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Low expression of $CD39^+/CD45RA^+$ on regulatory T cells (T_{reg}) cells in type 1 diabetic children in contrast to high expression of $CD101^+/CD129^+$ on T_{reg} cells in children with coeliac disease

K. Åkesson,*† A. Tompa,†‡§ A. Rydén¶** and M. Faresjö†‡§
*Department of Pediatrics, §Division of Medical Diagnostics, Ryhov County Hospital, †Futurum – the Academy for Health and Care in Jönköping County Council, ‡The Biomedical Platform, Department of Natural Science and Biomedicine, School of Health Sciences, Jönköping University, Jönköping, **Division of Paediatrics and Diabetes Research Centre, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden, and ¶Pacific Northwest Diabetes Research Institute and Type 1 Diabetes R&D Center, Novo Nordisk Inc., Seattle, WA, USA

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Correspondence: M. Faresjö, Department of Natural Science and Biomedicine, School of Health Sciences, Jönköping University, S-551 11 Jönköping, Sweden.
E-mail: maria.faresjo@hhj.hj.se

Summary

Type 1 diabetes (T1D) and coeliac disease are both characterized by an autoimmune feature. As T1D and coeliac disease share the same risk genes, patients risk subsequently developing the other disease. This study aimed to investigate the expression of T helper (Th), T cytotoxic (Tc) and regulatory T cells (T_{reg}) in T1D and/or coeliac disease children in comparison to healthy children. Subgroups of T cells (Th : $CD4^+$ or Tc : $CD8^+$); naive ($CD27^+CD28^+CD45RA^+CCR7^+$), central memory ($CD27^+CD28^+CD45RA^-CCR7^+$), effector memory (early differentiated; $CD27^+CD28^+CD45RA^-CCR7^-$ and late differentiated; $CD27^-CD28^-CD45RA^-CCR7^-$), terminally differentiated effector cells (TEMRA; $CD27^-CD28^-CD45RA^+CCR7^-$) and T_{reg} ($CD4^+CD25^+FOXP3^+CD127^-$) cells, and their expression of CD39, CD45RA, CD101 and CD129, were studied by flow cytometry in T1D and/or coeliac disease children or without any of these diseases (reference group). Children diagnosed with both T1D and coeliac disease showed a higher percentage of TEMRA $CD4^+$ cells ($P < 0{\cdot}05$), but lower percentages of both early and late effector memory $CD8^+$ cells ($P < 0{\cdot}05$) compared to references. Children with exclusively T1D had lower median fluorescence intensity (MFI) of forkhead box protein 3 (FoxP3) ($P < 0{\cdot}05$) and also a lower percentage of $CD39^+$ and $CD45RA^+$ within the T_{reg} population ($CD4^+CD25^+FOXP3^+CD127^-$) ($P < 0{\cdot}05$). Children with exclusively coeliac disease had a higher MFI of CD101 ($P < 0{\cdot}01$), as well as a higher percentage of $CD129^+$ ($P < 0{\cdot}05$), in the $CD4^+CD25^{hi}$ lymphocyte population, compared to references. In conclusion, children with combined T1D and coeliac disease have a higher percentage of differentiated $CD4^+$ cells compared to $CD8^+$ cells. T1D children show signs of low $CD39^+/CD45RA^+$ T_{reg} cells that may indicate loss of suppressive function. Conversely, children with coeliac disease show signs of $CD101^+/CD129^+$ T_{reg} cells that may indicate suppressor activity.

Keywords: coeliac disease, T cytotoxic cells, T helper cells, T regulatory cells, type 1 diabetes

Introduction

Type 1 diabetes (T1D) and coeliac disease are both characterized by an autoimmune feature. These autoimmune processes involve T cells ($CD4^+/CD8^+$) with both T helper (Th/$CD4^+$) and T cytotoxic (Tc/$CD8^+$) characteristics [1,2]. T1D is caused by the destruction of insulin-producing β cells in pancreas, an event leading to insufficient, or complete lack of, insulin production. Coeliac disease is caused by an immune response against gluten, a protein dominant in wheat and present in other major crops, leading to cross-reaction with small-bowel tissue. This leads to villous atrophy and crypt hyperplasia in the small intestine.

Memory $CD4^+$ and $CD8^+$ T cells are characterized based on CD27 and CD28 expression and subdivided further as naive and/or effectors by expression of $CD45RA^+$ [3]. CCR7 is a homing receptor important in T, B and dendritic cell migration into secondary lymphoid organs [4,5], with an important role in induction and maintenance of central and peripheral tolerance [6,7]. Thus, memory $CD4^+$ and $CD8^+$

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T cells can be subdivided based on CD27 and CD28 expression, and furthermore by differentiated expression of CD45RA and CCR7 into the subsets; naive ($CD27^+CD28^+$ $CD45RA^+CCR7^+$), central memory (T_{CM}, $CD27^+CD28^+$ $CD45RA^-CCR7^+$) effector memory (T_{EM}) by early differentiation ($CD27^+CD28^+CD45RA^-CCR7^-$) or late differentiation ($CD27^-CD28^-CD45RA^-CCR7^-$), or as terminally differentiated (TEMRA) ($CD27^-CD28^-CD45RA^+CCR7^-$) [8]. The primary purpose of this study was to investigate T cell ($CD4^+$ and $CD8^+$) subsets in children with T1D and/or coeliac disease, with a focus on naive-, central memory, early and late differentiated effector memory and terminally differentiated effector cells.

Regulatory T cells (T_{reg}) are important during an immune response in order to maintain self-tolerance and immune homeostasis and thereby avoid autoimmunity. A combination of $CD4^+$ and $CD25^+$ expression is used commonly for characterization of T_{reg} cells, regularly together with the transcription factor forkhead box P3 (FoxP3) [9,10]. Among humans, approximately 1–2% of the $CD4^+CD25^+$ T cells display the strongest regulatory function; this population is usually termed $CD4^+CD25^{high}$ [9]. In addition, CD39, an ectonucleotidase involved in suppression of inflammation, has been shown to be expressed on $FoxP3^+$ T_{reg} cells [11], named $CD39^+FoxP3^+$ T_{regs} [12]. CD101, expressed on, e.g. activated T cells [13], has been shown to be correlated highly with functional suppressor activity within $CD4^+CD25^+$ T_{reg} cells both *in vitro* and *in vivo* [14]. CD129 [interleukin (IL)-9R], also expressed on T cells, may be additionally important for regulation, as IL-9 has been shown to increase the suppressive function of T_{regs}, and this receptor is critical for the early stages of human intrathymic T cell development [15]. In contrast, as activated T_{reg} cells express low levels of CD45RA and CD127, both can be used to distinguish different types of T_{reg} cells [16,17]. Taken together, $CD4^+CD25^+FoxP3^+CD127^-$ T cells may be characterized further by the expression of CD39, CD45RA, CD101 and CD129.

