T1D and CeD comorbidity display decreased serum concentrations of CCL3 compared to healthy references [35], and that cell culture supernatants from peripheral blood mononuclear cells (PBMCs) derived from children with T1D have lower concentrations of both CCL3 and CCL4 compared to healthy references [36].

Due to the strong link between adaptive and innate immunity, shown as for example in the reliance of T cells upon antigen-presentation by, for example, dendritic cells (DCs) and the effect of cytokines produced by myeloid cells such as monocytes and macrophages, it was also of interest to investigate some innate

immune cells. Thus, included in the study was also analysis of the three monocyte subpopulations classical (CD14⁺CD16[−]), intermediate (CD14⁺CD16⁺) and nonclassical (CD14[−]CD16⁺) monocytes [37] and the three natural killer (NK) cell subsets CD56^{dim}CD16⁺, CD56^{dim}CD16[−] and CD56^{bright}CD16[−] NK cells [38]. Peripheral blood monocytes from patients with T1D have been shown to be more proinflammatory, have changed functionality and skewed ratios of their subpopulations [39–42]. Patients with T1D also tend to have NK cells with changed functionality and lower numbers [40, 43, 44]. In CeD peripheral blood monocytes have after culture and stimulation with gliadin, one of the components of gluten, been shown to produce more pro-inflammatory cytokines compared to healthy control subjects [45, 46].

The overall objective of this study was thus to characterise peripheral blood immune cells from children with only T1D, only CeD, or with a T1D and CeD comorbidity in an exploratory study context in order to facilitate understanding of the disease-causing mechanisms and potential routes for diagnosis and treatment. To this end, PBMCs from the three diagnosis groups and from age-matched healthy reference donors were immunophenotyped with flow cytometry using antibody panels covering various subgroups of immune cells, with a focus on T cells.

2 | Materials and Methods

This multi-center study includes children with T1D, CeD, or a T1D and CeD comorbidity, and references (age-matched healthy controls). The samples were collected at Linköping University Hospital, Linköping, Sweden and Ryhov County Hospital, Jönköping, Sweden, as previously described [47].

2.1 | Study Population

The cohort of this study included a total of 36 children: 9 children with T1D exclusively, 9 children with CeD exclusively, 9 children diagnosed with both T1D and CeD (T1D + CeD) and 9 reference children without these diseases (Reference). Information regarding age, sex, duration of disease (T1D and/or CeD) and tissue transglutaminase autoantibodies (tTGA) status is summarised in Table 1.

The children with T1D and CeD were recruited by convenience sampling at the Clinic of Paediatrics at Ryhov County Hospital, Jönköping, Sweden, and Linköping University Hospital, Linköping, Sweden. References (children without T1D and/or CeD) have been recruited by school nurses at schools in Linköping municipality informing about the study. Information on the study and voluntary participation was given both orally and in writing to all participants and their parents or responsible guardians. In agreement with the Declaration of Helsinki, all participants received convenient information adapted for their age. Informed consent was obtained from the children's guardian.

The general criterion for participation in the study was that children with or without diseases showed no signs of colds or other infections at the time of sample collection.

TABLE 1 | Characteristics of the study groups; children with combined type 1 diabetes and celiac disease (T1D + CeD), children with type 1 diabetes (T1D), children with celiac disease (CeD) and reference children (Reference).

	CeD	T1D	T1D + CeD	Reference
<i>n</i>	9	9	9	9
Sex B (<i>n</i>)/G (<i>n</i>)	5/4	4/5	3/6	3/6
Age (years)	10.0 (7.5–14.5)	10.0 (6.0–12.0)	10.5 (7.0–13.2)	11.0 (7.6–13.3)
Girls	10.0 (7.5–14.5)	10.3 (8.4–11.1)	10.8 (7.5–13.2)	11.2 (9.4–13.3)
Boys	10.0 (8.0–10.5)	10.0 (6.0–12.0)	10.5 (7.0–12.8)	8.0 (7.6–12.0)
Duration T1D (years)	—	5.4 (1.1–10.9)	3.6 (1.2–10.8)	—
Girls	—	5.4 (2.2–10.2)	3.6 (1.9–5.5)	—
Boys	—	3.8 (1.1–10.9)	3.6 (1.2–10.8)	—
Duration CeD (years)	5.8 (0.7–11.7)	—	1.9 (0.5–10.2)	—
Girls	7.1 (0.7–11.7)	—	3.3 (0.5–10.2)	—
Boys	2.6 (1.8–5.8)	—	0.8 (0.8–2.7)	—
tTGA status (pos/neg)	6/3	3/6	7/2	0/9

Note: Age and disease duration presented as median (minimum, maximum); B = Boys, G = Girls, tTGA = tissue transglutaminase autoantibodies.

The reference group consisted of healthy children, and neither the reference children nor their first-degree relatives displayed any clinical signs of T1D, CeD, or other autoimmune diseases.

T1D was diagnosed, according to the International Society for Paediatric and Adolescent Diabetes guidelines [48, 49]. Symptoms of diabetes plus casual plasma glucose concentration ≥ 11.1 mmol/L (200 mg/dL) or fasting plasma glucose ≥ 7.0 mmol/L (≥ 126 mg/dL) or 2-h post-load glucose ≥ 11.1 mmol/L (≥ 200 mg/dL) during an Oral Glucose Tolerance Test.

CeD was diagnosed according to the modified version of The European Society of Paediatric Gastroenterology and Nutrition criteria [50]. For CeD, the diagnosis date was the biopsy-confirmation date. The duration of T1D or CeD was calculated from the date of diagnosis until the date of sample collection. All children in the comorbidity group were diagnosed with T1D before diagnosing with CeD.

