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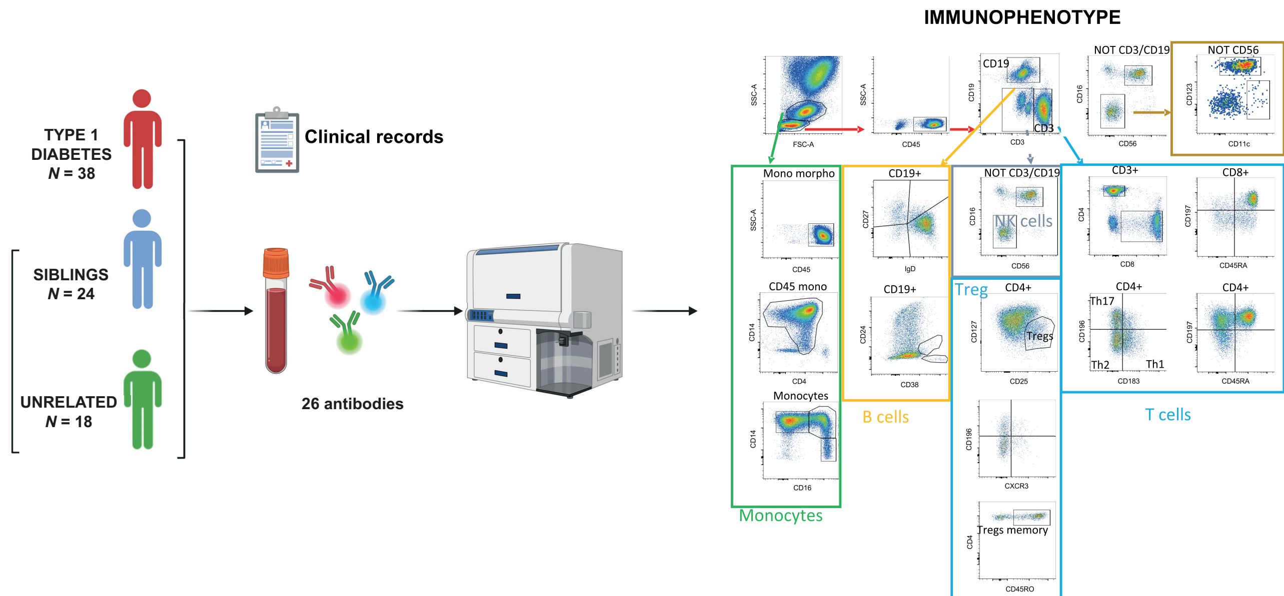

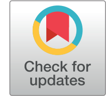

Memory Regulatory T Cells as a Biomarker of Early Type 1 Diabetes

Davide Raineri,[1,2] Silvia Savastio,[3] Simonetta Bellone,[1,3] Lorenza Scotti,[4] Camilla Barbero-Mazzucca,[1,2] Giuseppe Cappellano,[1,2] Flavia Prodam,[1,5] Erica Pozzi,[3] Ivana Rabbone,[1,3] and Annalisa Chiocchetti[1,2]

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IMMUNOLOGY AND TRANSPLANTATION

Type 1 diabetes (T1D) is the most common chronic autoimmune disease in children, driven by a breakdown in self-tolerance and T cell–mediated immune attack of pancreatic β-cells. There are no biomarkers to effectively diagnose autoimmunity before disease onset and clinical symptom development. Here, we applied deep multiparametric immunophenotyping to compare immune landscapes in 38 patients with new-onset T1D, 24 siblings, and 18 healthy control participants (HCs). Patients with T1D underwent clinical and metabolic evaluations. Immune populations in fresh whole-blood samples were analyzed using a panel of 26 antibodies, detecting 39 different cell populations. Memory regulatory T cells (memory Tregs) were significantly increased in patients with T1D ($P < 0.05$) and their siblings ($P < 0.01$) compared with HCs but not between patients with T1D and siblings. Memory Tregs were associated with disease status and age in multivariable analysis. There was a positive correlation between age and memory Tregs in the HC and sibling groups but not in patients with T1D. Baseline memory Treg levels in siblings resembled those of patients with T1D. These findings highlight the existence of an age-independent, disease-specific immune fingerprint that could serve as a minimally invasive biomarker for early diagnosis and personalized immunotherapy. Further studies using functional and single-cells analysis are needed to confirm memory Tregs as a pathogenic trait.

