

Uncoupling of Proliferation and Cytokines From Suppression Within the CD4+CD25+Foxp3+ T-Cell Compartment in the 1st Year of Human Type 1 Diabetes

Angela Hughson,¹ Irina Bromberg,¹ Barbara Johnson,² Sally Quataert,³ Nicholas Jospe,² and Deborah J. Fowell¹

OBJECTIVE—The mechanistic basis for the breakdown of T-cell tolerance in type 1 diabetes is unclear and could result from a gain of effector function and/or loss of regulatory function. In humans, the CD4+CD25+Foxp3+ T-cell compartment contains both effector and regulatory T cells, and it is not known how their relative proportions vary in disease states.

RESEARCH DESIGN AND METHODS—We performed a longitudinal study of CD4+CD25+ T-cell function in children with type 1 diabetes at onset and throughout the 1st year of disease. Function was assessed using single-cell assays of proliferation, cytokine production, and suppression. Type 1 diabetic individuals were compared with age-matched control subjects, and suppression was directly assessed by coculture with control T-cell targets.

RESULTS—We identify novel functional changes within the type 1 diabetes CD4+CD25+ compartment. Type 1 diabetic CD4+CD25+ cells exhibited a striking increase in proliferative capacity in coculture with CD4 T cells that was present at onset and stable 9–12 months from diagnosis. Elevated type 1 diabetes CD4+CD25+ cell proliferation correlated with increased inflammatory cytokines interleukin 17 and tumor necrosis factor- α but not γ -interferon. Type 1 diabetes CD4+CD25+ cytokine production occurred coincident with suppression of the same cytokines in the control targets. Indeed, enhanced proliferation/cytokines by CD4+CD25+ cells was uncoupled from their suppressive ability. Longitudinally, we observed a transient defect in type 1 diabetes CD4+CD25+ suppression that unexpectedly correlated with measures of improved metabolic function.

CONCLUSIONS—Type 1 diabetes onset, and its subsequent remission period, is associated with two independent functional changes within the CD4+CD25+ T-cell compartment: a stable increase in effector function and a transient decrease in regulatory T-cell suppression. *Diabetes* 60:2125–2133, 2011

From the ¹David H. Smith Center for Vaccine Biology and Immunology, Aab Institute of Biomedical Sciences, Department of Microbiology and Immunology, University of Rochester, Rochester, New York; the ²Division of Pediatric Endocrinology, University of Rochester Medical School, Rochester, New York; and the ³Human Immunology Center, David H. Smith Center for Vaccine Biology and Immunology, University of Rochester Medical School, Rochester, New York.

Corresponding author: Deborah J. Fowell, deborah_fowell@urmc.rochester.edu.

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A.H. and I.B. contributed equally to this study.

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Type 1 diabetes results from the immune destruction of insulin-producing β -cells in the pancreas. In humans, the breakdown in immune tolerance is evident years before disease onset with detectable autoantibodies to a number of β -cell antigens in first-degree relatives of subjects with diabetes (1,2). However, the immune changes that trigger progression to overt diabetes are not known. Animal models of autoimmune diabetes, namely, the spontaneously diabetic NOD mouse, have afforded the opportunity to manipulate regulatory T-cell (Treg) activity and have led to striking demonstrations that modulating Treg number can prevent or precipitate diabetes (3–5). However, there remains little evidence that β -cell destruction is preceded by a systemic loss in Treg number or function (6).

Immune tolerance is maintained in part through the action of Tregs, a specialized CD4+ T-cell lineage whose primary function is to suppress immunity (7–9). The transcription factor Foxp3 controls the development and function of Tregs, and loss of Foxp3 results in systemic multiorgan autoimmunity (10–12). Although a central role for Tregs in maintaining self-tolerance is evident from the pathologies of Foxp3 deficiency, there are limited reports that autoimmunity in humans is associated with a loss in Treg number or function (13–16).

In humans, the Foxp3+ Treg population is functionally heterogeneous (17), containing both resting and activated Tregs as well as nonregulatory effector cells (17,18). Indeed, human Foxp3 expression can be induced in CD4+ T cells during T-cell activation without conferring suppressive function (19–22). Furthermore, some Foxp3+ cells may remain functionally plastic, having both suppressive and proinflammatory functions, particularly interleukin (IL)-17-producing potential, depending on the type of inflammatory milieu at activation (18,23,24). Defining the relative proportion of these subsets in human disease settings is, thus, critical for appropriate therapeutic intervention.

There have been numerous cross-sectional studies on Tregs in human type 1 diabetes, but the data do not support a consensus (13). Qualitative changes in suppressive function have been demonstrated (25–28) but need careful interpretation because a defect could arise from a direct Treg defect and/or from an enhanced resistance to suppression by effector T cells (29,30). Regulatory defects in type 1 diabetes are not absolute (31,32), and discrepancies could come from heterogeneity within the Treg compartment and from variation in age, severity of illness, and time from diagnosis. To date, there have been no longitudinal

studies of CD4+CD25+ function from type 1 diabetes onset in children. Type 1 diabetes onset is followed within weeks by a transient remission phase or honeymoon period marked by improved metabolic function once insulin treatment is begun. Whether the metabolic changes are linked to immune system changes remains unclear (33,34) and is experimentally challenging because immune changes could be the cause or effect of metabolic changes. Nonetheless, gaining information on immune changes during this time is an essential first step. Modest changes in IL-1 receptor antagonist, IL-6, and CCR5 chemokines in the serum have been noted, but longitudinal functional data on CD4 T cells remains limited (35,36). The natural history of immunity during the first few years after diagnosis remains poorly understood but may hold clues regarding the nature of remission and where to direct therapy.

This report focuses on a longitudinal study of children with type 1 diabetes from diagnosis through the first 9–12 months, addressing changes in phenotype and function within the CD4+CD25+Foxp3+ compartment. We identify for the first time two independent variables in the function of the CD4+CD25+Foxp3+ compartment in the early stages of clinical disease. The CD4+CD25+ population from type 1 diabetes subjects, but not age-matched control subjects, showed increased proliferative capacity in coculture with CD4 T cells from healthy donors, which correlated with an increase in IL-17 and tumor necrosis factor- α (TNF- α) cytokine production. This proliferative phenotype was found at diagnosis and remained constant throughout the first 9 months. In contrast, independent defects in Treg suppression were observed that were variable over the time course studied. No suppressive defects were observed at type 1 diabetes onset, but individuals displayed a surprising defect in suppressive activity 3–6 months postdiagnosis at a time when glycosylated hemoglobin (HbA_{1c}) measurements were reduced, in keeping with disease remission. Normal Treg function was generally restored by 9 months. In healthy control subjects, there was a strict inverse correlation between the degree of suppressive activity and the degree of CD4+CD25+ proliferation and cytokine production, suggesting the two functions are mutually exclusive. In contrast, in type 1 diabetic subjects, the correlation was lost with concomitant high cytokine production and suppression. We thus reveal that the development of type 1 diabetes is marked by two independent changes in the CD4+CD25+ compartment: a stable gain of effector function that is uncoupled from a transient loss of regulatory function.