The study was reviewed and approved by the Research Ethics Committee of the Faculty of Health Sciences, Linköping University, Linköping, Sweden and the Regional Ethics Committee for Human Research, Linköping (approval number: M89-2006 and complementary r. 2012/27-32).

2.2 | Sample Collection

Blood samples were collected in Vacutainer tubes with sodium-heparin (BD Biosciences, San Jose, CA, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples with Ficoll-Paque (Sigma-Aldrich AB, Stockholm, Sweden) density gradient centrifugation. The PBMCs were isolated and cryopreserved within 24 h after sampling. For cryopreservation, the cells were resuspended in 4°C freezing media consisting of 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich), 40% fetal calf

serum (FCS) and 50% RPMI-1640. One milliliter aliquots, that is, 5×10^6 PBMC/mL in cryotubes (Nalgene, VWR International, Bristol, UK), were placed into a freezing container 'MrFrosty' (Nalgene) and kept overnight at -80°C before being transferred to -150°C for long-term storage.

2.3 | Flow Cytometry

Flow cytometric phenotyping of immune cell subsets was performed on cryopreserved and thawed PBMCs. Briefly, cryopreserved PBMC samples were thawed in a 37°C water bath and resuspended in RPMI-1640 cell medium (Sigma-Aldrich) supplemented with 2% FCS (Gibco, Darmstadt, Germany). Thereafter, the cells were washed twice with phosphate-buffered saline (PBS, Gibco), supplemented with 2% FCS, and finally resuspended in RPMI-1640 medium supplemented with 2% FCS. Cell viability in all PBMC samples was determined with the TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA) and was $\geq 90\%$. The cells were allowed to rest for 1 h before staining intra- and extracellular markers with fluorochrome-conjugated monoclonal antibodies (mAbs) purchased from BD Biosciences, presented in Table S1. $100\mu\text{L}$ PBMC samples ($2.0\text{--}2.5 \times 10^5$ immune cells) and $50\mu\text{L}$ BD Horizon Brilliant Stain buffer (BD Biosciences) were added to each staining tube. For the staining of the extracellular markers, titrated amounts of fluorochrome-conjugated mAbs were added to samples and incubated for 30 min at room temperature, protected from light. After incubation, the samples were washed and resuspended in $500\mu\text{L}$ of PBS.

For staining of the intracellular markers, the cells were permeabilized with BD Pharmingen Transcription Factor Buffer Set (BD Biosciences), in accordance with the manufacturer's instructions. After permeabilization, fluorochrome-conjugated mAbs were added to samples for staining of the intracellular markers (FoxP3, Perforin) and incubated for 30 min. Post-incubation, the

cells were washed and finally resuspended in flow cytometry stain buffer.

Data collection on BD FACSCanto II Flow cytometer (BD Biosciences) using BD FACSDiva software version 8.0 (BD Biosciences) were performed within 2h after staining. Further data analysis was performed with Kaluza Analysis 2.2 software (Beckman Coulter, Indianapolis, IN, USA). Gating strategies are presented in Figure S1 for CD4⁺ T cells, Figure S2 for CD8⁺ T cells, and Figure S3 for NK cells and monocytes.

2.4 | Statistics

Statistical analyses were performed in GraphPad Prism 10 (GraphPad Software, Boston, MA, USA). Differences in median values between the four groups were tested with nonparametric Kruskal–Wallis tests followed by nonparametric, unpaired Mann–Whitney tests with an α -threshold of 0.05. *p* values from Kruskal–Wallis can be found in Table S2. Due to the exploratory nature of this study, no adjustments for multiple comparisons were made. In addition, *p* values <0.1 are also presented in the

figure graphs in order to capture findings of interest. *p* values presented in graphs in regular fonts are <0.05, while *p* values presented in *italics* are from 0.05 to 0.1.

3 | Results

3.1 | Children With Both Type 1 Diabetes and Celiac Disease Have a Skewed CD4⁺/CD8⁺ Ratio

Analysis of T cells shows that while the percentage of CD4⁺ T cells does not differ between the groups (Figure 1A), children with a T1D and CeD comorbidity tend to have a higher proportion of CD8⁺ T cells compared to healthy references (Figure 1B). Furthermore, dividing cytotoxic T cells into two populations based on the expression of CD8, with highly expressing cells as CD8⁺ and cells with a lower expression as CD8^{dim} (Figure S2), shows that children with a comorbidity tend to have a higher percentage of CD8^{dim} cells compared to children diagnosed with only T1D (Figure 1C). This is also apparent when analysing the ratio between CD4⁺ to CD8⁺ and CD8^{dim} T cells, respectively (Figure 1D,E).