ARTICLE HIGHLIGHTS

- There are more memory regulatory T cells (Tregs) in individuals with type 1 diabetes (T1D) and siblings than in healthy control (HC) individuals.
- Individuals with T1D and their siblings share an immunological profile, with siblings displaying an intermediate phenotype that overlaps with both T1D and HC individuals.
- Memory Tregs increased with age in HC individuals and siblings but not in individuals with T1D.
- Diabetic ketoacidosis status had no impact on immune cell populations in patients with T1D.

Type 1 diabetes (T1D) is a multifactorial autoimmune disease characterized by the progressive, immune-mediated destruction of insulin-producing pancreatic β-cells, ultimately leading to absolute insulin deficiency (1). This process is driven by complex immune mechanisms, including cytotoxic autoreactive $CD8^+$ T cells and sustained release of proinflammatory cytokines that contribute to β-cell dysfunction and loss (2–4). Genome-wide association studies have identified approximately 60 risk loci, many of which are enriched in genes critical for regulatory T cell

[1]Department of Health Sciences, Interdisciplinary Research Center of Autoimmune Diseases, University of Eastern Piedmont, Novara, Italy
[2]Center for Translational Research on Autoimmune and Allergic Diseases, University of Eastern Piedmont, Novara, Italy
[3]Division of Paediatrics, Department of Health Sciences, University of Eastern Piedmont, Novara, Italy
[4]Department of Translational Medicine, University of Eastern Piedmont, Novara, Italy
[5]Endocrinology, Department of Translational Medicine, University of Eastern Piedmont, Novara, Italy

Corresponding author: Annalisa Chiocchetti, annalisa.chiocchetti@med.uniupo.it

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I.R. and A.C. share senior authorship.

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(Treg) development and function (5–7). Despite these advances, the functional impact of most risk variants remains unclear, leaving important gaps in understanding how genetic susceptibility translates into pathogenic immune pathways during the preclinical phase of the disease. Although T1D has long been considered a T helper 1 (Th1) cell–mediated autoimmune disorder, growing evidence points to the involvement of a broader network of both innate and adaptive immune cells (8). Nevertheless, comprehensive immunoprofiling at early onset of T1D remains limited. Most existing studies have been conducted after disease onset and have reported marked age-dependent variability in immune cell composition (9). Moreover, the use of multiple antibody panels, while reducing blood volume requirements, often limits the ability to detect rare or phenotypically overlapping immune cell populations (10,11). Importantly, although age plays a central role in development of T1D, the independent contributions of age, disease status, and age-related factors such as BMI are frequently insufficiently accounted for in statistical analyses (12).

In the present study, we applied high-dimensional multiparametric flow cytometry to systematically profile peripheral immune cell populations with the goal of identifying early immunological biomarkers of T1D while rigorously controlling for potential confounders. Importantly, we also included siblings of individuals with T1D, who are at increased genetic and immunological risk, to identify shared immune signatures that may precede the loss of immune tolerance, rather than reflecting established autoimmunity (13).

RESEARCH DESIGN AND METHODS

Study Populations

This prospective observational study was conducted at the Division of Pediatrics, University of Piemonte Orientale (Novara, Italy), between 1 December 2020 and 30 April 2023. Forty-two children and adolescents with newly diagnosed T1D were enrolled at disease onset, prior to treatment, and underwent peripheral blood collection for immunophenotyping, along with anamnestic evaluation, physical examination, and assessment of metabolic parameters (14).