T_{reg} cells have been implicated as being part of the disease process in T1D. In non-obese diabetic (NOD) mice, $CD4^+FoxP3^+$ T_{regs} decrease during the disease progress, in correlation with a decreased level of IL-2 [18]. In humans, data are conflicting with regard to the numbers of T_{regs} in T1D patients. Kukreja *et al.* [19] showed that the numbers of T_{regs} are decreased. In contrast, Lindley *et al.* showed that the numbers of $CD4^+CD25^+$ cells were normal but with a defect in the suppressor activity [20], as did Lawson *et al.*, who demonstrated that the frequency of $CD4^+CD25^{high}$ is similar to healthy controls, as well as the difference in CD127 and FoxP3 expression [21]. Putnam *et al.* [22] have even shown that $CD4^+CD127^{lo/-}CD25^+$ T cells represent a viable cell population for cellular therapy in patients with T1D. Also in coeliac disease, T_{reg} cells are thought to contribute to immunological failure. A higher frequency of $CD4^+CD25^+FoxP3^+$ cells has been reported in active coeliac disease, in comparison to healthy controls and patients with treated coeliac disease [23].

T1D and celiac disease share the same risk genes (DR3–DQ2), with an increased risk of subsequently developing the other disease. FoxP3 mRNA expression, as well as the frequency of $FoxP3^+$ with simultaneous expression of CD4 and CD25 markers, is shown to be increased in mucosa from patients with coeliac disease compared to healthy subjects, and even more pronounced in patients with both coeliac disease and T1D [24]. We have shown previously that patients with T1D have a reduced FoxP3 mRNA expression in peripheral blood mononuclear cells compared to children with coeliac disease and children with a combination of T1D and coeliac disease [25].

The second purpose of this study was thus to examine a panel of different combinations of T_{reg}-associated markers for the characterization of T_{reg} cells: FoxP3, CD127, CD39, CD45RA, CD101 and CD129 in the total $CD4^+CD25^+$ population, as well as in the strict $CD4^+CD25^{high}$ population, in children with a combination of T1D and coeliac disease in comparison to children with either T1D or coeliac disease, as well as healthy children.

Materials and methods

Study population

Children diagnosed with T1D ($n = 14$, seven females, seven males, mean age 14·9 years, median age 14·5 years), coeliac disease ($n = 6$, four females, two males, mean age 12·3 years, median age 11·5 years) or both diagnoses ($n = 7$, three females, four males, mean age 10·8 years, median age 8 years) were included in this study (Table 1). Healthy children ($n = 19$, 10 females, nine males, mean age 12 years, median age 11 years) without any of these diseases were included as a reference group. The material included children with T1D and coeliac disease from the Clinic of Paediatrics, Ryhov County Hospital, Jönköping, Sweden, and healthy control subjects recruited by convenience sampling at the Clinic of Paediatrics at Ryhov County Hospital and Linköping University Hospital, Linköping, Sweden.

Diagnostic criteria and examination procedures

T1D was diagnosed according to the International Society for Pediatric and Adolescent Diabetes (ISPAD) guidelines [26]: 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 (OGTT).

Coeliac disease was diagnosed according to the modified version of the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) criteria [27]. Neither the reference children nor their first-degree relatives displayed

Table 1. Characteristica of study cohort.

Diagnosis	Gender	Age	Duration (years since diagnosis) (T1D)	Duration (years since diagnosis) (C)
Type 1 diabetes and celiac disease	Male	16	4	3
	–	12	10	2
	–	11	4	2
	–	8	5	1
	Female	8	5	4
	–	8	5	4
	–	8	1	5
Type 1 diabetes	Male	17	13	
	–	17	6	
	–	17	3	
	–	15	7	
	–	15	4	
	–	15	2	
	–	15	2	
	Female	17	13	
	–	17	5	
	–	14	7	
	–	14	6	
	–	14	4	
	–	13	9	
	–	8	6	
Celiac disease	Male	10		1
	–	8		1
	Female	17		16
	–	17		1
	–	13		8
	–	10		6
Healthy controls	Male	16		
	–	15		
	–	14		
	–	14		
	–	13		
	–	10		
	–	10		
	–	8		
	–	7		
	Female	17		
	–	17		
	–	16		
	–	14		
	–	11		
	–	10		
	–	10		
	–	9		
	–	9		
	–	8		

any signs of T1D, other autoimmune disease or coeliac disease. Further, none of the children showed signs of colds or other infections at the time of sample collection and none of the children were considered allergic. Blood samples, supplemented with ethylenediamine tetraacetic acid (EDTA), were collected from all children.

Staining for flow cytometry

Staining for flow cytometry was performed in whole blood within 24 h of sampling.

In samples stained exclusively for extracellular markers, staining was performed as follows: blood samples were stained with antibodies for extracellular markers (Table 2) for 15 min at room temperature, then incubated for 10 min with 0·5 ml Optilyse C (Beckman Coulter, Bromma, Sweden), followed by 5 min incubation with the addition of 0·5 ml phosphate-buffered saline (PBS; Life Technologies, Stockholm, Sweden) + 0·5% bovine serum albumin (BSA; Life Technologies) and finally washed twice in PBS + 0·5% BSA.

For samples stained for extra- and intracellular markers, staining was performed as follows: blood samples were stained with antibodies for extracellular markers (Table 2) for 15 min at room temperature and then washed in PBS + 0·5% BSA. After discarding cell supernatant, cells were resuspended and incubated with fixation/permeabilization buffer (eBioscience, San Diego, CA, USA) for 30 min. Suspensions were then centrifuged for 10 min at 500 ***g***, cell supernatants discarded and cells washed with permeabilization buffer (eBioscience). Cells were resuspended in permeabilization buffer, and incubated for 30 min with antibody for intracellular marker (Table 2). Cells were washed with PBS + 0·5% BSA and the cell supernatants were discarded.

After staining, cells were resuspended with PBS + 0·5% BSA and kept at 4°C in darkness until analysis. Analysis was performed on a Gallios flow cytometer (Beckman Coulter).

Gating strategy and analysis

Lymphocytes were gated based on forward- (FSC) and side-scatter (SSC).

Th/Tc

To analyse subgroups of T cells (Table 3), $CD4^+$ (Th) or $CD8^+$ (Tc) cells were gated from the lymphocyte gate. From these gates, naive cells were gated as $CD27^+CD28^+CD45RA^+CCR7^+$. Central memory cells were gated as $CD27^+CD28^+CD45RA^-CCR7^+$. Early differentiated effector memory cells were gated as $CD27^+CD28^+CD45RA^-CCR7^-$ and late differentiated effector cells as $CD27^-CD28^-CD45RA^-CCR7^-$. Furthermore, terminally differentiated effector cells (TEMRA) were gated as $CD27^-CD28^-CD45RA^+CCR7^-$ [8,28,29].

T_{regs}

From the lymphocyte gate, $CD4^+$ cells were gated, followed by gating for $CD25^+$ cells. From this population, a strict

Table 2. Antibodies.