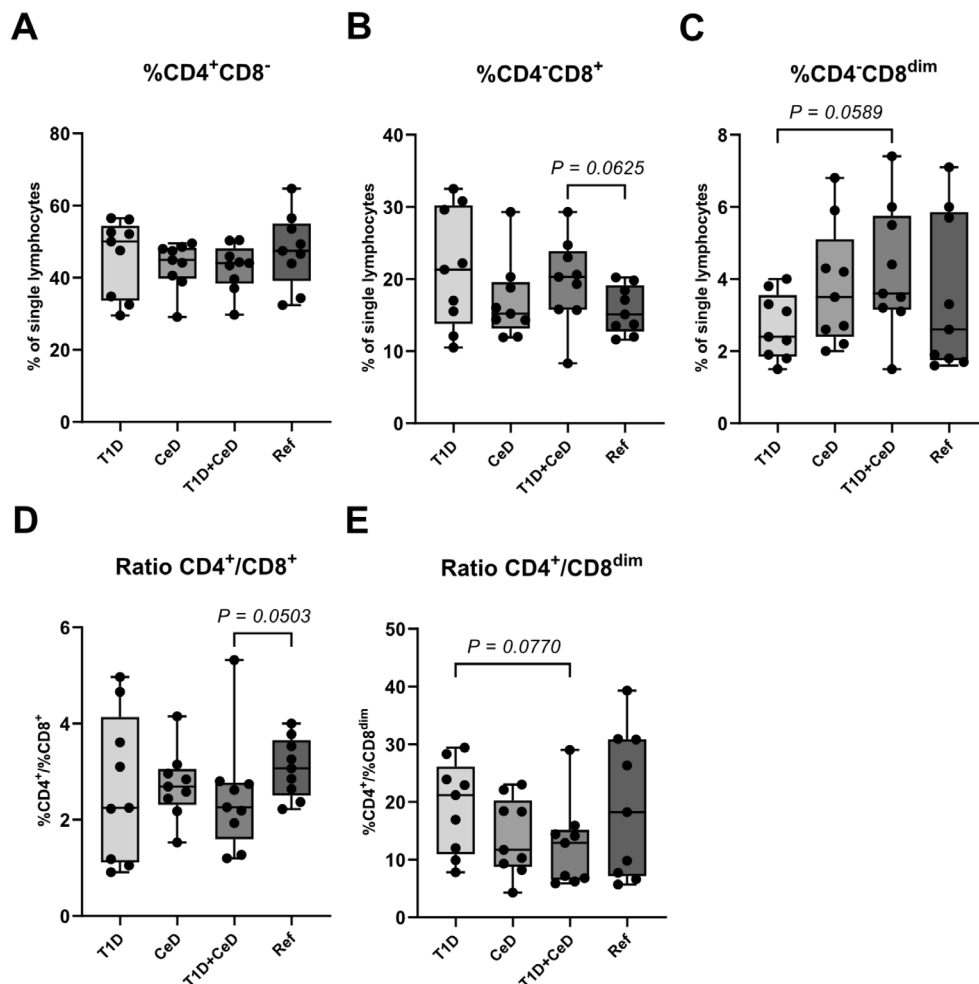


FIGURE 1 | Fractions of CD4⁺, CD8⁺ and CD8^{dim} T cells. Percentage of (A) CD4⁺CD8⁻, (B) CD4⁺CD8⁺ and (C) CD4⁺CD8^{dim} T cells among lymphocytes in the peripheral blood of children with T1D, CeD, T1D and CeD comorbidity, or healthy references, as measured by flow cytometry. Ratios of CD4⁺ T cells and (D) CD8⁺ and (E) CD8^{dim} among the different diagnosis groups. CeD, celiac disease; Ref, reference; T1D, type 1 diabetes. Mann–Whitney test, *n* = 9.

3.2 | Activated CD4⁺ T Helper Cells in Children With Celiac Disease

Further characterisation of subpopulations of CD4⁺ T cells shows that all three diagnosis groups (T1D, CeD, T1D and CeD comorbidity) have a higher proportion of T regulatory cells (Tregs), when defined as CD4⁺CD25⁺CD127^{low}, compared to healthy references (Figure 2A). The same trend is seen, although not statistically significant, when the expression of FoxP3 is added to the definition of Tregs, that is, CD4⁺CD25⁺CD127^{low}FoxP3⁺ (Figure 2B). There are no differences between the groups for the median fluorescence intensity (MFI) values of FoxP3 on CD4⁺, CD4⁺CD25⁺CD127^{low} and CD4⁺CD25⁺CD127^{low}FoxP3⁺ T cells (Table S3).

Analysis of individual markers on the CD4⁺ T cells shows that children with CeD have higher expression of the receptor PD-1 compared to the reference group (Figure 2C). Furthermore, if the expression of CD26 on CD4⁺ T cells is divided into two groups depending on the intensity of expression, that is, CD26⁺ and CD26⁺⁺ (Figure S1), it can be seen that while all groups contain similar percentages of CD4⁺CD26⁺ cells (Figure 2E), children with CeD have a higher proportion of CD4⁺CD26⁺⁺ cells compared to both children with T1D and CeD comorbidity and healthy references (Figure 2G).

3.3 | Activated CD8⁺ T Cytotoxic Cells in Children With Celiac Disease

Similar analyses of CD8⁺ T cells reveal that children with CeD also have a higher proportion of CD8⁺PD-1⁺ cells compared to healthy references (Figure 2D). While no differences are found between the different groups for CD8⁺CD26⁺ cells (Figure 2F), children with CeD tend to have a higher proportion of CD8⁺CD26⁺⁺ cells compared to children with T1D (Figure 2H). Children with CeD do also have a higher percentage than reference children of CD8⁺ cells expressing CD39 (Figure 2I).

Expression of CD122 on CD8⁺ and CD8^{dim} cells shows that while children with T1D have a lower fraction of CD8⁺CD122⁺ cells than healthy references (Figure 2J), no differences can be found among the groups for CD8^{dim}CD122⁺ cells (Figure 2K). Analysis of the MFI values for CD122 reveals that while no differences can be found for CD8⁺ and CD8^{dim} cells (Figure S4A,B), and for their CD26⁺ derivatives (Figure S4C,D), the CD122 MFI on CD8⁺CD26⁺⁺ and CD8^{dim}CD26⁺⁺ differs between the groups. Children with CeD have lower CD122 MFI on CD8⁺CD26⁺⁺ cells than children with T1D and lower MFI on CD8^{dim}CD26⁺⁺ cells than the reference group (Figure S4E,F). Additionally, children with a T1D and CeD comorbidity show decreased CD122 MFI values on CD8⁺CD26⁺⁺ cells compared to references (Figure S4E). Of note, reference children have higher CD122 MFI compared to both T1D and CeD when analysed on CD4⁺CD26⁺⁺ cells (Figure S4G).