Evaluations included diabetic ketoacidosis (DKA) status, HbA_{1c}, insulin requirements, 25-hydroxyvitamin D (25OHD), thyroid function, autoantibodies (namely, GAD antibody, insulin autoantibody, IA-2, islet cell antibody, ZnT8), celiac disease antibodies, and lipid profile, measured by immunoradiometric assays with coefficients of variation of 13%, 8.4%, and 13%. DKA was assessed according to International Society for Pediatric and Adolescent Diabetes criteria, with severe DKA defined as pH <7.1 or serum bicarbonate <5 mmol/L. Height and weight were measured using standardized instruments, and BMI was calculated as weight (kg) divided by the square of height (m). Participants completed a questionnaire on ethnicity and family history of immune diseases. The study included 25 asymptomatic siblings of patients with T1D and 19 age- and sex-matched healthy control (HC) participants. Exclusion criteria were acute illness, eating disorders, genetic diseases, or treatments affecting immune function. No participants had vascular complications at enrollment. T1D diagnosis followed American Diabetes Association criteria (15). The study was approved by the local ethics committee (Maggiore della Carità Hospital Ethical Committee, approval CE 117/19), and written informed consent was obtained from all participants. Immunophenotyping was performed in a subset of the study cohort, including 38 individuals with T1D, 24 siblings, and 18 HC participants. Blood samples from the remaining participants (4 individuals with T1D, 1 sibling, and 1 HC participant) were unavailable for this specific analysis due to insufficient sample volume and were therefore not included.

Flow Cytometry Analysis

We used a panel of 26 antibodies to identify 39 immune cell types, including total $CD4^+$ and $CD8^+$ T cells and their subsets, such as Th1, Th2, and Th17, memory and naïve Tregs, and memory $CD4^+$ T cells (Supplementary Fig. 1), as described in our previous report (16).

Statistical Analysis

Continuous variables are presented as mean ± SD and categorical variables as counts and percentages; normality was verified using the Shapiro–Wilks test. Immune population frequencies were compared by ANOVA among patients with T1D, their siblings, and HC participants, and between T1D subgroups using Student *t* test. For multiple comparisons, *P* values were adjusted using the positive false discovery rate, and Tukey post hoc test was applied

Table 1—Characteristics of the study cohort (*N* = 80)

Variable	T1D (*n* = 42) Mean (SD)	Siblings (*n* = 25) Mean (SD)	HCs (*n* = 19) Mean (SD)	*P* value by ANOVA	*P* value for FDR	*P* value (Tukey adjustment)		
						T1D vs. S	T1D vs. HC	S vs. HC
BMI	17.31 (3.23)	20.07 (5.39)	15.57 (1.43)	0.0012	0.0065	0.0144	0.2932	0.0016
Height (m)	1.34 (0.26)	1.46 (0.25)	1.21 (0.25)	0.0099	0.0377	0.1317	0.2071	0.0078
Age	8.9 (4.26)	11.02 (3.89)	7.07 (3.99)	0.0129	0.0417	0.1081	0.3032	0.0115
Weight (kg)	33.1 (16.42)	46.42 (23.22)	23.67 (10.71)	0.0006	0.0044	0.0122	0.1958	0.0006

FDR, false discovery rate; S, siblings.

when ANOVA was significant. Multivariable linear regression, adjusted for age, sex, and BMI, was used to assess associations between disease status and immune cell frequencies; an interaction term was included for memory Tregs to evaluate age-related effects. Statistical significance was set at $P < 0.05$, and analyses were performed using SAS, version 9.4.

Data and Resource Availability

Data are available from the corresponding author on reasonable request.

RESULTS

Demographic and Clinical Data in T1D and Control Groups

There were no significant differences in age or sex distribution between individuals with T1D and control participants (i.e., siblings and HC participants). On the contrary, siblings were significantly older than HC participants and had a higher BMI compared with both individuals with T1D and HC participants. Baseline demographic and clinical characteristics are summarized in Table 1. Among siblings, 5 of 25 tested positive for at least one diabetes-associated autoantibody, including two who were positive for two autoantibodies. All children with T1D were positive for disease-specific autoantibodies, whereas all HC participants were negative.