	Antibody	Fluorochrome	Manufacturer
T_{reg}	CD39 (extracellular)	FITC	Biolegend (San Diego, CA, USA)
	FoxP3 (intracellular)	PE	BD Biosciences (San José, CA, USA)
	CD45RA (extracellular)	PerCP-Cy5·5	Biolegend
	CD25 (extracellular)	PE-Cy7	Biolegend
	CD127 (extracellular)	APC	BD Biosciences
	CD4 (extracellular)	APC-Cy7	Biolegend
	CD129 (extracellular)	PE	Biolegend
	CD101 (extracellular)	APC	Biolegend
Th/Tc	CD45RA (extracellular)	FITC	BD Biosciences
	CCR7 (extracellular)	PE	BD Biosciences
	CD28 (extracellular)	PerCP-Cy5·5	BD Biosciences
	CD8 (extracellular)	PE-Cy7	BD Biosciences
	CD27 (extracellular)	APC	Biolegend
	CD4 (extracellular)	APC-Cy7	Biolegend

T_{reg} = regulatory T cells; Th: T helper cells; Tc: cytotoxic T cells; FITC = fluorescein isothiocyanate; PE = phycoerythrin; PerCP = peridinin-chlorophyll proteins; Cy = cyanin; APC = allophycocyanin; FoxP3=forkhead box protein 3.

$FoxP3^+CD127^-$ gate was applied. This population was then examined for the expression of CD39 and CD45RA.

$CD4^+$ cells were also gated from the lymphocyte gate, followed by gating for $CD25^+$ or $CD25^-$ cells. From these populations, a strict $CD127^-CD45RA^+$ gate was applied with or without further gating of $CD39^-$. These populations were then examined for the expression of FoxP3.

From the $CD4^+CD25^+$ gate, the 2% with the highest CD25 expression, $CD4^+CD25^{hi}$, were determined. The $CD4^+CD25^{hi}$ lymphocyte population was then examined further for the expression of CD101 and CD129.

Results were either expressed as frequency of expressed marker (%) or as median fluorescence intensity (MFI), equivalent to the amount of receptors on the cell. All analyses were blinded and performed with the software package Kaluza version 1·2 (Beckman Coulter, Manchester, UK).

Statistics

As the T_{reg}-associated markers were not distributed normally, the Kruskal–Wallis test for unpaired observations was used as a pretest for comparison of three groups or more and, if significant ($P \leq 0{\cdot}05$), two groups were analysed further with the Mann–Whitney *U*-test for unpaired observations. A probability level of $<0{\cdot}05$ was considered statistically significant. To correct for multiple statistical comparisons, the probability level was adjusted by Sidak correction to = 0·01. Statistical analysis was performed with GraphPad Prism version 5·01 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Table 3. Subgroups of Th, $CD4^+$/Tc, $CD8^+$ cells, according to expression of CD27, CD28, CD45RA and CCR7.

Subgroups of Th($CD4^+$)/Tc($CD8^+$)	Marker expression
Naive	$CD27^+CD28^+CD45RA^+CCR7^+$
Central memory	$CD27^+CD28^+CD45RA^-CCR7^+$
Effector memory (early diff)	$CD27^+CD28^+CD45RA^-CCR7^-$
Effector memory (late diff)	$CD27^-CD28^-CD45RA^-CCR7^-$
TEMRA	$CD27^-CD28^-CD45RA^+CCR7^-$

Th: T helper cells; Tc: cytotoxic T cells; TEMRA: terminally differentiated; diff = differentiated.

Ethics

This study was approved by the Research Ethics Committee of the Faculty of Health Sciences, Linköping University, Linköping Sweden in concordance with the Helsinki Declaration. Prior to blood sampling, informed consent was received from all responsible guardians and adolescents, and all children also received oral and written information adapted for their age.

Results

Th and Tc cells in children with T1D and/or coeliac disease

Initially, the proportions of $CD4^+$ (Th) to $CD8^+$ (Tc) T cells within the lymphocyte population were compared between the study groups. It was found that children diagnosed with coeliac disease had a higher proportion of $CD4^+$ T cells to $CD8^+$ T cells compared to healthy controls ($P < 0{\cdot}01$, Fig. 1a).

In contrast, children with coeliac disease had a lower percentage of central memory ($CD4^+CD27^+CD28^+CD45RA^-CCR7^+$) Th cells in the $CD4^+$ lymphocyte population compared to healthy controls as well as T1D children ($P < 0{\cdot}01$ and $P < 0{\cdot}01$, respectively, Fig. 1b). Additionally, there was a higher percentage of terminally differentiated ($CD4^+CD27^-CD28^-CD45RA^+CCR7^-$) Th cells in the $CD4^+$ lymphocyte population in the children with a combined diagnosis of T1D and coeliac disease compared to healthy controls ($P < 0{\cdot}05$, Fig. 1c).

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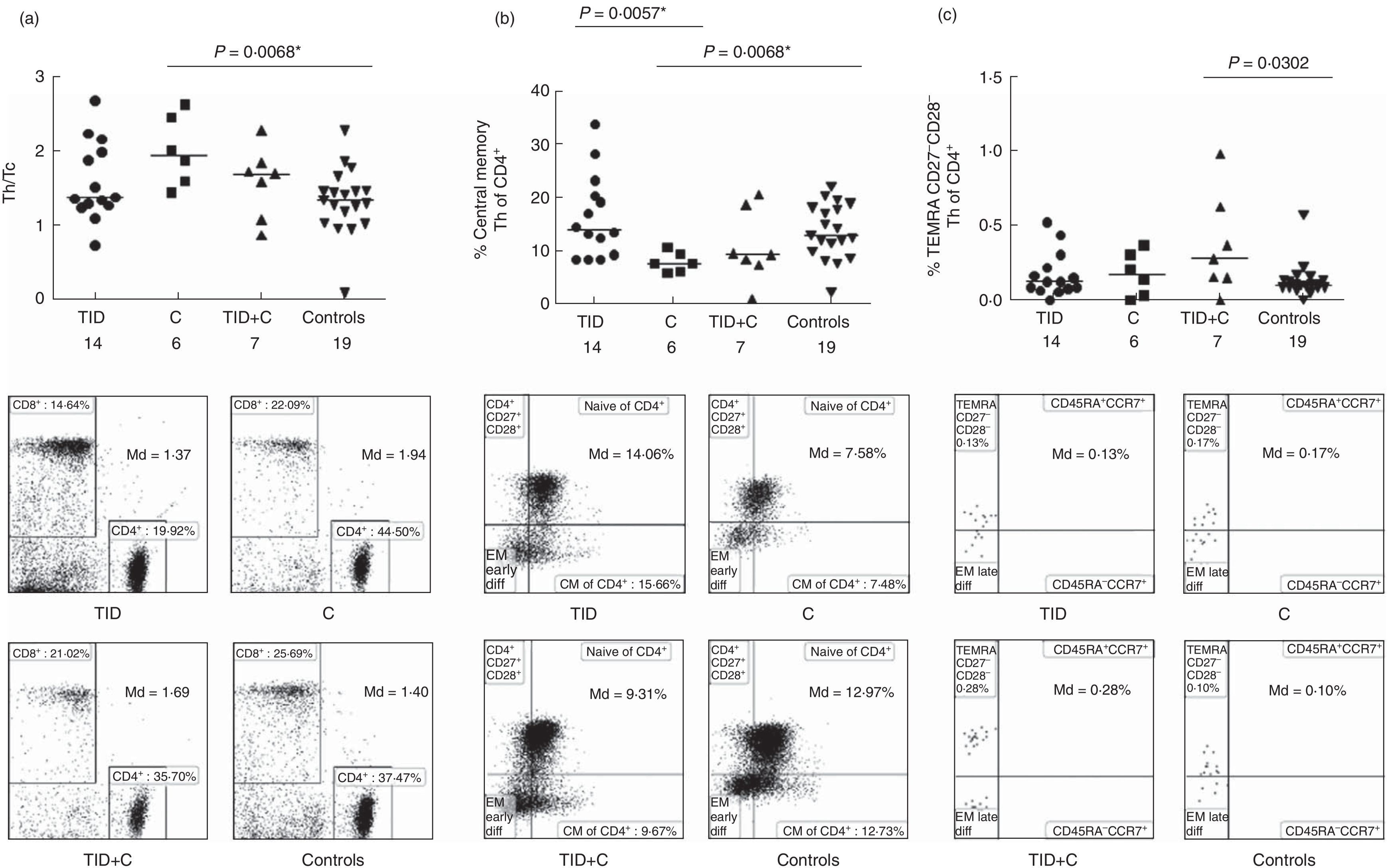