Analyses were also performed on the memory subpopulations of CD8⁺ T cells and on intracellular perforin expression. Simultaneous expression of CD45RA and CCR7 on CD8⁺ T cells shows a tendency to lower percentages of naïve/stem cell memory (CD45RA⁺CCR7⁺) cells in both children diagnosed with only CeD and in children with a T1D and CeD comorbidity, compared to references (Figure S5A). Contrary to this, no differences in CD8⁺

memory subpopulations (central memory CD45RA⁺CCR7⁺, effector memory CD45RA⁺CCR7⁺, effector memory cells re-expressing CD45RA, TEMRA CD45RA⁺CCR7⁺) could be seen between any of the study groups (Figure S5B–D).

Children with solely CeD showed trends towards a higher expression of intracellular perforin compared to healthy references for both total CD8⁺ T cells and CD8⁺ effector memory cells (Figure S6A,D). No differences in perforin expression were found for CD8⁺ naïve/stem cell memory cells or the other memory subpopulations (Figure S6B,C,E).

3.4 | High Expression of Chemokine Receptors in Children With Celiac Disease

The expression of the chemokine receptors CCR5 and CCR10 on CD4⁺ and CD8⁺ T cells was also investigated. Children with CeD have increased percentages of CD4⁺CCR5⁺ and CD8⁺CCR5⁺ cells compared to healthy references, while children with T1D and CeD comorbidity also have a higher fraction of CD8⁺CCR5⁺ compared to the same group (Figure 3A,B). Similar trends could be seen for the MFI values of CCR5 on both CD4⁺ and CD8⁺ T cells (Table S4).

Furthermore, both CeD and the comorbidity group have a higher percentage of CD4⁺CCR10⁺ cells compared to references (Figure 3C). Interestingly, this is not reflected in the MFI values for CCR10 on CD4⁺ T cells where CeD and the comorbidity group do not differ compared to the reference, but children with T1D instead do (Table S4). No differences between the groups are found for the percentage of CD8⁺CCR10⁺ cells (Figure 3D) or the CCR10 MFI on CD8⁺ T cells (Table S4).

3.5 | No Major Differences in Percentages of NK Cells and Monocytes Between the Study Groups

There was no observable difference in the fraction of total NK cells (CD3⁺CD19⁺CD56⁺) between the different groups (Figure 4A). While children diagnosed with T1D did not differ from the references, children with CeD did show an increased percentage of CD56^{dim}CD16⁺ NK cells compared to children with a T1D and CeD comorbidity (Figure 4B). This was accompanied by a corresponding decrease in the percentage of CD56^{dim}CD16⁺ NK cells for CeD compared to the comorbidity group (Figure 4C), while the fraction of CD56^{bright}CD16⁺ NK cells remained the same for all groups (Figure 4D).

Analysis of monocytes shows no differences between the different groups in terms of the percentage of the subpopulations classical (CD14⁺CD16⁺) (Figure 4E), intermediate (CD14⁺CD16⁺) (Figure 4F) and nonclassical (CD14⁺CD16⁺) monocytes (Figure 4G).

4 | Discussion

Type 1 diabetes and celiac disease are two autoimmune diseases commonly diagnosed during childhood years. Altered immune profiles are found in the affected organs, that is, pancreas and small intestine, but also in the peripheral blood of patients