RESEARCH DESIGN AND METHODS

Human samples. Blood was obtained from 21 children with newly diagnosed type 1 diabetes (8–18 years old: 6 girls, 15 boys), 22 nondiabetic age-matched control subjects (8–18 years old), and 70 healthy adult control subjects (20–58 years old) (Supplementary Fig. 2). Onset was defined as within 48 h of diagnosis. Blood samples were obtained with informed consent following the protocols underlined by the institutional review board.

Cell purification. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphocyte Separation Media (Cellgro, Manassas, VA). Regulatory T cell Isolation Kit (Miltenyi Biotec, Auburn, CA) was used to isolate CD4+CD25– and CD4+CD25+ cells according to manufacturer's instructions (see Supplementary Fig. 1 for purity). The CD4-negative fraction of PBMC was used as antigen presenting cells (APCs) and irradiated (2,500 rad).

Suppression assays. CD4+CD25– T cells (10^7 /mL) were incubated with 5 μ M carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen, Carlsbad, CA) at room temperature for 5 min. CD4+CD25+ T cells (30×10^6 /mL) were incubated with 2×10^{-6} mol/L PKH26 (Sigma-Aldrich, St. Louis, MO) at room temperature for 4 min and washed three times before cell culture. Comparable results were obtained with CD4+CD25+ T cells labeled with PKH or CFSE. Next, 5×10^4 CD4+CD25– target cells were cultured in

RPMI 1640 medium (Lonza, Walkersville, MD)/10% heat inactivated FCS with 5×10^4 APCs with/without CD4+CD25+ in round bottom 96-well plates. CD4+CD25+ cells were titrated from 1:2 (25+:25–) to 1:64. The cells were stimulated with 1 μ g/mL anti-CD3 (HIT3a) or 1 μ g/mL anti-CD3 and 10 ng/mL anti-CD28 (CD28.2). Stimuli were chosen to compare Treg function under conditions that may facilitate suppression (anti-CD3) or that may provide signals to the targets to resist suppression (anti-CD3/CD28) (13). On day 5, cells were stained with 7AAD (BD Biosciences, San Jose, CA) and anti-human CD4 (RPA-T4, Ebioscience, San Diego, CA) and analyzed by flow cytometry (FACSCalibur, BD Biosciences). Cells were gated on live (7AAD–) CD4+ expression and then PKH (Tregs) and CFSE (targets) (see Supplementary Fig. 1 for gating strategy). Percent suppression was [(% CFSE dilution CD25– alone – % CFSE dilution CD25– CC) \div % CFSE dilution CD25– alone \times 100]. To correct for variability in proliferation of target T cells between individuals, and for variability in suppression of proliferation depending on the degree of target T-cell proliferation, we normalized within each experiment to be able to compare type 1 diabetic subjects assayed on separate days with different targets. We developed a suppressive index (SI); SI equals percent suppression by type 1 diabetic CD4+CD25+ cells divided by the percent suppression by control CD4+CD25+ cells on the same control targets on a given day. We report this SI for control subjects using percent suppression by control CD4+CD25+ on a given day divided by the average of all control subjects in the study ($n = 70$).

Flow cytometry. Multicolor analysis for CD4+CD25+ subsets using the LSRII (BD Biosciences) included PE-Cy7 anti-CCR4 (1G1), PE-Cy5 anti-CD16 (3G8), PE anti-CD127 (hIL-2R-M21), APC-Cy7/PE-Cy7 anti-CD25 (M-A251), and APC anti-CD152 (BN13) from BD Biosciences; PE-Cy5.5 anti-CD3 (S4.1), Tri color anti-CD14 (TUK4), and PE-Texas Red anti-CD4 (S3.5) from Invitrogen; Pacific Orange SA-anti HLA-DR biotin (L243), Pacific Blue anti-CD45RA (HI100), and Alexa 700 anti-CD62 L from Biolegend (San Diego, CA); and Alexa 488 anti-Foxp3 (PCH101) from Ebioscience. The analysis used FlowJo (Tree Star, Ashlan, OR) and SPICE (version 4.3, from Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

PBMCs and cells after purification were stained with PE anti-CD25 (M-A251) (BD Biosciences), PE-Cy5 anti-CD127 (eBioRdr5), and PE-Cy7 anti-CD4 (RPA-T4) (Ebioscience). For Foxp3, cells were fixed and permeabilized using the Foxp3 Staining Buffer Set (Ebioscience) and stained with APC anti-Foxp3 (236A/E7) or APC mouse IgG1 (Ebioscience).

Cytokine analysis. Supernatants from day 5 cultures were analyzed for cytokines using the 9-Plex Human Cytokine Kit and BioPlex Cytokine Reagent Kit (Bio-Rad Laboratories, Hercules, CA) and measured with the BioPlex 200 (Bio-Rad Laboratories).

Intracellular cytokine staining was performed on day 4. Brefeldin A at 5 μ g/mL (BD Biosciences) and monensin at 1 μ mol/L (Sigma-Aldrich) were added 6 h prior to cytokine analysis. Cells were fixed and permeabilized using the Cytofix/Cytoperm Set (BD Biosciences) and stained with Live/Dead Aqua Fixable Dead Cell Stain (Invitrogen), Q-dot 605 anti-human CD3 (UCHT1) (Invitrogen), PerCP/Cy5.5 anti-human TNF- α (MAB11), Pacific Blue anti-human IL-17a (BL168), and APC/Cy7 anti-human CD69 (FN50) (Biolegend). For some analyses, PMA/ionomycin (50 ng/mL and 1 μ mol/L, respectively) was added with the Brefeldin.

Statistical analysis. For most analyses, nonparametric unpaired Mann-Whitney *U* tests or paired Wilcoxon rank sum tests were performed; *P* values of 0.05 or less were considered significant. All tests were two-tailed. Bar graphs denote mean \pm SEM.