Multiparametric Flow Cytometry Reveals Alterations in Different Immune Cell Subsets in T1D

Immune cell frequencies expressed as percentages of the total acquired events of the parent population are shown in Supplementary Fig. 1. Among $CD4^+$ T subsets, the percentages of Th1 cells were significantly lower in both the T1D and HC groups compared with siblings (Fig. 1). Although total Treg numbers did not differ, memory Tregs were increased in individuals with T1D and in siblings, compared with HC participants. (Fig. 1).

There were no differences in total $CD8^+$ T cells between patients with T1D and either siblings or HCs. However, between the control groups, percentages of $CD8^+$ effector memory and naïve Tregs were significantly increased and decreased, respectively, in siblings compared with HCs (Supplementary Fig. 2).

In the innate cell compartment, intermediate monocytes were significantly increased in the T1D group compared with both sibling and HC groups (Fig. 1). Monocyte-derived dendritic cells (mDCs) were significantly decreased in the T1D group compared with siblings (Fig. 1).

Memory Tregs Are Influenced by Disease Status and Age

We next examined whether the differences observed between the T1D and control groups were due to baseline differences among the three groups (Table 2) by performing multivariable linear regression adjusting for age, sex, and BMI. Interestingly, the multivariable regression model revealed that disease status and age were significantly associated with increased frequency of memory Tregs (Table 3).

Memory Tregs Are Not Correlated With Age in T1D

To further evaluate if the association between age and memory Treg cells differed in the three groups, we added an interaction term to the linear model to determine relationships between the two variables for each group (Fig. 1*B*). Although the interaction term did not produce statistically significant differences, a similar positive relationship between age and memory Tregs was observed in siblings and HCs (β = 2.09 and 2.12, respectively), which was not seen in patients with T1D (P = 0.1340 for β parameter). Moreover, siblings had baseline memory Treg levels similar to those of individuals with T1D, suggesting early immune alterations.

DKA Status Does Not Influence the Immune Cell Populations in Patients With T1D

In our cohort, at disease onset, 16 of 38 patients (42%) with T1D developed DKA, which was severe in 8 patients (21%). As expected, patients with DKA had higher levels of ketone bodies and lower pH, bicarbonate ($P < 0.0001$), and C-peptide ($P < 0.05$) than individuals without this adverse event (Supplementary Table 2). At discharge, the total daily insulin dose (IU/kg/day) was higher in patients with DKA than without DKA ($P < 0.01$). Patients with DKA had lower hemoglobin levels, lymphocyte percentages ($P < 0.01$), and HDL levels ($P < 0.05$) and higher neutrophils and triglyceride values ($P < 0.05$) than those without DKA ($P < 0.05$). Patients with severe DKA had higher glucose levels (52 vs. 394.6 mg/dL; $P < 0.01$) and lower 25OHD (34.5 vs. 48.7 nmol/L; $P < 0.05$) levels than patients without severe DKA (Supplementary Table 2). There were no significant differences in any immune cell population when individuals were stratified according to the presence of DKA (Supplementary Table 3).

DISCUSSION

Our results in individuals with T1D define a distinct immunological landscape characterized by an expansion of memory Tregs compared with HC participants. A similar increase was also observed in their unaffected siblings, who nonetheless exhibited a unique intermediate immune phenotype marked by increased mDCs and $CD8^+$ effector memory T cells, together with reduced intermediate monocytes and naïve Tregs. Collectively, these findings suggest a shared immunological background between individuals with T1D and their siblings, with siblings occupying a transitional state between immune health and overt disease. The enrichment of T1D-associated risk polymorphisms in genes regulating Treg biology among siblings supports a genetically driven contribution to these immune alterations (17). By including siblings alongside unrelated HCs, we were able to disentangle the effects