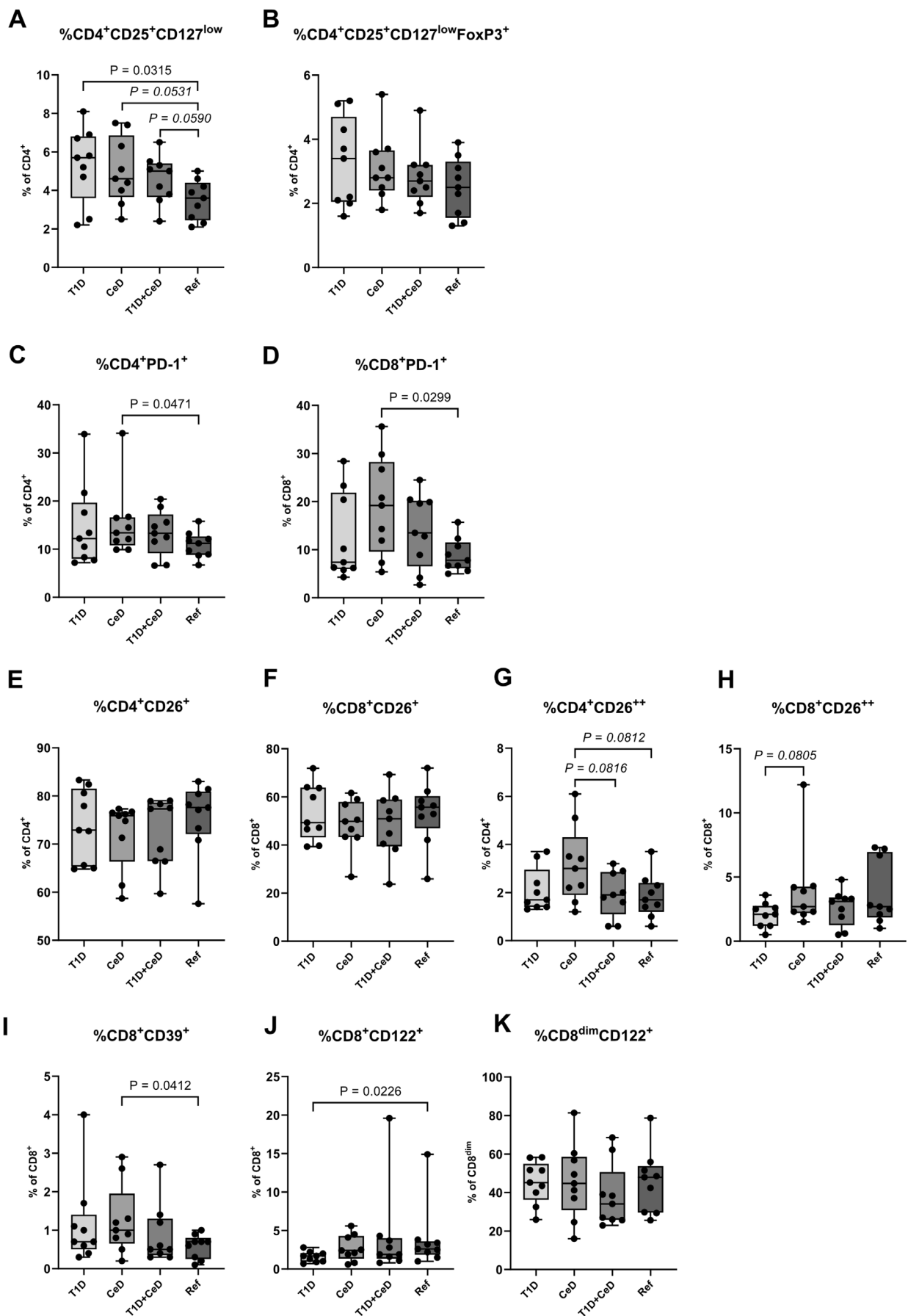


FIGURE 2 | Legend on next page.

FIGURE 2 | Tregs and activation markers on CD4⁺ and CD8⁺ T cells. Percentage of (A) CD4⁺CD25⁺CD127^{low}, (B) CD4⁺CD25⁺CD127^{low}FoxP3⁺, (C) CD4⁺PD-1⁺, (E) CD4⁺CD26⁺ and (G) CD4⁺CD26⁺⁺ cells among CD4⁺ T cells in the peripheral blood of children with T1D, CeD, T1D and CeD comorbidity, or healthy references, as measured by flow cytometry. Percentage of (D) CD8⁺PD-1⁺, (F) CD8⁺CD26⁺, (H) CD8⁺CD26⁺⁺, (I) CD8⁺CD39⁺, (J) CD8⁺CD122⁺ cells among CD8⁺ T cells, and percentage of (K) CD8^{dim}CD122⁺ cells among CD8^{dim} T cells. CeD, celiac disease; Ref, reference; T1D, type 1 diabetes. Mann–Whitney test, *n* = 9.

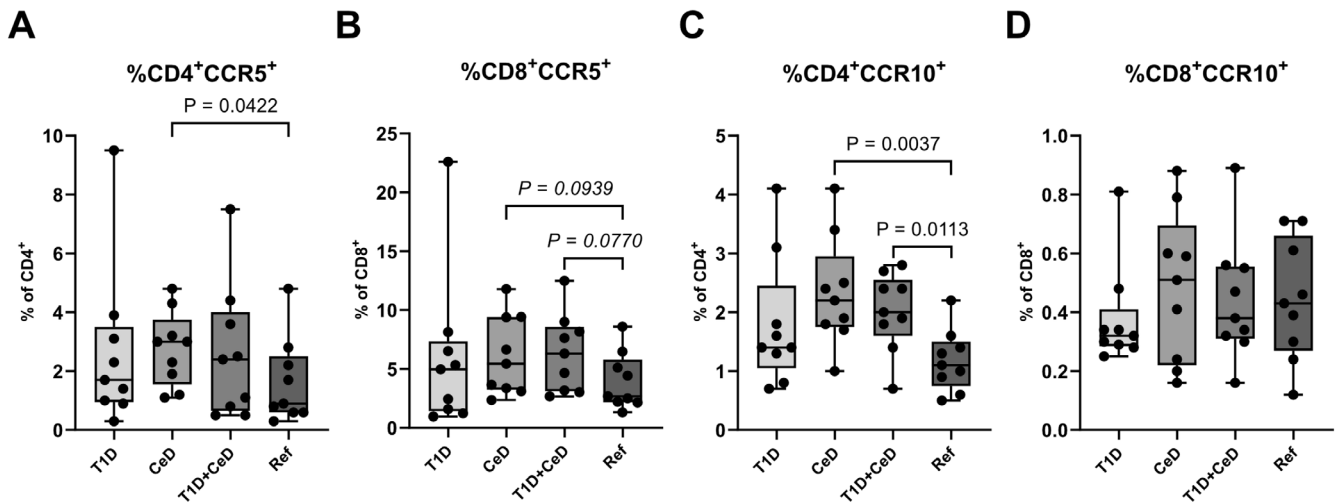


FIGURE 3 | Expression of CCR5 and CCR10 on CD4⁺ and CD8⁺ T cells. Percentage of (A) CD4⁺CCR5⁺ and (C) CD4⁺CCR10⁺ cells among CD4⁺ T cells in the peripheral blood of children with T1D, CeD, T1D and CeD comorbidity, or healthy references, as measured by flow cytometry. Percentage of (B) CD8⁺CCR5⁺ and (D) CD8⁺CCR10⁺ cells among CD8⁺ T cells. CeD, celiac disease; Ref, reference; T1D, type 1 diabetes. Mann–Whitney test, *n* = 9.

with these diseases [51, 52]. Immunophenotyping of peripheral blood cells can thus be used to investigate the immunological landscape of T1D and CeD. In this study, we analysed isolated PBMCs with flow cytometry from paediatric patients with only T1D, only CeD, or with a T1D and CeD comorbidity. With a focus on circulating lymphocytes, we found differences in various T cell subpopulations between the analysed groups and could in particular observe a tendency towards a higher degree of activated T cells in children with a sole diagnosis of CeD compared with healthy references.