RESULTS

Increased proliferative capacity within the CD4+CD25+ compartment of type 1 diabetic subjects. To study both the effector and suppressive function of the CD4+CD25+ population, we designed a single-cell assay that enabled analysis of the CD4+CD25+ cells in coculture with CD4+CD25– target T cells by labeling each subset with a different fluorescent dye (PKH-labeled Tregs and CFSE-labeled targets) (Supplementary Fig. 1). To limit analysis to a single variable, CD4+CD25+ cells from type 1 diabetic or age-matched control subjects were cocultured with CD4+CD25– targets from control subjects. This experimental design introduced an allostimulus for both age-matched and type 1 diabetes cultures, but comparison of syngeneic and allogeneic cultures revealed the antibody stimulation was the dominant stimulus (Supplementary

Fig. 1). Children with type 1 diabetes were analyzed at onset and at regular intervals during the first 12 months of disease (see Supplementary Fig. 2 for clinical data). Our first observation was a striking increase in the proliferative capacity of the CD4+CD25+ compartment in type 1 diabetic subjects (Fig. 1A and B). The increased proliferation was specific to the type 1 diabetic CD4+CD25+ population; it was not seen when type 1 diabetic targets were cocultured with control Tregs (Fig. 1B) or with age-matched CD4+CD25+ T cells and control targets (Fig. 1B).

The proliferating cells could come from contaminating nonregulatory (Foxp3⁻) cells within the CD25+ population; however, the proliferating compartment was predominantly CD4+Foxp3⁺ (Fig. 1C) and retained suppressive function when repurified from coculture and tested for inhibition of fresh CD4+ targets (Supplementary Fig. 3). We also found no correlation between proliferation and frequency of input CD25+CD127⁻ cells (Fig. 1D). Thus, the degree of proliferation is not a function of differences in the purity of the CD4+CD25+ T cells. However, to determine whether an increased frequency of nonregulatory Foxp3⁺ T cells may contribute to the higher proliferative capacity, we further phenotyped the CD4+CD25+ compartment. CD45RA⁻Foxp3^{lo} phenotype marks a nonregulatory subpopulation, elevated in active systemic lupus erythematosus (17) and

recently in type 1 diabetes (37). However, we found little difference between type 1 diabetic and age-matched control subjects in the frequency of the RA⁻Foxp3^{lo} subset collectively (Fig. 1E) or longitudinally (data not shown). Both healthy and type 1 diabetic children did have an increase in RA⁺Foxp3^{lo} cells—resting Tregs—and a reduction in RA⁻Foxp3^{lo} nonregulatory cells (Fig. 1E) that may reflect the more limited antigen exposure in children versus adults, but these changes were not type 1 diabetes specific.

We also used a multiparameter flow cytometry panel targeting key molecules known to functionally subdivide the CD4+CD25+Foxp3⁺ population (CCR4, CD45RA, CD62L, CD127, CD152, and HLA-DR) (Supplementary Fig. 4). The CD4+CD25^{hi}Foxp3^{hi} population was phenotypically more heterogeneous than the CD4+CD25^{lo}Foxp3^{lo} population, possibly indicating the presence of activated Tregs in the former (17). Nevertheless, we found no significant differences in the surface phenotype of Foxp3⁺ subsets from type 1 diabetic and age-matched control subjects. There were significant differences between type 1 diabetic (mean age 12.81 years) and the control subject group (mean age 35.14 years), but these could be accounted for solely by differences in CD45RA expression (Supplementary Fig. 4) (31). Thus, with the markers used, we found no changes in surface molecules within the CD4+CD25+ compartment in