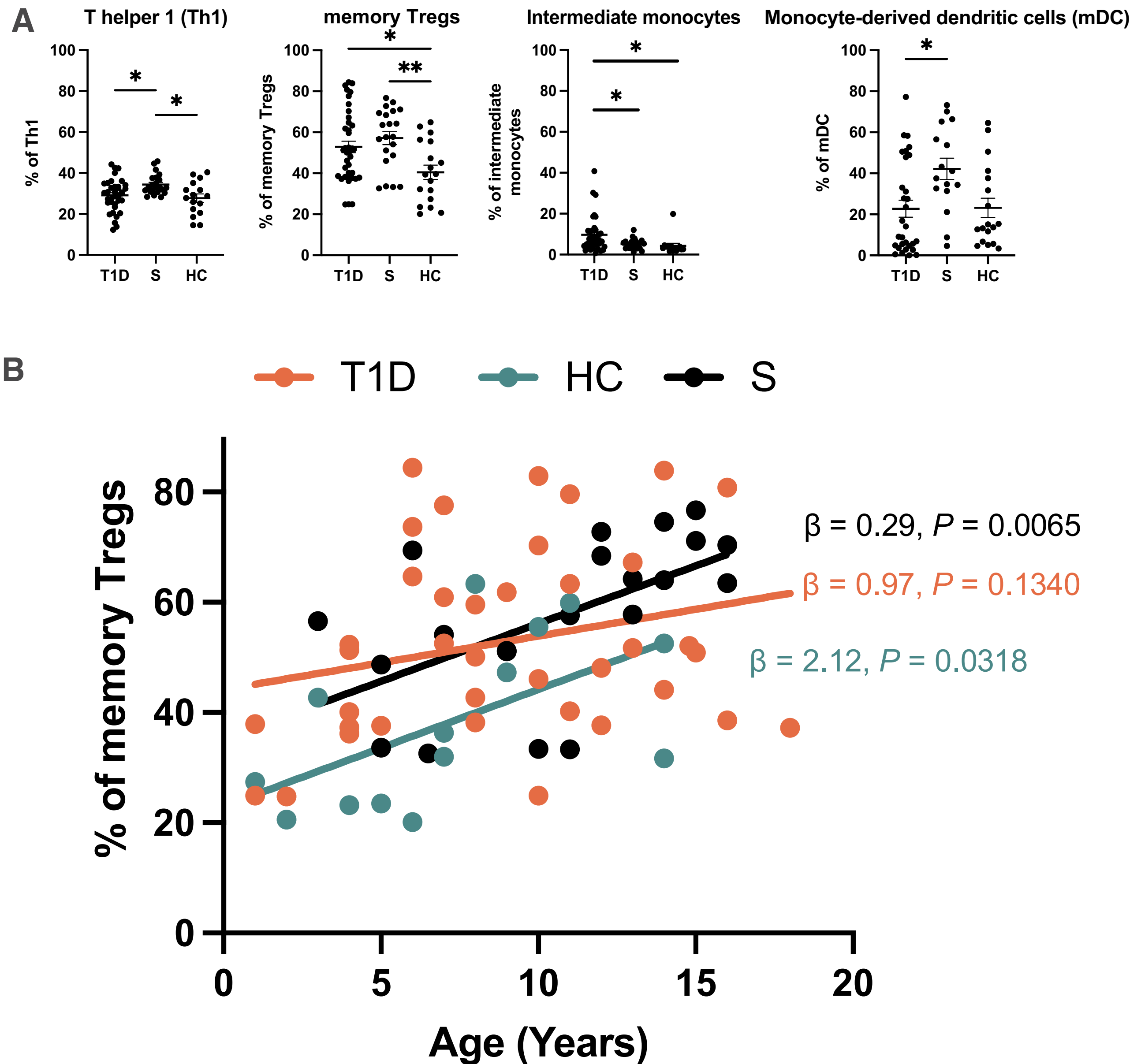

Figure 1—*A*: CD4+ Th1 cells, memory Tregs, intermediate monocytes, and mDCs were modulated into different groups. Dot plots show the percentage of the evaluated immune populations. $*P < 0.05$, $**P < 0.01$. Kruskal-Wallis test or one-way ANOVA was used to test differences between groups. *B*: Scatter plots showing correlations between age and percentage of memory Tregs in different study groups: HCs, siblings (S), and individuals with T1D. The plot shows the β value of the regression and the corresponding *P* values.