Fig. 1. Dot-plot showing (a) the quotas of T helper (Th) cells *versus* T cytotoxic (Tc) cells, (b) percentages of central memory Th cells in the CD4-positive lymphocyte population and (c) percentages of terminally differentiated Th cells (TEMRA) in the CD4-positive lymphocyte population. Study groups consisting of children diagnosed with type 1 diabetes (T1D), coeliac disease (C), both T1D and C (T1D+C) and healthy controls. Bars display median. *P*-values represent comparison between groups by Mann–Whitney *U*-test. Significance after correction for multiple statistical comparisons by Sidak correction is presented as * ≤ 0·01. Flow cytometric plots show a representative plot of one individual, representing the median (Md) in each study group.

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The $CD8^+$ lymphocyte population contained a higher percentage of naive ($CD8^+CD27^+CD28^+CD45RA^+CCR7^+$) Tc cells in T1D children compared to healthy controls ($P < 0{\cdot}05$, Fig. 2a). Children diagnosed with both T1D and coeliac disease had lower percentages of effector memory Tc cells, early differentiated ($CD8^+CD27^+CD28^+CD45RA^-CCR7^-$) and late differentiated ($CD8^+CD27^-CD28^-CD45RA^-CCR7^-$), in comparison to healthy controls ($P < 0{\cdot}05$, Fig. 2b and $P = 0{\cdot}01$, Fig. 2c, respectively).

No differences were detected between the study groups in the percentage of naive ($CD4^+CD27^+CD28^+CD45RA^+CCR7^+$), early differentiated ($CD4^+CD27^+CD28^+CD45RA^-CCR7^-$) or late ($CD4^+CD27^-CD28^-CD45RA^-CCR7^-$) differentiated effector memory $CD4^+$ cells in the lymphocyte population (data not shown).

T regulatory cells in children with T1D and/or coeliac disease

In examining possible differences in the T_{reg} population between the study groups, no differences in the percentages of $CD4^+CD25^+FoxP3^+CD127^-$ T_{regs} were detected, either within the total $CD4^+$ population or the $CD4^+CD25^+$ population. However, T1D children had a lower MFI of CD25 in the $CD25^{hi}$ population in comparison to healthy controls ($P < 0{\cdot}05$, Fig. 3a); there was also a lower MFI of CD25 in children diagnosed with coeliac disease when compared to children diagnosed with both T1D and coeliac disease ($P < 0{\cdot}01$, Fig. 3a). Further, both T1D children and children with coeliac disease had lower MFI of FoxP3 in the $CD4^+CD25^+FoxP3^+CD127^-$ T_{reg} population compared to healthy controls ($P < 0{\cdot}05$ for both, Fig. 3b). Moreover, T1D children were found to have lower percentages of $CD4^+CD25^+FoxP3^+CD127^-$ T_{regs} positive for both CD39 and CD45RA, in comparison to both healthy controls and children with coeliac disease ($P < 0{\cdot}05$ for both, Fig. 3c).

Similarly, children with T1D had a lower MFI of FoxP3 in the $CD4^+CD25^+CD127^-CD45RA^+$ Treg population ($P < 0{\cdot}01$, Fig. 4a), as well as in the strict $CD4^+CD25^+CD127^-CD45RA^+CD39^-$ T_{reg} population, in comparison to healthy controls ($P = 0{\cdot}01$, Fig. 4b). Further, T1D children had also lower MFI of FoxP3 in the $CD4^+CD25^-CD127^-CD45RA^+CD39^-$ non-T_{reg} population in comparison with children with coeliac disease and healthy controls ($P = 0{\cdot}01$ and $P < 0{\cdot}05$, respectively, Fig. 4c).

Children diagnosed with coeliac disease had a higher MFI of CD101 in the $CD4^+CD25^{hi}$ lymphocyte population compared to both healthy controls and children diagnosed with both T1D and coeliac disease ($P < 0{\cdot}05$ for both, Fig. 5a). Moreover, there were a higher percentage of $CD129^+$ cells in the $CD4^+CD25^{hi}$ lymphocyte population of children with coeliac disease compared to healthy controls ($P < 0{\cdot}05$, Fig. 5b). However, there were no differences in MFI of CD129 in the $CD4^+CD25^{hi}$ lymphocyte population between the study groups (data not shown).

Discussion

Th and Tc cells in children with T1D and/or coeliac disease

The primary purpose of this study was to investigate T cell ($CD4^+$ and $CD8^+$) subsets in children with T1D and/or coeliac disease, with a focus on naive, central memory, early and late differentiated effector memory and terminally differentiated effector cells. With varying outcome, a number of studies have attempted to evaluate circulating T cell subsets in human T1D and, to our knowledge, there is still a shortage of knowledge of T cell subsets in human coeliac disease.

Central memory T cells home to lymph nodes, lack potent effector functions and mount rapid secondary responses upon re-exposure to antigens. It has been found previously that adult T1D patients have a lower absolute lymphocyte count of $CD4^+$ and $CD8^+$ $CCR7^+CD45RA^-$ central memory cells [30]. In our cohort, we observed that children diagnosed with exclusively coeliac disease had a significantly lower percentage of $CD4^+$ central memory cells, and tended to have a lower percentage of $CD8^+$ central memory cells, compared to healthy children but also compared to children with T1D. Because it has been described previously that T_{CM} cells have the unique ability to differentiate in an antigen-independent, but not antigen-dependent, fashion into $CD8^+CD45RA^+CCR7^+$ effector cells [31], we may speculate that the reduction of T_{CM} in children with coeliac disease can be a sign of an impaired immune response.