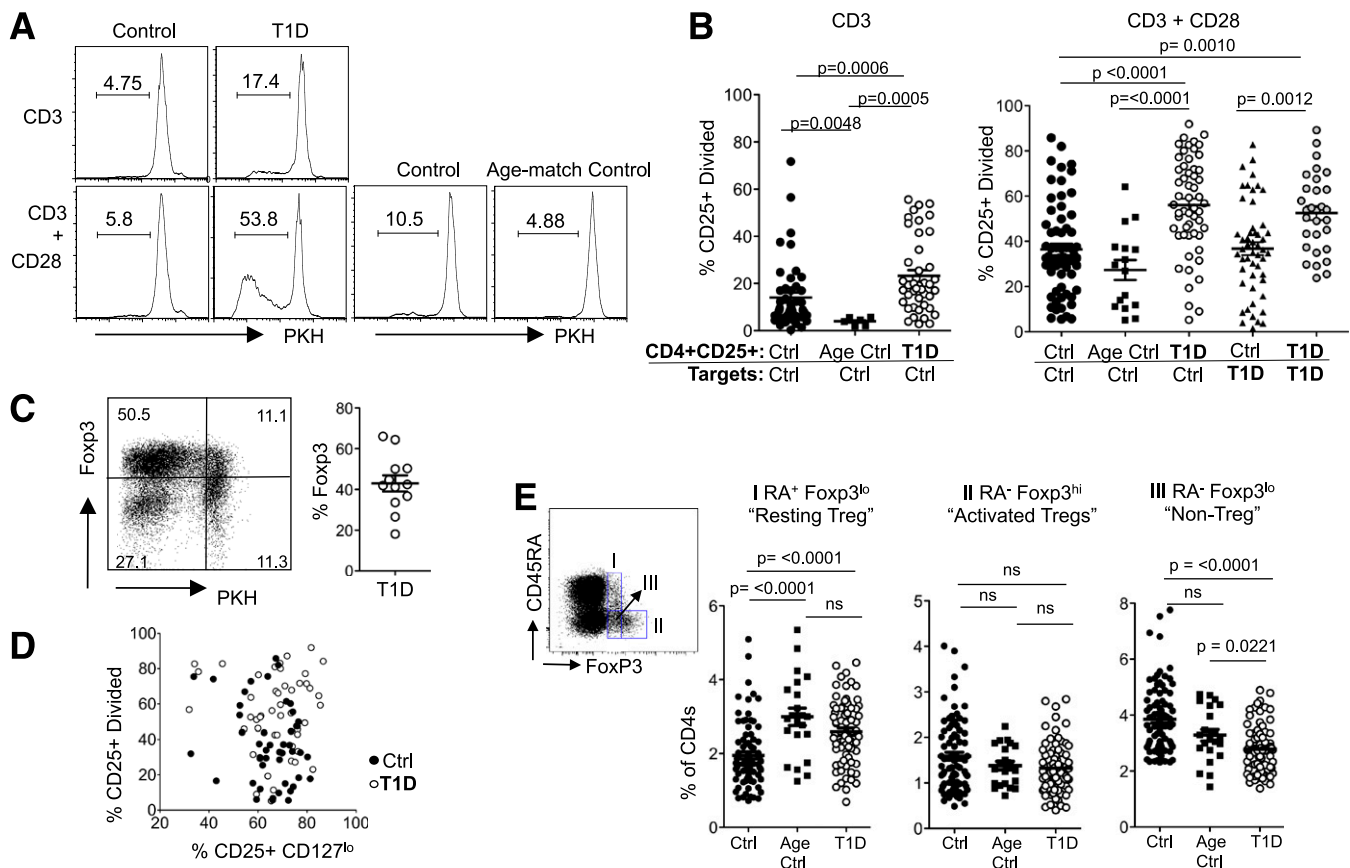


FIG. 1. Increased proliferative capacity of type 1 diabetic CD4+CD25+ T cells. Control CD4+CD25+ targets were labeled with CFSE, and control, age-matched control, or diabetic CD4+CD25+ T cells were labeled with PKH and stimulated with control APCs and anti-CD3 or anti-CD3/CD28 for 5 days and analyzed by fluorescence-activated cell sorter. **A:** Representative plots of PKH expression in CD25+ T cells in coculture with control CD25⁻ T-cell targets, day 5. **B:** Collective analysis (all data shown from all time points in the study) of control, age-matched control, and diabetic CD4+CD25+ T-cell proliferation to APCs and anti-CD3 (left) or anti-CD3/CD28 (right). Statistics by two-tailed Mann-Whitney *U* test. **C:** Foxp3 and PKH expression on gated live CD4+CD25+ T cells after 5-day anti-CD3/CD28 stimulation, numbers equal percentage of CD4+CD25+ cells (left). Cumulative data on proliferating Foxp3⁺ cells, percentage of CD4+CD25+ cells (right). **D:** Correlation between percentage of CD4+CD25+ divided versus percentage of CD25+CD127^{lo} input cells. No statistical correlation by two-tailed Spearman rank correlation test. **E:** Collective analysis of CD45RA/Foxp3-expressing subsets within the gated CD4+CD25+ population. Gates designated on dot plot (left). Statistics by two-tailed Mann-Whitney *U* test. Ctrl, control subjects; T1D, type 1 diabetes. (A high-quality color representation of this figure is available in the online issue.)

children with type 1 diabetes that might account for the heightened proliferative response.

Stable increase in proliferative capacity. Because few longitudinal studies on immunity in type 1 diabetes have been reported, we elected to follow children with type 1 diabetes prospectively from onset to 9–12 months post-diagnosis ($n = 21$) (Supplementary Fig. 2). The key focus was to track changes in CD4+CD25+ cells during the honeymoon or remission period. The increased proliferation within the type 1 diabetes CD4+CD25+ compartment that was present at diagnosis remained remarkably stable through the first 9 months (Fig. 2A). We also followed longitudinally a small cohort of control subjects, over a similar timeframe, to determine if our observations were type 1 diabetes specific. We determined how many times the function of individual type 1 diabetic or control subjects deviated over time from the 99% CI around the mean for all control subjects ($n = 43$). The majority of type 1 diabetic individuals, but not control subjects, exhibited elevated CD4+CD25+ proliferation at multiple times during the first 9 months from diagnosis (Fig. 2B and C). Thus, the type 1 diabetes CD4+CD25+ compartment has a heightened and prolonged proliferative response in coculture at early stages of disease.

Type 1 diabetes CD4+CD25+ proliferation is associated with elevated IL-17 and TNF- α . The functional relevance of this in vitro proliferation of CD4+CD25+ cells to type 1 diabetes is not clear, but it could point to changes in effector potential within the type 1 diabetic CD4+CD25+ population. Indeed, we found highly significant increases in IL-17 and TNF- α in cocultures containing type 1 diabetic CD4+CD25+ cells compared with control CD4+CD25+ cells (Fig. 3A; for paired analysis, see Fig. 3B). In contrast, we found no increase in IL-2 or γ -interferon (IFN- γ) (Fig. 3A). Longitudinally, elevated IL-17 and TNF- α were detected at all time points (Supplementary Fig. 5). Cytokine production positively correlated with increased CD4+CD25+ proliferation (Fig. 3C), suggesting either that proliferation of the type 1 diabetic CD4+CD25+ cells attenuated their suppressive activity or that proliferation was linked to enhanced effector cytokine production by the CD4+CD25+ cells themselves.

Using intracellular cytokine staining, we revealed that although the coculture supernatants contained elevated cytokines in the presence of type 1 diabetic CD4+CD25+ cells (Fig. 3A and B), the target (CD4+CD25-) T-cell source of IL-17 and TNF- α was actively suppressed (Fig. 3D). In contrast, the CD4+CD25+ cells from type 1 diabetic subjects contained a significantly higher frequency of IL-17 producers in coculture (Fig. 3E), consistent with the CD4+CD25+ population being a potent source of IL-17 in healthy control subjects (17,18,23,24) and type 1 diabetic subjects (37,38). Roughly 60% of the IL-17 producers also expressed Foxp3 in both age-matched and type 1 diabetic subjects, with IL-17+Foxp3+ cells significantly elevated in type 1 diabetic subjects compared with age-matched control subjects (Fig. 3F). A large proportion of these IL-17 producers had the capacity to also make TNF- α (but not IFN- γ) on a brief restimulation in coculture (Fig. 3G). Therefore, the type 1 diabetic CD4+CD25+ population contains a distinct subset of effector T cells that appears to secrete cytokine in the face of ongoing Treg function.

Transient loss of Treg function. Treg suppression of proliferation and cytokines can be independently regulated (16). Therefore, the ability of type 1 diabetic CD4+CD25+ T cells to suppress cytokines (Fig. 3D) prompted us to further examine suppression of proliferation by type 1 diabetic CD4+CD25+ T cells. CD4+CD25+ cells from type 1 diabetic children or age-matched control subjects were cultured with healthy donor targets and APCs (Supplementary Fig. 1). An SI was calculated against internal controls (see RESEARCH DESIGN AND METHODS) with an SI of <1 representing less suppression relative to controls. Consistent with published data, we found modest but significant defects in suppression of target CD4 T-cell proliferation by type 1 diabetic CD4+CD25+ T cells under anti-CD3 or anti-CD3/CD28 stimulation conditions in collective analysis (Fig. 4A; Supplementary Fig. 1). Longitudinally, the defects in proliferative suppression were not seen at onset but were restricted to an early window 3–6 months from diagnosis, with suppression restored by 9 months (Fig. 4B). The decrease in suppression was not associated with a loss in the absolute number of CD4+CD25^{hi}Foxp3^{hi} cells