While the role of T cells in the pathogenesis of T1D and CeD has been established, published studies have not reached a clear consensus about the relative distribution of various T cell subpopulations in peripheral blood in affected patients. Most studies show no differences in the percentage of CD4⁺ and CD8⁺ T cells in peripheral blood from T1D and CeD patients compared to healthy controls [36, 43, 53–55], which is in agreement with what we present here, but there are also reports of increased fractions of both CD4⁺ [56] and CD8⁺ T cells [57] in T1D.

We could also show that children with a combined diagnosis of T1D and CeD, a rarely studied diagnosis group, have a higher percentage of CD8⁺ T cells compared to healthy references, and a higher percentage of CD8^{dim} T cells compared to children with a sole diagnosis of T1D. This is reflected in the lower ratios between CD4⁺ T cells and the two different CD8 subpopulations. In a previous study by our group on the same cohorts, but not the same individuals, there were no discernible differences in the CD4⁺/CD8⁺ ratios between children

with a T1D and CeD comorbidity and the other groups [58]. Instead, children with a sole diagnosis of CeD had a higher CD4⁺/CD8⁺ ratio compared to healthy references [58]. The discrepancy between these two studies may be due to differences in methodological factors, such as analyses of whole blood versus isolated PBMCs and the usage of different instruments, reagents, and operators. It has, for example, been shown that the density gradient centrifugation used for purifying PBMCs from whole blood can introduce a slight but consistent bias towards naïve CD8⁺ T cells in PBMCs compared to whole blood [59]. This clearly displays how different methodological set-ups give different results, indicating that the differences seen between various studies may be due to more than biologically relevant factors.

A deeper analysis of CD4⁺ T cell subpopulations revealed that children in all three diagnosis groups (exclusively T1D, exclusively CeD, or T1D and CeD comorbidity) had higher fractions of cells with Treg-like phenotypes compared to healthy references. While the differences were statistically significant for cells defined as CD4⁺CD25⁺CD127^{low}, the same pattern could be seen, albeit not significant, for cells with the same markers with the addition of FoxP3, that is, CD4⁺CD25⁺CD127^{low}FoxP3⁺. Other studies utilising the same definition of Tregs as we do have shown similar results with a higher percentage in patients with T1D [60, 61], but also no differences [58, 62] or a lower percentage [63] in T1D patients. This is reflected in studies in patients with a sole diagnosis of CeD, where reports have shown both a higher percentage [64] of Tregs (defined as CD4⁺CD25⁺FoxP3⁺) in CeD compared to control subjects, but also a lower percentage [65] or no difference [66]. One study, utilising the same Treg