Children diagnosed with both T1D and coeliac disease had a higher percentage of $CD4^+$ TEMRA cells compared to healthy controls. This is in concordance with a previous observation showing that in adult T1D patients the percentage and number of terminally differentiated effector memory cells (TEMRA, $CD45RA^+CCR7^-$) were markedly increased [30]. They postulated that the considerable accumulation of TEMRA T cells in patients suggests lifelong stimulation by protracted antigen exposure or a homeostatic defect in the regulation/contraction of immune responses [30]. This suggestion fits well with our finding in children with a combination of two immunological diseases with a frequent exposure to several immune-stimulatory antigens and autoantigens.

Children diagnosed with exclusively T1D showed a higher percentage of naive ($CD8^+CD27^+CD28^+CD45RA^+CCR7^+$) Tc cells in comparison to healthy children. We have previously reported a higher percentage of $CD8^+$ lymphocyte expression $CD45RA^+$ as well as $CCR7^+$ in T1D children, especially in children with short disease duration, compared to healthy children [32]. This indicates an increased activation of naive $CD8^+$ cells in children with T1D.

In contrast, children diagnosis with both T1D and coeliac disease had a lower percentage of effector memory Tc cells,

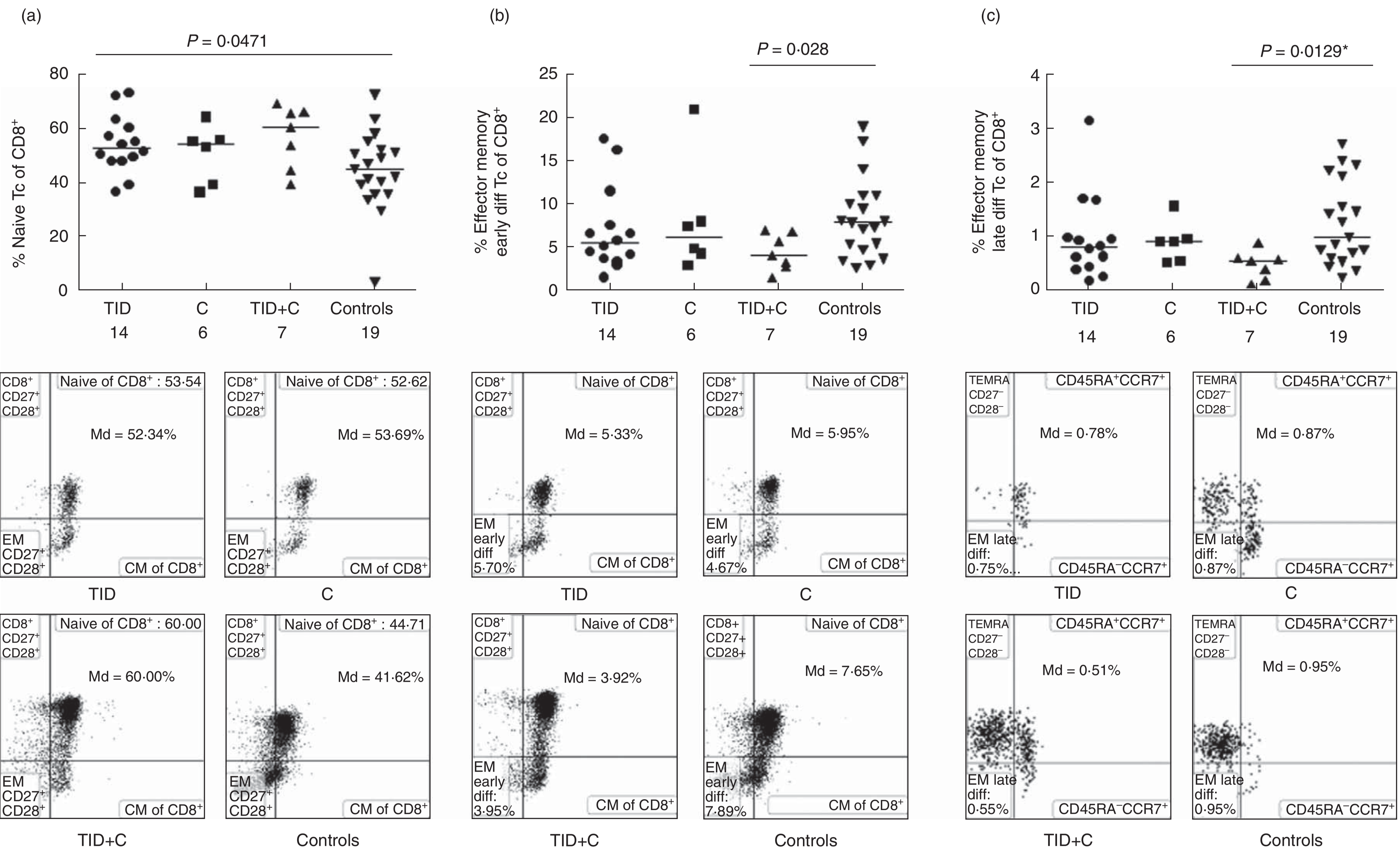

Fig. 2. Dot-plots showing percentages (a) of naive cytotoxic T (Tc) cells, (b) early-differentiated effector memory Tc cells and (c) late-differentiated effector memory Tc cells within the CD8-positive lymphocyte population. Study groups consisting of children diagnosed with type 1 diabetes (T1D), coeliac disease (C), both T1D and C (T1D+C) and healthy controls. Bars display median. *P*-values represent comparison between groups by Mann–Whitney *U*-test. Significance after correction for multiple statistical comparisons by Sidak correction is presented as * ≤ 0·01. Flow cytometric plots show a representative plot of one individual, representing the median (Md) in each study group.