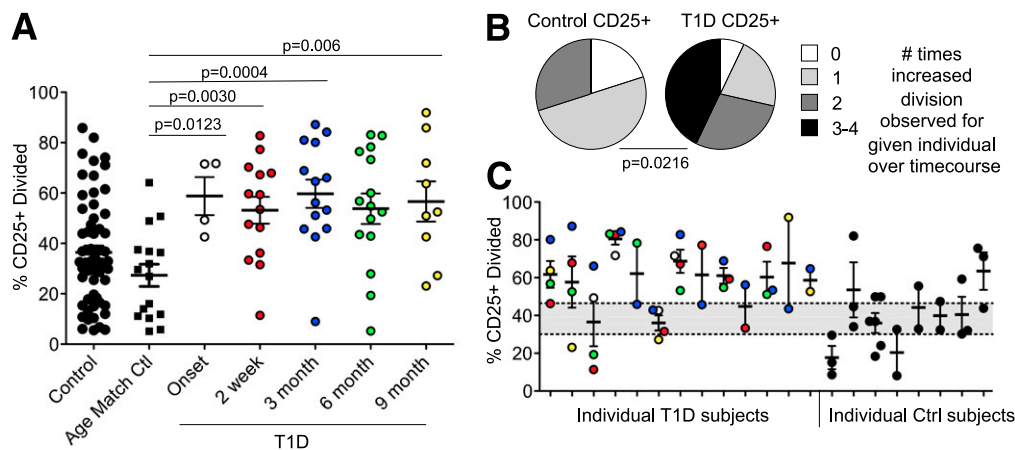


FIG. 2. Increased proliferative capacity present at onset and stable for the first 9 months of disease. **A:** Type 1 diabetes CD4+CD25+ proliferation in coculture with control CD4+CD25- targets for individuals assayed at onset, 2 weeks, and 3, 6, and 9 months after diagnosis. Statistics by two-tailed Mann-Whitney U test; control subjects were significantly different from type 1 diabetes at all time points ($P < 0.05$). **B:** Pie charts depict the number of times the proliferation of CD25+ cells from an individual type 1 diabetic donor or individual control donor rose above the 99% CI around the mean of all control CD25+ samples ($n = 57$). Type 1 diabetic ($n = 16$) and control ($n = 10$) subjects tested at intervals over a 9-month time period. Statistics by two-tailed Mann-Whitney U test. **C:** Graph of variation in percentage of CD25+ cells divided in individuals over time. Colors match time of analysis as depicted in A. Gray area between dotted lines is within the 99% CI around the mean of all controls. Ctrl, control subjects; T1D, type 1 diabetes.