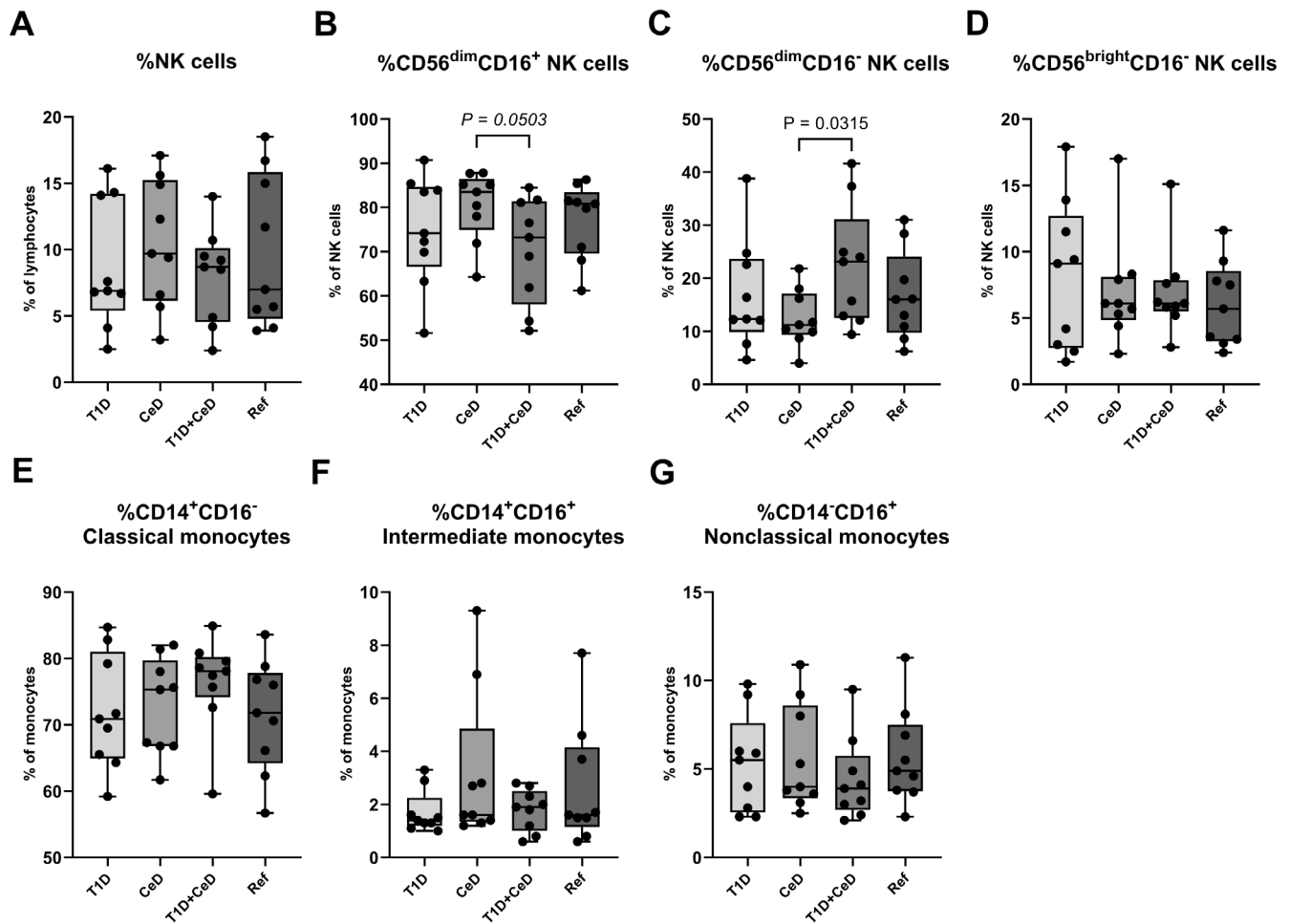


FIGURE 4 | NK cell and monocyte subpopulations. Percentage of (A) CD3⁻CD56⁺ NK cells among all lymphocytes and percentages of (B) CD56^{dim}CD16⁺, (C) CD56^{dim}CD16⁻ and (D) CD56^{bright}CD16⁻ NK cells among total NK cells in the peripheral blood of children with T1D, CeD, T1D and CeD comorbidity, or healthy references, as measured by flow cytometry. Percentage of (E) CD14⁺CD16⁻ classical, (F) CD14⁺CD16⁺ intermediate and (G) CD14⁻CD16⁺ nonclassical monocytes among total monocytes. CeD, celiac disease; Ref, reference; T1D, type 1 diabetes. Mann–Whitney test, $n = 9$.

definition as we do (CD4⁺CD25⁺CD127^{low}FoxP3⁺), also showed no difference in the fraction of Tregs between CeD patients and healthy controls [67]. While it is known that Tregs are important players in the pathogenesis of T1D and CeD, their exact function and regulation are not as easily understood.

Even though the fraction of Tregs differed in all diagnosis groups compared to healthy reference subjects, children with sole diagnosis of CeD stand out when it comes to T cell activation markers. Compared to the reference group, children with CeD have higher fractions of CD4⁺ and CD8⁺ T cells expressing PD-1, CD39, perforin, and CCR5. All are markers upregulated on activated and/or exhausted T cells [25–28, 68–70], which is reflected in the lower fraction of CD8⁺ naïve/stem cell memory cells seen in CeD. It has been shown in another study that gluten-specific CD4⁺ T cells express higher levels of, for example, PD-1 and CD39 in peripheral blood than non-gluten-specific CD4⁺ T cells for at least 1 year after initiation of a gluten-free diet [71]. Moreover, the same group has also shown that clones of gluten-specific CD4⁺ T cells remain in blood as memory cells for several years after initiation of a gluten-free diet, and that these cells expand upon re-exposure to gluten [72].

Elevated expression levels of CD26 on CD4⁺ and CD8⁺ T cells were also present in children with a sole diagnosis of CeD. If the cells positive for CD26 were divided into two groups, with medium to high expression denoted as CD26⁺ and very high expression as CD26⁺⁺, it was evident that all groups showed similar levels of CD26⁺, but only children with CeD had increased fractions of CD26⁺⁺ T cells. While the enzymatic activity of CD26 has been investigated in tissue biopsies and in serum from CeD patients [73, 74], the literature on the surface expression of CD26 on T cells in CeD is very sparse. On the contrary, similar studies have been performed on T cells from peripheral blood in T1D patients. One study confirms our findings that the main CD4⁺ and CD8⁺ T cell populations from T1D patients show similar expression levels of CD26 as healthy controls [75].

Another interesting T cell marker, which does not follow the pattern of a higher fraction in CeD, is CD122. Children with a sole diagnosis of T1D showed a lower percentage of CD8⁺CD122⁺⁺ T cells, but not of CD8^{dim}CD122⁺, compared to healthy references. While it is known that CD122 is a part of the receptors for IL-2 and IL-15 [30], it is unclear what physiological role CD8⁺ T cells expressing CD122 have in humans. Since CD22 has been shown

to be expressed on a CD8⁺ regulatory T cell population in mice [31], but is also upregulated on cells with memory-like phenotypes [76], expression of CD122 on CD8⁺ T cells might define several different T cell subpopulations.

In addition to various T cell subpopulations, we also analysed monocytes and NK cells. We could not observe any differences in the percentages of various monocyte subpopulations between the different groups, which is in accordance with other studies [53, 77, 78]. There are also reports where T1D patients showed both higher and lower fractions of the different monocyte subpopulations compared to healthy controls [41, 42], partly depending on disease duration.

While we could not observe any difference in the percentage of the main NK cell population between the groups, as also seen by at least one other study for T1D [42], previous literature reports a lower percentage of total NK cells for both T1D and CeD compared to healthy control subjects [43, 44, 55, 56, 62]. NK cells are often divided into two groups based on the expression of CD16 and CD56; the more cytotoxic CD56^{dim}CD16⁺, and CD56^{bright}CD16⁻ which are more proficient cytokine producers [38]. We could see no difference in percentage of CD56^{bright}CD16⁻ NK cells between the analysed groups, also reported in [53, 56] for T1D and healthy references, and a tendency towards a higher degree of CD56^{dim}CD16⁺ NK cells in CeD compared to the comorbidity group. In addition to these two subpopulations, we also analysed the lesser-known CD56^{dim}CD16⁻ population. Inversely reflecting the CD56^{dim}CD16⁺ population, children with a sole diagnosis of CeD had a lower fraction of CD56^{dim}CD16⁻ cells compared to the comorbidity group, with no statistical differences to T1D. Other studies investigating the CD56^{dim} NK cell subpopulations in T1D have shown that patients with T1D have lower percentages of CD56^{dim}CD16⁺ than controls [44, 56], with one group reporting a higher percentage of CD56^{dim}CD16⁻ [44] and another reporting lower percentages [56].