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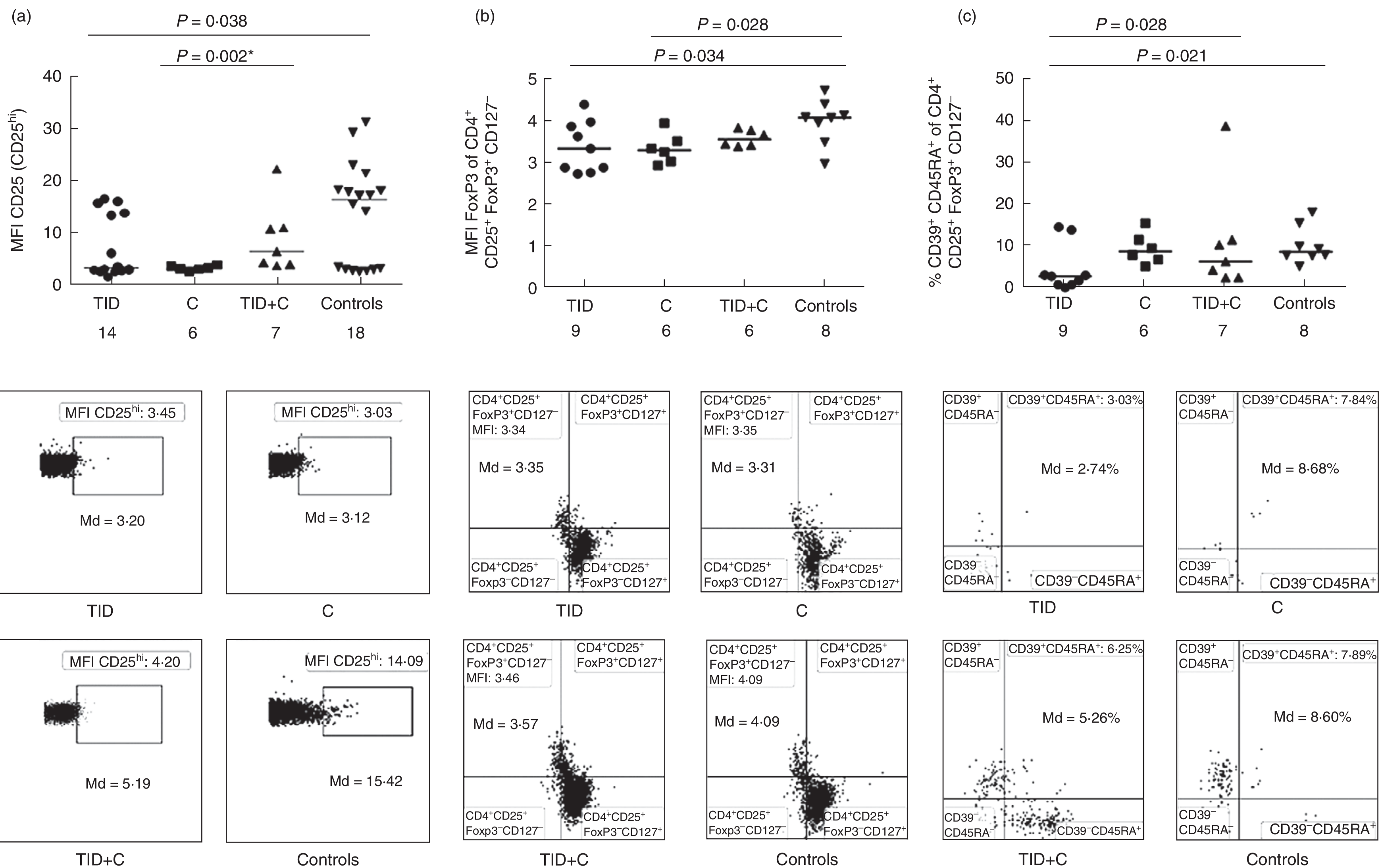

Fig. 3. Dot plots displaying (a) mean fluorescence intensity (MFI) of CD25 expression in the $CD4^{+}CD25^{hi}$ lymphocyte population, (b) MFI of forkhead box protein 3 (FoxP3) in the $CD4^{+}CD25^{+}FoxP3^{+}CD127^{-}$ regulatory T cell (T_{reg}) population and (c) the percentages of $CD4^{+}CD25^{+}FoxP3^{+}CD127^{-}$ T_{reg} cells expressing both CD39 and CD45RA in children diagnosed with type 1 diabetes (T1D), coeliac disease (C), both T1D and C (T1D+C) and healthy controls. Bars display median. *P*-values represent comparison between groups by Mann–Whitney *U*-test. Significance after correction for multiple statistical comparisons by Sidak correction is presented as $* \leq 0{\cdot}01$. Flow cytometric plots show a representative plot of one individual, representing the median (Md) in each study group.