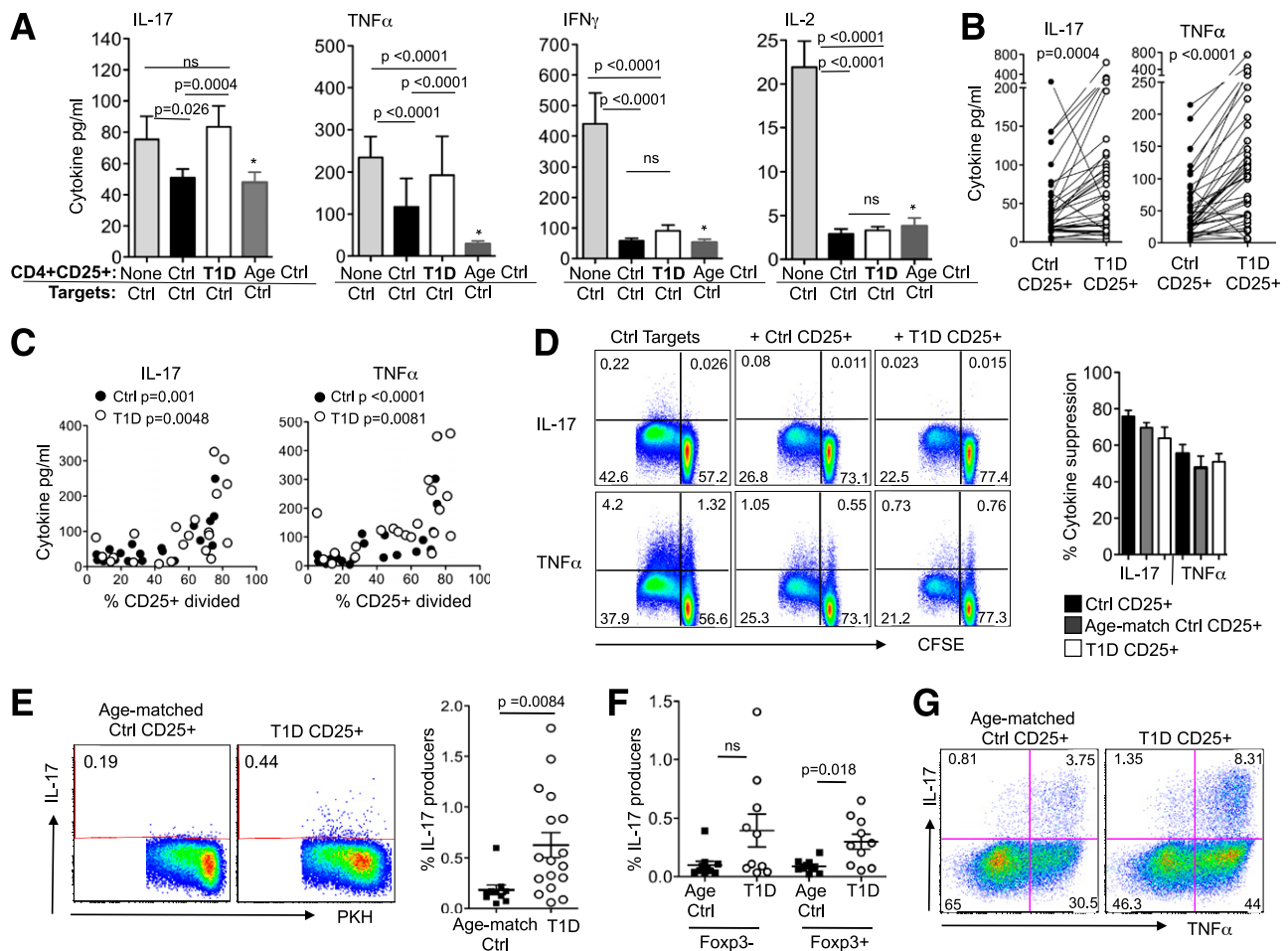


FIG. 3. Elevated IL-17 and TNF- α in cocultures with type 1 diabetic CD4+CD25+ cells. **A:** Cytokines in supernatants from 5-day cocultures of control CD4+CD25- targets with/without control, age-matched, or diabetic CD4+CD25+ cells. Statistics by paired two-tailed Wilcoxon rank sum test. Cultures containing only type 1 diabetic CD4+CD25+ cells, average are indicated as follows: IL-17, 25.54 pg/mL; TNF- α , 2.62 pg/mL; IFN- γ , 51.29 pg/mL; and IL-2, 0.82 pg/mL. * $P < 0.05$, paired two-tailed Wilcoxon rank sum test for age-matched CD4+CD25+ cocultures with control targets compared with control targets alone. **B:** Pairs indicate cytokines (as in **A**) from cocultures containing the same control target cells. **C:** Correlation plots for cytokine production versus percentage of CD4+CD25+ divided. Statistics by two-tailed Spearman rank correlation test. **D:** Suppression of control CD4+CD25- target T-cell production of IL-17 and TNF- α in coculture with control or type 1 diabetic CD4+CD25+ cells by intracellular cytokine staining on day 4. Dot plots are gated on CFSE-labeled control targets. Percent suppression was calculated from the frequencies of cytokine-producing target T cells alone or in coculture; statistically not significant by two-tailed Mann-Whitney U test. **E:** Representative dot plots (*left*) of CD4+CD25+ cells in coculture, day 4. Frequency of IL-17 producers (*right*) from CD4+CD25+ cells in coculture, gated on PKH+CD3+CD69+; statistics by two-tailed Mann-Whitney U test. **F:** Frequency of IL-17 cells in (**E**) subdivided based on Foxp3 expression; statistics by two-tailed Mann-Whitney U test. **G:** Representative plots of cytokines from type 1 diabetic or age-matched CD4+CD25+ T cells in coculture with control targets. Stimulated with PMA/ionomycin for 6 h on day 4 of coculture. Ctrl, control subjects; T1D, type 1 diabetes. (A high-quality color representation of this figure is available in the online issue.)

(Supplementary Fig. 6). The transient loss of suppression in type 1 diabetes 3–6 months postdiagnosis correlated with improved β -function during type 1 diabetes remission as measured by serum HbA_{1c} (Fig. 4C) and insulin dependency (Supplementary Fig. 2). Individual type 1 diabetic subjects exhibited a significantly greater number of Treg defects in suppression over time than individual control subjects (Fig. 4D and E), consistent with two distinct groups of type 1 diabetic subjects showing either a transient loss of suppression within the first 3–6 months or a stable loss of Treg function throughout the first 12 months (Supplementary Fig. 7). In contrast, we found no decrease in type 1 diabetes target suppression by control CD4+CD25+ cells collectively or longitudinally (Supplementary Fig. 8), unlike recently published studies (29,30). Although the mechanisms behind clinical remission are unclear (33,34), this pattern is counterintuitive, given that Treg activity is predicted to be protective. However, the results are in

line with a cross-sectional study of antigen-specific T-cell responses in type 1 diabetes (39) in which IL-10 was higher at onset but unexpectedly lower during partial remission.

Uncoupling of proliferation and cytokine production from suppression in type 1 diabetes. Functional heterogeneity within the CD4+CD25+ compartment revealed the presence of both effector T cells (enriched for IL-17 producers) and Tregs. In many studies, effector function and suppression are mutually exclusive (18,23,24). Indeed, for the control subjects, there was a highly significant inverse correlation between the proliferative capacity and cytokine production of the CD4+CD25+ T cells and their suppressive activity (Fig. 5A and C). This correlation was lost for CD4+CD25+ T cells from type 1 diabetic subjects (Fig. 5B and C). Therefore, increased proliferation and cytokine production appear to be temporally uncoupled from suppressive function within the CD4+CD25+ compartment in the early stages of type 1 diabetes.