of shared genetics and environment from disease-specific immune changes, thereby capturing early immunological deviations that may precede clinical onset. Importantly, our analyses rigorously accounted for age, sex, and BMI, key confounders that often are overlooked, strengthening the specificity of the observed associations with T1D-related immune biology.

Univariable analyses revealed differences in memory Tregs, Th1 cells, intermediate monocytes, and mDCs in T1D. However, after multivariable adjustment, memory Tregs emerged as the only immune population independently associated with disease status, regardless of DKA. This finding aligns with previous reports describing increased yet functionally impaired memory Tregs in T1D, as well as elevated intermediate monocytes with enhanced antigen-presenting capacity in recent-onset disease (18).

The central role of T-cell regulation highlighted by our data is further supported by therapeutic evidence. Immunomodulatory strategies targeting effector–regulatory T-cell balance, such as treatment with alefacept and teplizumab, have demonstrated the ability to preserve β-cell function or delay disease progression by selectively modulating effector and memory T cells while sparing or reshaping regulatory compartments (19,20).

Table 2—Differences in immune population percentages between individuals with T1D and the pooled control participants

	T1D (*n* = 38) Mean (SD)	Siblings (*n* = 24) Mean (SD)	HC participants (*n* = 18) Mean (SD)	*P* value	*P* value for pFDR[a]
$CD3^+$	67.89 (6.32)	65.9 (9.6)	63.06 (7.31)	0.0921	0.0923
$CD8^+$ CM	5.47 (3.97)	4.53 (4.8)	4.77 (4.56)	0.6980	0.6980
$CD8^+$ naïve	56.52 (16.7)	47.29 (15.81)	58.83 (14.53)	0.0393	0.0713
$CD8^+$ EM	**18.09 (12.09)**	**23.41 (13.76)**	**12.35 (8.03)**	**0.0144**	**0.0384**
$CD8^+$ TEMRA	19.44 (15.19)	26.34 (12.42)	24.07 (13.42)	0.1642	0.1642
$CD4^+$	57.69 (8.9)	53.95 (8.63)	51.27 (14.77)	0.0851	0.0923
$CD4^+$ CM	25.21 (13.99)	26.44 (10.39)	22.01 (9.48)	0.5294	0.5294
$CD4^+$ naïve	57.28 (21.25)	59.9 (13.77)	66.03 (12.88)	0.2655	0.2655
$CD4^+$ EM	12.27 (12.54)	12.34 (8.61)	9.12 (6.28)	0.5586	0.5586
$CD4^+$ TEMRA	5.22 (15.89)	1.33 (1.06)	2.84 (2.88)	0.4292	0.4292
Th17	22 (7.92)	19.49 (5.12)	20.68 (5.81)	0.3897	0.3897
Th17-Th1	14.32 (6)	16.99 (5.85)	14.81 (8.35)	0.3112	0.3112
Th2	34.59 (12.2)	29.12 (8.17)	36.79 (13.54)	0.0969	0.0969