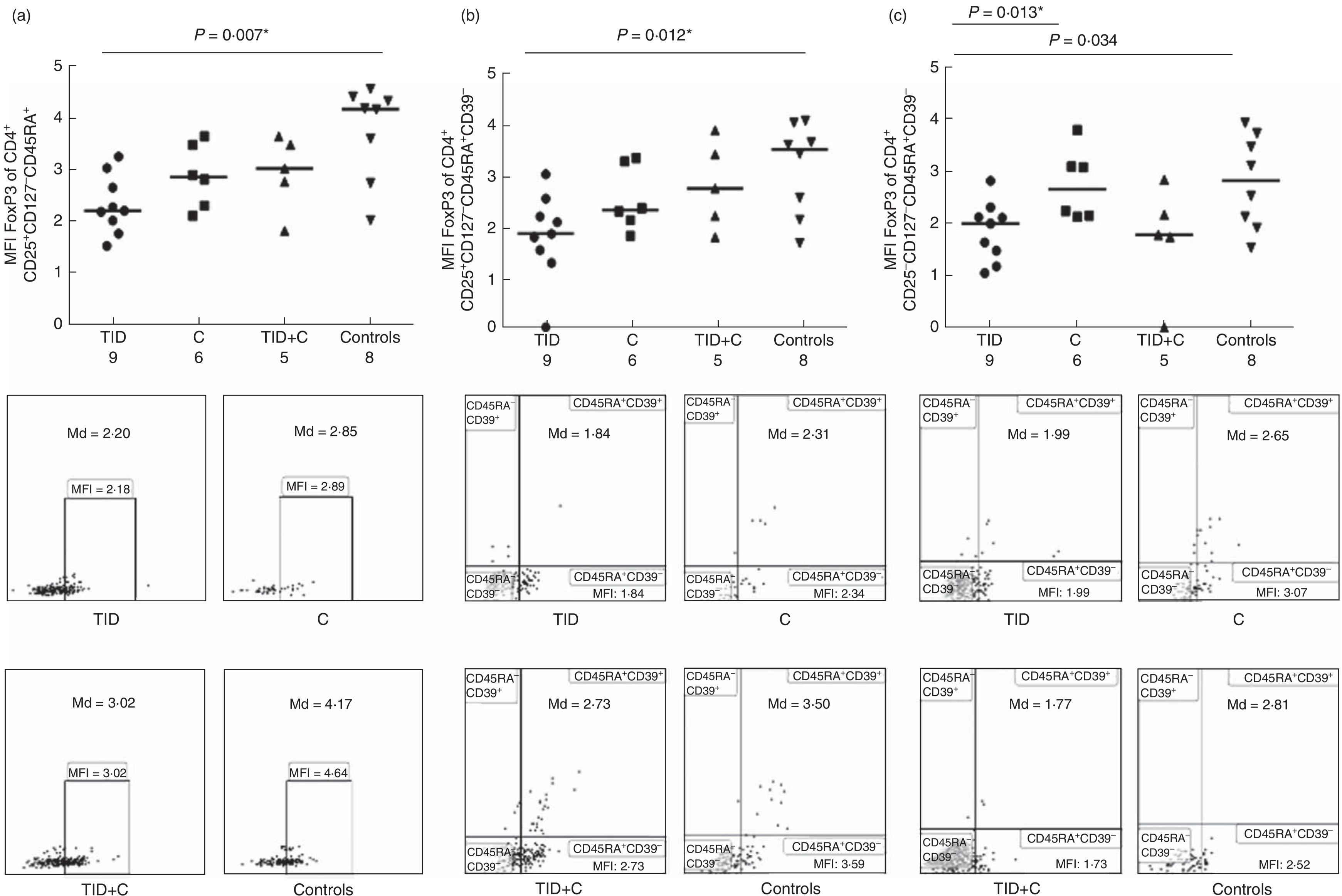

Fig. 4. Dot-plots showing (a) mean fluorescence intensity (MFI) of forkhead box protein 3 (FoxP3) in the $CD4^+CD25^+CD127^-CD45RA^+$ regulatory T cell (T_{reg}) population, (b) MFI of FoxP3 in the $CD4^+CD25^+CD127^-CD45RA^+CD39^-$ strict T_{reg} population and (c) MFI of FoxP3 in the $CD4^+CD25^-CD127^-CD45RA^+CD39^-$ non-T_{reg} population. Study groups consisting of children diagnosed with type 1 diabetes (T1D), coeliac disease (C), both T1D and C (T1D+C) and healthy controls. Bars display median. *P*-values represent comparison between groups by Mann–Whitney *U*-test. Significance after correction for multiple statistical comparisons by Sidak correction is presented as * ≤ 0·01. Flow cytometric plots show a representative plot of one individual, representing the median (Md) in each study group.

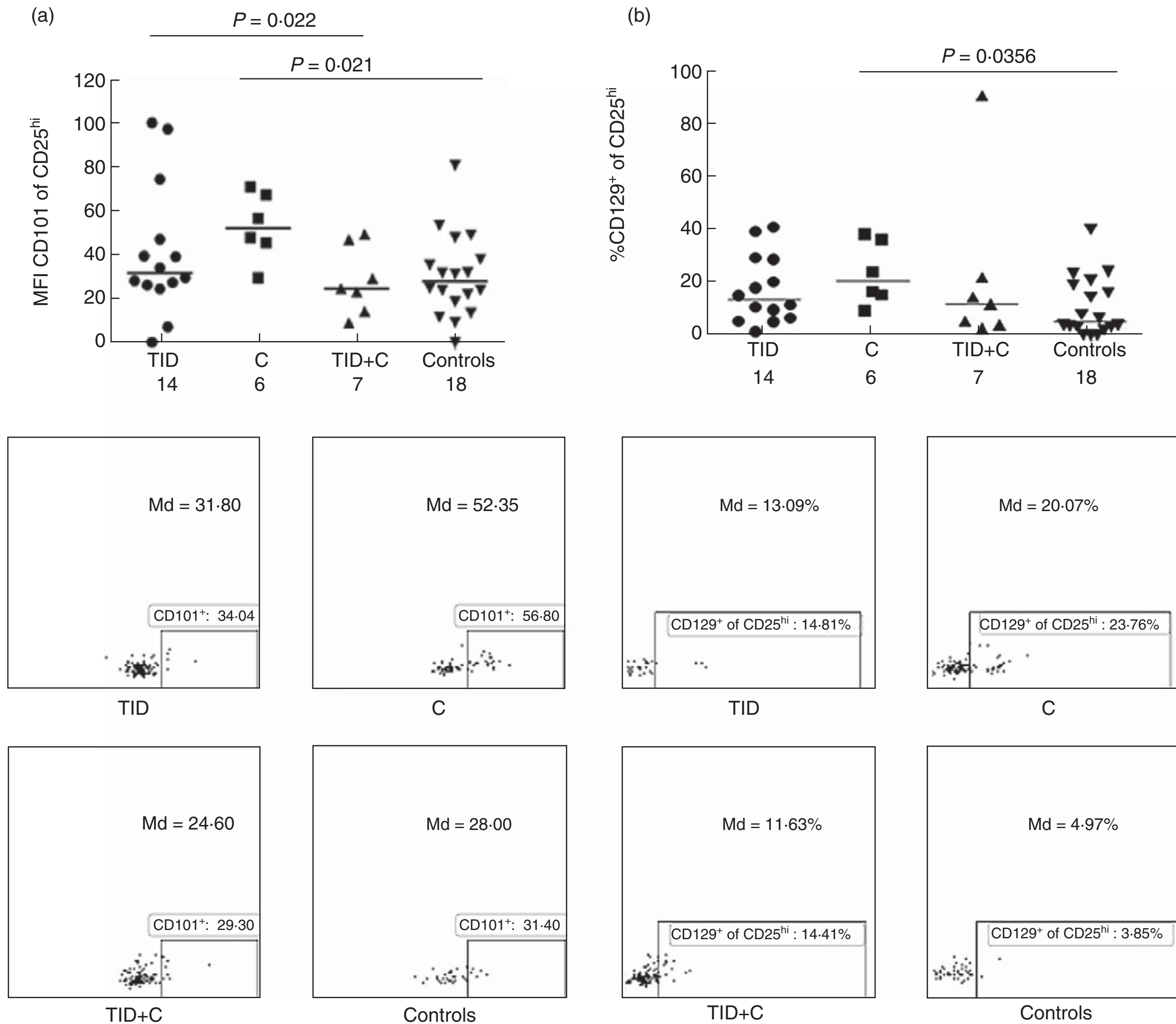

Fig. 5. Dot-plots displaying (a) mean fluorescence intensity (MFI) of CD101 expression in the $CD4^+CD25^{hi}$ lymphocyte population and (b) the percentages of CD129 expressing cells in the $CD4^+CD25^{hi}$ lymphocyte population. Study groups consisting of children diagnosed with type 1 diabetes (T1D), coeliac disease (C), both T1D and C (T1D+C) and healthy controls. Bars display median. *P*-values represent comparison between groups by Mann–Whitney *U*-test. Flow cytometric plots show a representative plot of one individual, representing the median (Md) in each study group.

early ($CD8^+CD27^+CD28^+CD45RA^-CCR7^-$) as well as late differentiated ($CD8^+CD27^-CD28^-CD45RA^-CCR7^-$), in comparison to healthy controls. It has been shown in pancreas grafts that a large population of $CD8^+$ pancreas-infiltrating lymphocytes lacks expression of CD28 [33]. This may indicate a reduced proportion of late differentiated effector memory T cells in T1D children that can support our finding of reduced percentages of differentiated T_{EM} cells.

T regulatory cells in children with T1D and/or coeliac disease

The second purpose of this study was to study a panel of different combinations of T_{reg}-associated markers for characterization of T_{reg} cells (FoxP3, CD127, CD39, CD45RA, CD101 and CD129) in the total $CD4^+CD25^+$ population as well as in the strict $CD4^+CD25^{hi}$ population, in children diagnosed with both T1D and coeliac disease, in comparison to children with either T1D or coeliac disease, as well as in healthy children.