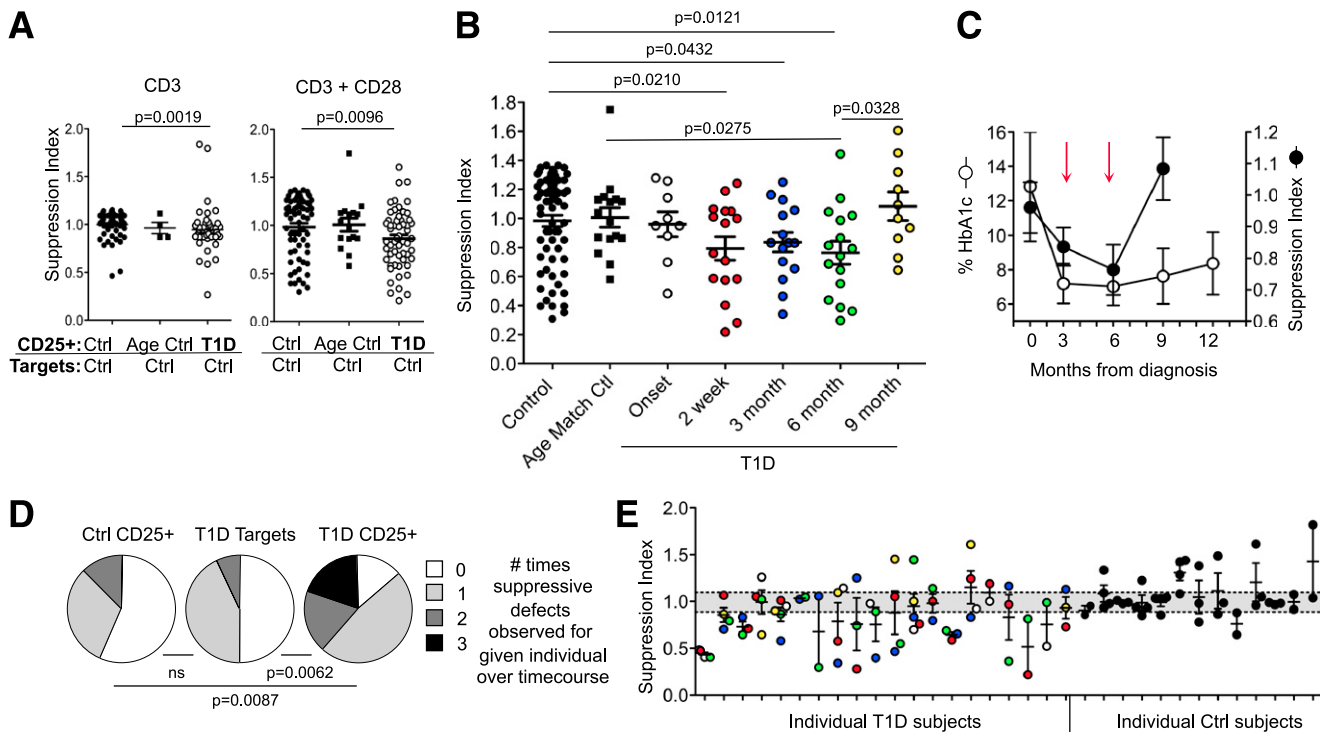


FIG. 4. Transient defect in proliferative suppression by type 1 diabetic CD4+CD25+ T cells. Control CD4+CD25- targets were labeled with CFSE, and control, age-matched control, or diabetic CD4+CD25+ cells were labeled with PKH and stimulated with control APCs and anti-CD3 or anti-CD3/CD28 for 5 days and analyzed by fluorescence-activated cell sorter as in Fig. 1 and Supplementary Fig. 1. **A:** Collective analysis of suppression of proliferation by CD4+CD25+ cells from type 1 diabetic subjects using an SI where 1 equals mean suppression of all historical control cultures, >1 equals enhanced suppression compared with mean of controls, and <1 equals reduced suppression compared with mean of controls (99% CI around the mean, 1.096–0.887). Statistics by two-tailed Mann-Whitney *U* test. **B:** Longitudinal analysis of proliferative suppression, SI for individuals at time points indicated. Statistics by two-tailed Mann-Whitney *U* test. **C:** Longitudinal comparison of clinical score (percent HbA_{1c}, left *y*-axis) and suppressive activity (SI, right *y*-axis); mean ± SD. Red arrows indicate percentage of HbA_{1c} levels <7.5, defining partial remission. **D:** Pie charts depict the number of times the suppression by CD4+CD25+ cells from the same individual fell below the 99% CI around the mean of CD4+CD25+ suppression for all controls (*n* = 65). Type 1 diabetic (*n* = 21) and control (*n* = 14) subjects tested at intervals over a 9-month period. Statistics by two-tailed Mann-Whitney *U* test. **E:** Graph of variation in suppression in individuals over time. Colors match time of analysis as depicted in **B**. Gray area between dotted lines is within the 99% CI around the mean of all controls. Ctrl, control subjects; T1D, type 1 diabetes.

DISCUSSION

Loss of tolerance to self-antigens could be accounted for by the loss of suppressive mechanisms and/or a gain of effector function. The CD4+CD25+Foxp3+ population contains both suppressive and effector cell types, and the relative

balance between these subsets in health and disease remains unclear (17,18,24). Here, we reveal functional differences within the CD4+CD25+ compartment in children with newly diagnosed type 1 diabetes. Two independent variables were identified: a stable increase in CD4+CD25+

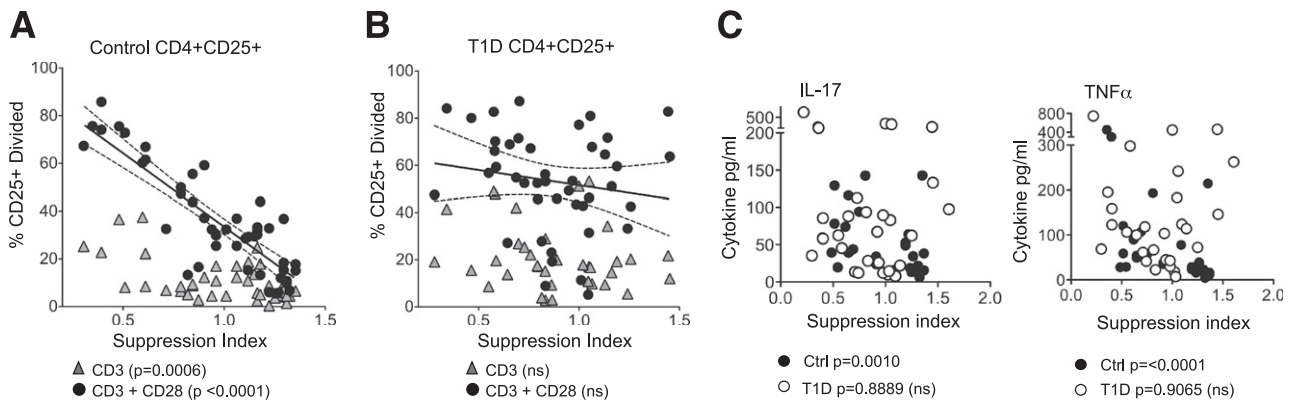


FIG. 5. CD25+ proliferation and suppression are independent functional variables in type 1 diabetes. Control (**A**) and type 1 diabetes correlation plots (**B**) between the percentage of PKH-labeled CD4+CD25+ T-cell proliferation and CD4+CD25- target T-cell proliferative suppression. Mean for CD3/CD28 stimulation, 95% CI (dotted lines), linear regression, *r*²: control CD3/CD28, 0.7345; type 1 diabetes CD3/CD28, 0.027. Correlation statistics by two-tailed Spearman rank correlation test. **C:** Correlation plots between cytokines in cocultures with type 1 diabetic or control CD4+CD25+ cells and proliferative suppression of CD4+CD25- targets. Correlation statistics by two-tailed Spearman rank correlation test. Ctrl, control subjects; T1D, type 1 diabetes.

proliferative capacity and cytokine production—IL-17 and TNF- α —and a transient loss of suppressive function during the first 9 months from diagnosis. Thus, recent onset type 1 diabetes is marked by increased effector function within the CD4+CD25+ compartment but without lasting disruption of Treg function.

Future studies on the functional heterogeneity of the CD4+CD25+Foxp3+ compartment will be aided by phenotypic definitions of regulatory and nonregulatory cells such as those based on CD45RA/CD25 (17), CCR6 (24), and HLA-DR/CD25 (18). We did not see changes in the frequency of putative nonregulatory CD45RA-CD25+Foxp3^{lo} cells or HLA-DR- cells, including additional multiple phenotypic subset analyses. Because we detected no change in the absolute numbers of CD4+CD25^{hi}Foxp3^{hi}, we speculate that the functional makeup of that subset may change either in suppressive capabilities or in the ability to traffic to inflammatory sites. Phenotypic heterogeneity may be more evident in an islet-specific population and/or within the pancreas itself. It is interesting to note that changes in number or function of circulating CD25+ subsets in autoimmunity have mainly been seen during acute episodes or flares of autoimmune activity in systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis. In type 1 diabetes onset, there may simply not be acute immune alterations, or these may predate clinical manifestations. Although our studies in type 1 diabetes did not reveal differences in individual subsets between diabetic and healthy control subjects, the enhanced degree of heterogeneity within the CD4+CD25^{hi}Foxp3^{hi} compartment (Supplementary Fig. 4) was notable and appears to reflect the more complex nature of the phenotype of the CD45RA-Foxp3^{hi} activated Tregs. The functional significance of this phenotypic heterogeneity deserves further study.