Despite the existence of numerous publications on the immune landscape in patients with T1D and CeD, with the majority published on T1D, no clear consensus can be reached about the exact details of the immune cells investigated. This might be due to multiple factors, both demographic and clinical, such as the age of patients and the duration of disease, and also methodological, for example, analysis in whole blood versus isolated PBMCs, as well as the choice of reagents and instruments. Thus, more data is needed to facilitate a better understanding of the immunological pathogenesis behind these two diseases. In this study, we characterised peripheral blood from children with T1D and CeD by flow cytometry with extensive panels covering multiple immune cell subpopulations. The studied cohort is rather unique in that it contains children with sole diagnoses of T1D and CeD, but also children with a T1D and CeD comorbidity.

To conclude, this study brings forth information about the peripheral immunological milieu in children with T1D and CeD, and in the rare group of children with a comorbidity of both these diseases. This aids the development of better tools for diagnosis and monitoring and can facilitate the introduction of immune-based treatment regimes for children with these common but still incurable autoimmune disorders.

Author Contributions

Andrea Tompa: conceptualization, methodology, investigation, writing – original draft preparation, writing – review and editing, funding acquisition. **Junko Johansson:** formal analysis, writing – original draft preparation, writing – review and editing, visualisation. **Maria Faresjö:** conceptualization, methodology, writing – review and editing, supervision, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Monoclonal antibodies used for staining for flow cytometric analysis of different peripheral immune cells and their subsets. All antibodies were from BD Biosciences. FITC=fluorescein-isothiocyanate, PE=phycoerythrin, PerCP=peridininchlorophyll-protein, Cy=Cyanine; APC=allophycocyanin, BV=Brilliant Violet, BB=Brilliant Blue. **Table S2:** *p* values from Kruskal-Wallis tests for each separate figure present in the article and in the Supporting Information. *p* values presented in bold font are ≤ 0.05 , while *p* values in italics are 0.05–0.1. **Table S3:** FoxP3 MFI (median fluorescence intensity) values for the different CD4+ T cell subpopulations. Data show median MFI (min MFI–max MFI). T1D=Type 1 diabetes, CeD=Celiac disease, Ref=Reference. Mann-Whitney test, $n=9$, no statistically significant differences. **Table S4:** CCR5 and CCR10 MFI (median fluorescence intensity) values for

CD4⁺ and CD8⁺ T cells. Data show median MFI (min MFI—max MFI). T1D=Type 1 diabetes, CeD=Celiac disease, Ref=Reference. Mann-Whitney test, $n=9$. **Figure S1:** Gating strategy for CD4⁺ T cells. Flow cytometric gating strategy for the subpopulations of CD4⁺ T cells with a patient sample as a representative example. **Figure S2:** Gating strategy for CD8⁺ T cells. Flow cytometric gating strategy for the subpopulations of CD8⁺ T cells with a patient sample as a representative example. **Figure S3:** Gating strategy for monocytes and NK cells. Flow cytometric gating strategy for monocytes and NK cells with a patient sample as a representative example. **Figure S4:** CD122 MFI on CD4⁺ and CD8⁺ T cells. MFI (median fluorescence intensity) values for CD122 on A) CD4⁺CD8⁺, B) CD4⁺CD8^{dim}, C) CD8⁺CD26⁺, D) CD8^{dim}CD26⁺, E) CD8⁺CD26⁺⁺, F) CD8^{dim}CD26⁺⁺ and G) CD4⁺CD26⁺⁺ T cells in the peripheral blood of children with T1D, CeD, T1D and CeD comorbidity, or healthy references, as measured by flow cytometry. T1D=Type 1 diabetes, CeD=Celiac disease, Ref=Reference. Mann-Whitney test, $n=9$. **Figure S5:** Memory subpopulations of CD8⁺ T cells. Percentage of A) CD8⁺CD45RA⁺CCR7⁺ naïve/stem cell memory, B) CD8⁺CD45RA⁺CCR7⁺ central memory, C) CD8⁺CD45RA⁺CCR7⁺ effector memory and D) CD8⁺CD45RA⁺CCR7⁺ TEMRA (CD45RA⁺ effector memory) cells among all CD8⁺ T cells in the peripheral blood of children with T1D, CeD, T1D and CeD comorbidity, or healthy references, as measured by flow cytometry. T1D=Type 1 diabetes, CeD=Celiac disease, Ref=Reference. Mann-Whitney test, $n=9$. **Figure S6:** Expression of perforin on CD8⁺ T cells. MFI (median fluorescence intensity) values for perforin on A) CD8⁺, B) CD8⁺CD45RA⁺CCR7⁺ naïve/stem cell memory, C) CD8⁺CD45RA⁺CCR7⁺ central memory, D) CD8⁺CD45RA⁺CCR7⁺ effector memory and E) CD8⁺CD45RA⁺CCR7⁺ TEMRA (CD45RA⁺ effector memory) T cells in the peripheral blood of children with T1D, CeD, T1D and CeD comorbidity, or healthy references, as measured by flow cytometry. T1D=Type 1 diabetes, CeD=Celiac disease, Ref=Reference. Mann-Whitney test, $n=9$.