Th1	**29.1 (7.76)**	**34.4 (4.94)**	**27.71 (8.2)**	**0.0081**	**0.0384**
Tregs	7.31 (2.83)	8.05 (2.68)	6.62 (2.48)	0.2450	0.2450
Treg memory	**52.85 (17.37)**	**57.39 (14.42)**	**40.79 (14.96)**	**0.0058**	**0.0384**
Treg naïve	**47.12 (17.44)**	**42.42 (14.67)**	**57.03 (13.91)**	**0.0183**	**0.0407**
Treg naïve $ICOS^+$	6.91 (8.83)	3.48 (2.27)	10.74 (13.62)	0.0517	0.0766
Treg $ICOS^+$	19.04 (14.12)	16.48 (9.11)	20.48 (9.87)	0.5343	0.5343
Treg $CCR6^+$	16.24 (9.92)	16.71 (4.5)	16.82 (9.54)	0.9669	0.9669
Treg $CCR6^+/CXCR3^+$	5.81 (3.37)	7.36 (2.94)	5.46 (3.53)	0.1393	0.1393
Treg $CCR6^-/CXCR3^-$	64.36 (13.4)	60.25 (9.91)	66.58 (14.41)	0.2869	0.2869
Treg $CXCR3^+$	13.59 (7.8)	15.65 (5.5)	10.89 (5.77)	0.1104	0.1104
$CD4^+$ memory	22.93 (9.72)	22.67 (7.01)	17.83 (4.54)	0.0777	0.0923
$CD19^+$	17.75 (5.97)	15.41 (5.7)	17.36 (6.61)	0.3211	0.3211
Class switched memory	10.91 (10.06)	10.87 (5.95)	11.08 (6.63)	0.9966	0.9966
Nonclassical switched memory	7.19 (10.76)	7.13 (5.68)	5.15 (3.83)	0.6654	0.6654
Naïve B cells	78.44 (15.16)	76.22 (8.45)	74.17 (20.02)	0.6211	0.6211
Not naïve B cells	37.59 (15.85)	39.92 (12.68)	30.98 (12.04)	0.1219	0.1219
Transitional B cells	9.29 (7.15)	9.2 (5.69)	9.89 (4.67)	0.9290	0.9290
Plasmablasts	2.45 (3.1)	1.67 (1.88)	2.01 (1.27)	0.4825	0.4825
Cytotoxic T cells	31.25 (8.23)	33.93 (7.16)	31.57 (7.25)	0.3924	0.3924
NK cells	47.26 (16.55)	60.22 (18.65)	48.65 (20.09)	0.0428	0.0713
NKT	3.29 (2.45)	3.73 (2.73)	3.34 (3.23)	0.8431	0.8431
Activated NK cells	1.64 (1.91)	0.77 (0.53)	1 (1.01)	0.0844	0.0923
DC	23.75 (16.69)	23.91 (14.94)	27.78 (13.1)	0.6423	0.6423
mDC	**22.73 (22.67)**	**42.16 (20.84)**	**23.47 (20.07)**	**0.0121**	**0.0384**
pDC	17.35 (18.02)	22.72 (15.08)	15.54 (13.01)	0.3451	0.3451
Monocytes	12.87 (7.55)	9.87 (3.77)	11.01 (6.52)	0.2103	0.2103
Classical monocytes	81.28 (10.2)	86.13 (7.49)	82.21 (9.09)	0.1442	0.1442
Intermediate monocytes	**9.69 (8.92)**	**5.15 (2.43)**	**4.33 (4.57)**	**0.0115**	**0.0384**
Nonclassical monocytes	3.47 (3.07)	3.58 (2.51)	4.93 (2.95)	0.2617	0.2617

DC, dendritic cell; EM, effector memory; NK, natural killer; pFDR, positive false discovery rate; ICOS, Inducible Co-Stimulator; NKT, natural killer T cells; pDC, plasmacytoid dendritic cells; TEMRA, T effector memory RA+. [a]Bold values indicate statistical significance ($P < 0.05$).