Most striking were our observations that the CD4+CD25+ compartment from type 1 diabetic subjects had increased proliferative capacity that correlated with increases in select cytokines—IL-17 and TNF- α but not IFN- γ . Unlike healthy control subjects, this change in function was uncoupled from suppressive activity. IL-17 producers have been implicated in the pathology of a number of autoimmune diseases (40), perhaps reflecting their relative resistance to Treg suppression (41,42). We find that IL-17 and TNF- α from healthy control subject CD4+CD25- targets can be readily inhibited by type 1 diabetic CD4+CD25+ cells (Fig. 3), suggesting that IL-17/TNF- α producers within the CD4+CD25+ population in type 1 diabetes are particularly resistant to suppression. Thus, in type 1 diabetes, as disease progresses, there may emerge distinct effector populations, such as IL-17/TNF- α producers, that may be less effectively controlled by Tregs. Future studies determining the stability of Foxp3 expression, using measures of Foxp3 methylation, and its relationship to IL-17 production in the type 1 diabetic CD4+CD25+ population will be important in light of current discussions on functional plasticity (43). IL-17 is the subject of intensifying study in type 1 diabetes (37,38). Indeed, anti-IL-17 treatment of older NOD mice with established insulinitis, but not young mice prior to insulinitis, prevented diabetes (44). It will be important to determine when these cytokine-producing cells emerge during type 1 diabetes development and also whether they are enriched for distinct islet specificities.

In most settings, the production of IL-17 and suppressive function are mutually exclusive (45). The generation of

induced Tregs and Th17 subsets is driven by shared factors, such as transforming growth factor- β , but regulated by distinct cofactors (46,47). Alternatively, clonal analysis suggests that CD4+CD25+ cells can be functionally flexible, producing IL-17 or being suppressive, depending on the cytokine milieu (18,23,24). The inflammatory cytokines IL-1 β and IL-6 have been shown to potentiate IL-17 production (18,24). It is interesting to note that IL-1 β and IL-6 appear elevated in monocytes from type 1 diabetic subjects (48), and we also found elevated IL-6 in cocultures with type 1 diabetic CD4+CD25+ cells and control targets and APCs (data not shown). Therefore, in type 1 diabetes, these IL-17 producers could originate from a distinct nonregulatory anti-islet effector subset or be more easily induced from a previously suppressive population. Our investigations uniquely identify that the elevated IL-17 and TNF- α production associated with type 1 diabetic CD4+CD25+ cells was not associated with a concomitant loss in suppression of CD4+CD25- target proliferation or cytokine production.

Our study reveals a novel functional dysregulation in the CD4+CD25+ compartment in children with type 1 diabetes. The type 1 diabetic CD4+CD25+ population not only contains cells with the ability to suppress proliferation and cytokine production but also contains cells with heightened capacity to produce specific proinflammatory cytokines IL-17 and TNF- α . This prospective longitudinal study reveals a temporal change from diagnosis in Treg control of CD4+ T-cell proliferation. Children had normal regulatory function at diabetes onset, which declined between 2 weeks and 6 months and returned to normal levels by 9 months (Fig. 4B). This timeframe parallels the clinical remission in type 1 diabetes, which is a period that follows diagnosis by a few weeks and is denoted by a decline in HbA_{1c} that lasts through 9–12 months (49) (Fig. 4C). The underlying mechanism of remission remains unknown. A loss of regulatory function during remission appears counterintuitive to an improvement in β -cell function if these changes in immunity are indeed causally linked to the metabolic changes (33,34). There are a number of ways in which this apparent discrepancy could be explained and tested experimentally. Changes in the peripheral blood may reflect a temporary but beneficial mobilization of Tregs to the pancreas, as speculated by others (39), possibly in response to the alleviation of metabolic stress after insulin treatment. Although we were unable to find differences in surface marker expression, the loss of regulatory function in the CD4+CD25+ compartment could still reflect a change in the effector/regulatory balance in favor of effector T cells. This might be explained by a change in antigen presenting function in the less metabolically stressed pancreas that could change the sequestration of effectors in the tissue. Alternatively, although the kinetics of metabolic and immune changes appear synchronous, they may not be synchronous in their downstream effects. Thus, the transient decline in Treg function during remission may precede, or predict, the exit from the honeymoon period. Such a scenario would fit with models of diabetes as a relapsing/remitting disease (33,34) and might suggest that other waves of decline and gain in circulating Treg function may have occurred prior to clinical disease onset. Natural history studies are currently focusing on immune function prior to the final loss of β -cell function that precipitates the disease and will be essential to our understanding of disease development.

On the other hand, the metabolic and immune changes may well be distinct. Indeed, our analysis highlights that the entry into clinical remission is characteristically homogeneous within our patient group (Supplementary Fig. 2), whereas the changes in regulatory function are variable. Some individuals exhibit no Treg defects, whereas others exhibit transient defects or have a stable Treg defect during the remission period (Supplementary Fig. 6). Extended analysis past 12 months might reveal a linkage between these functional differences and the timing of exit from remission. However, studies of metabolic function and immunity remain challenging, as tight metabolic control is a necessary clinical goal in the management of type 1 diabetes. This necessity underscores the challenge of combining independent strategies for controlling metabolic function and anti-islet immunity (34). Regardless, the remission period needs to be better understood from both metabolic and immune function standpoints. Despite limitations in our ability to discern this interplay, our demonstration of immune changes from diabetes onset in children with type 1 diabetes provides a platform for future immune intervention.

Understanding the developmental and functional relationship between the Tregs and nonregulatory T cells within the CD4+CD25+ compartment in type 1 diabetes, as in other autoimmune states, will inform decisions and provide insight into therapeutic strategies. Immune intervention trials for type 1 diabetes are performed, to date, in this early window of time after diagnosis. The identification of enhanced IL-17 expression within the CD4+CD25+ population could add a wrinkle to Treg-based therapy because these effector cells could be preferentially expanded in cultures of type 1 diabetic CD4+CD25+ T cells and may also be relatively resistant to Treg control. In a similar manner, the identification of some patients with transient decreases in Treg activity in a defined window 3–6 months postdiagnosis could alter the efficacy of certain immune interventions in these particular individuals. In conclusion, our results provide new insight into the basal immune activity and kinetic changes within the CD4+CD25+ compartment of children during the first year of type 1 diabetes.

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