It has been shown repeatedly that T_{reg} cells are affected in T1D patients, but the results are conflicting with regard to, for example, the frequency of T_{reg} cells. In our cohort, we observed that children with either T1D or coeliac disease had a lower MFI of T_{reg} cells ($CD4^+CD25^{hi}$) compared to healthy controls or children diagnosed with both these diseases in combination. A decreased frequency of T_{regs} ($CD4^+CD25^+$) in T1D patients has also been observed

previously [19,34,35]. Currently, there is no precise definition of T_{reg} cells in humans. However, in order to further specify T_{reg} cells ($CD4^+CD25^{hi}$) in our cohort, FoxP3 and CD127 expression was detected. CD127 has been reported to be correlated negatively with FoxP3 and its expression is low on T_{reg} cells [16]. Previously, we found lower percentages of the $CD4^+CD25^+CD127^{lo/-}$ population in children with T1D [36]. In this cohort, we also observed that children diagnosed with either T1D or coeliac disease had a lower MFI of FoxP3 within the T_{reg} population ($CD4^+CD25^+FoxP3^+CD127^-$) compared to healthy controls. Together, these results point towards an affected frequency of T_{regs} in children with autoimmune diseases.

In terms of additional markers for delineating cells of T_{reg} lineage, CD39 and CD45RA have been included as possible markers for detection of T_{reg} cells. Thus far, it has been shown that T_{reg} cells, defined as $CD4^+FoxP3^+$, express more CD45RO but less CD45RA in T1D patients compared to healthy controls [37]. Also in fulminant T1D patients, the frequency of $CD45RA^-FoxP3^{high}$ activated T_{reg} cells is shown to be significantly lower compared to patients with type 1A diabetes [38]. Further, the suppressive functional capacity of $CD45RA^-FoxP3^{high}$ activated T_{reg} cells is impaired and related to residual insulin-secretion capacity [38]. The proportions of $CD39^+$ cells appear highly variable in humans [39], and decreased frequency and function of $CD39^+$ T_{regs} has been reported in, for example, multiple sclerosis [40] and ryegrass allergy [41]. We also found a lower percentage of $CD4^+CD25^+FoxP3^+CD127^-$ T_{regs}, positive for both CD39 and CD45RA, in T1D children in comparison to children diagnosed with coeliac disease or without any of these diseases. Consequently, our data indicate that T1D children show signs of low expression of CD39 and CD45RA within the T_{reg} population that may indicate loss of suppressive function.

Recently, the role of FoxP3 as the optimal marker to pinpointing human T_{reg} cells has been questioned. In fact, FoxP3 mRNA expression is not fully confined to $CD4^+CD25^+$ T_{reg} cells in humans [42]. In humans, in contrast to mice, activated T cells up-regulate FoxP3 transiently without acquiring a T_{reg} cell phenotype and function [43–46]. A minor population of non-regulatory $FoxP3^+$ T cells, exhibiting promiscuous and transient FoxP3 expression, which give rise to $FoxP3^-$ Th cells and selective accumulation in inflammatory milieus, has been identified recently [47]. In order to define true T_{reg} cells, an attempt to expand $CD4^+CD25^+CD127^{lo/-}CD45RA^+$ cells resulted in high-yield, functional T_{regs} that maintained higher FoxP3 expression [22]. Thus, in order to elucidate further the population of T_{reg} cells in our cohort of children with autoimmune diseases, we analysed the FoxP3 expression in $CD4^+CD25^+CD127^-CD45RA^+CD39^-$ (T_{reg} cells) and $CD4^+CD25^-CD127^-CD45RA^+CD39^-$ (non-T_{reg} cells). We observed that children with T1D had lower MFI of FoxP3 in the $CD4^+CD25^+CD127^-CD45RA^+CD39^-$ T_{reg} population in comparison to healthy controls. T1D children also had a lower MFI of FoxP3 in the $CD4^+CD25^-CD127^-CD45RA^+CD39^-$ non-T_{reg} population in comparison to both children with coeliac disease and healthy controls. Impaired function of T_{reg} cells has been observed previously in T1D patients, indicating a defective ability to suppress proliferation of autologous effector T cells [20,48]. Recently, it was suggested that the effector T cell population in T1D can actually resist regulatory activity of T_{reg} cells [21,49]. These observations are in line with our own findings, indicating that children with T1D, besides low expression of CD39 and CD45RA, have a lower expression of FoxP3 within the T_{reg} population compared to children without autoimmune diseases.

Recently, two other cell surface receptors, CD101 and CD129, have been suggested to be associated with T_{reg} cells. CD101 cell surface expression is correlated highly with functional suppressor activity within $CD4^+CD25^+T_{reg}$ both *in vitro* and *in vivo* [14]. IL-9, produced by activated T cells, supports the growth of Th clones and has been shown to increase the suppressive function of T_{regs} [50]. Its receptor, CD129, may be of importance for the regulation of early stages of human intrathymic T cell development [15]. Recently, it was also found that intestinal $CD4^+CD25^+$ T cells from patients with potential or active coeliac disease exerted suppressive effects on T responder cells [51,52]. We observed that children diagnosed with coeliac disease had a higher MFI of CD101 and a higher percentage of $CD129^+$ cells in the $CD4^+CD25^{hi}$ lymphocyte population, compared to both healthy controls and children diagnosed with both T1D and coeliac disease. Also an increased frequency of $CD62L^+$ natural $FoxP3^+$ T cells has been seen in adult coeliac disease patients treated with a gluten-free diet, but not in paediatric patients [53], which may reflect an ongoing chronic inflammation. Thus, our finding of expression of CD101 and CD129 on T_{reg} cells in children with coeliac disease may indicate suppressor activity and thereby suppressive capacity related to systemic inflammation.

In summary, the Th/Tc profile in children diagnosed with T1D showed a reduced proportion of late-differentiated effector memory T cells, indicating reduced percentages of differentiated T_{EM} cells. Children diagnosed with exclusively coeliac disease had a reduced proportion of T_{CM} cells, which may be indicative of an impaired immune response. Furthermore, children with combined T1D and coeliac disease had a higher percentage of TEMRA Th cells in the $CD4^+$ lymphocyte population, thus a higher percentage of differentiated Th cells, compared to Tc cells.

Focusing on T_{reg} cells, children with T1D had a low MFI of CD25 in the $CD25^{hi}$ population, low percentages of $CD39^+$ and $CD45RA^+$ and low expression of FoxP3 within the T_{reg} population, which may indicate a loss of suppressive function. In contrast, children diagnosed with coeliac disease showed signs of a $CD101^+$ and $CD129^+$ T_{reg} cell population that may indicate suppressor activity. Due to

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multiple comparisons, the probability level was adjusted, resulting in the loss of some significant differences in immune marker expression between the studied groups. Thus, these findings are not conclusive, and continuous studies are needed to investigate further the impact of T_{reg} cells in autoimmune diseases, e.g. T1D and coeliac disease.

In conclusion, our results hint that T_{regs} in T1D children may lose their suppressive activity, whereas T_{regs} in children with coeliac disease still show signs of a suppressive feature.

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Disclosure

There were no conflicts of interest in the presented study neither regarding the collection-, analysis- and interpretation of data nor the writing of the report, the decision to submit for publication nor any financial and commercial conflicts.

Author contributions

M. F. was the principal investigator of this paper and designed the study together with K. Å.; K. Å. was the medical adviser and was responsible for contact with all patients and healthy children included in the study. A. T. and A. R. were responsible for laboratory analysis, gating strategy and data analysis. All authors contributed to the preparation of this paper.

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