Table 3—Multivariable regression adjusting for BMI, age, and sex

		Intermediate monocytes			Th1 cells			Memory Tregs[a]			mDCs		
		β (SE)	*P* value	*P* value for F test	β (SE)	*P* value	*P* value for F test	β (SE)	*P* value	*P* value for F test	β (SE)	*P* value	*P* value for F test
T1D	T1D vs. HC	5.35 (2.48)	0.0347	0.0704	0.87 (2.38)	0.7156	0.1625	**12.2 (4.83)**	**0.0139**	**0.0354**	1.02 (7.04)	0.8850	0.0827
	S vs. HC	2.50 (2.77)	0.3705		4.65 (2.79)	0.1002		**12.9 (5.72)**	**0.0274**		16.1 (8.56)	0.0655	
BMI		−0.22 (0.23)	0.3415	0.3415	0.49 (0.25)	0.0528	0.0528	0.21 (0.52)	0.6868	0.6868	0.17 (0.88)	0.8517	0.8517
Age		−0.18 (0.22)	0.4067	0.4067	−0.16 (0.23)	0.4743	0.4743	**1.32 (0.49)**	**0.0087**	**0.0087**	1.25 (0.73)	0.0933	0.0933
Sex	Female vs. male	−2.53 (1.77)	0.1576	0.1576	1.00 (1.89)	0.5994	0.5994	3.73 (3.81)	0.3312	0.3312	3.45 (5.83)	0.5570	0.5570

S, siblings. [a]Bold values indicates statistical significance ($P < 0.05$).

Thus, our observation of increased memory Tregs in patients with T1D and their at-risk siblings underscores the potential relevance of early immune intervention, before irreversible β-cell loss occurs. Whether Treg dysregulation in T1D represents a primary driver or a downstream consequence of disease remains debated. Genetic associations with key Treg-related genes support a causal role, becauses such variants may compromise Treg development and function prior to clinical onset (21). This interpretation is strongly supported by our data showing elevated memory Tregs not only in individuals with T1D but also in their siblings, suggesting that Treg alterations arise early and possibly are linked to genetic susceptibility rather than established autoimmunity alone. At the same time, the inflammatory milieu characteristic of T1D, rich in cytokines such as IFN-γ, IL-17, and TNF-α (22), may further destabilize Tregs, indicating that genetic and inflammatory mechanisms likely converge along the disease continuum.

Across autoimmune diseases, circulating Treg frequencies and function vary widely and do not consistently correlate with disease status (23). Consistently, we observed no differences in total circulating Tregs, but we did find a selective expansion of memory Tregs in patients with T1D and their siblings, pointing to early, preclinical immune remodeling. Notably, increased memory Treg numbers are also a hallmark of immune aging, reflecting reduced naïve Treg generation and a shift toward memory phenotypes (24). Accelerated immune aging has been proposed in T1D (11); however, our observation of this pattern in siblings suggests such changes may occur before clinical disease onset. Indeed, memory Tregs were increased with age in HCs but not in patients with T1D, whereas siblings had elevated baseline levels independent of age, indicating both genetic influence and disease-related disruption of normal Treg maturation trajectories.

This study has limitations, including the relatively small sample size and the lack of functional Treg assays, which precludes drawing conclusions regarding suppressive capacity. Genetic analyses were not performed in HC participants, because of ethical constraints, and Tregs were defined using surface markers without transcription factor validation. Nevertheless, age-adjusted multivariable modeling allowed control of key confounders, preserving analytical power while minimizing bias. As such, this work represents an initial, hypothesis-generating framework rather than a mechanistic investigation.

In conclusion, our findings identify memory Tregs as a potential early biomarker of T1D, reflecting altered age-dependent immune trajectories linked to genetic risk. Elucidating the mechanisms underlying these changes may inform targeted immunotherapies aimed at preserving β-cell function and delaying or preventing disease onset.

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Author Contributions. A.C. conceived the study. D.R. and C.B.-M. performed the experiment and data analysis. S.S. performed data analysis, recruited study participants, and collected their clinical data. E.P. recruited study participants and collected their clinical data. L.S. performed statistical analysis. A.C., I.R., G.C., S.B., and F.P. edited the manuscript. D.R. 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.

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