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Circulating CXCR5$^+$PD-1$^+$ICOS$^+$ Follicular T Helper Cells Are Increased Close to the Diagnosis of Type 1 Diabetes in Children With Multiple Autoantibodies

Diabetes 2017;66:437–447 | DOI: 10.2337/db16-0714

Although type 1 diabetes (T1D) is primarily perceived as a T cell–driven autoimmune disease, islet autoantibodies are the best currently available biomarker for autoimmunity and disease risk. These antibodies are produced by autoreactive B cells, the activation of which is largely dependent on the function of CD4$^+$CXCR5$^+$ follicular T helper cells (Tfh). In this study, we have comprehensively characterized the Tfh- as well as B-cell compartments in a large cohort of children with newly diagnosed T1D or at different stages of preclinical T1D. We demonstrate that the frequency of CXCR5$^+$PD-1$^+$ICOS$^+$–activated circulating Tfh cells is increased both in children with newly diagnosed T1D and in autoantibody-positive at-risk children with impaired glucose tolerance. Interestingly, this increase was only evident in children positive for two or more biochemical autoantibodies. No alterations in the circulating B-cell compartment were observed in children with either prediabetes or diabetes. Our results demonstrate that Tfh activation is detectable in the peripheral blood close to the presentation of clinical T1D but only in a subgroup of children identifiable by positivity for multiple autoantibodies. These findings suggest a role for Tfh cells in the pathogenesis of human T1D and carry important implications for targeting Tfh cells and/or B cells therapeutically.

Type 1 diabetes (T1D) is caused by an autoimmune process that leads to the destruction of the insulin-producing β-cells in the pancreas (1). Although T1D is thought to be a primarily T cell–driven disease, a pathogenic role for B cells has been demonstrated by multiple studies in the NOD mouse model (2,3). In humans, autoantibodies produced by B cells are the best currently available biomarker for early β-cell autoimmunity. Subjects with multiple islet autoantibodies have an extremely high risk of developing T1D, with a 5-year risk of ~50% and 15-year risk of >80% (4), and this risk is further increased to almost 90% within 2 years in individuals who also develop impaired glucose

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Received 6 June 2016 and accepted 1 October 2016.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-0714/-/DC1.

See accompanying article, p. 258.

tolerance (IGT) (5). The partial preservation of β-cell function with anti-CD20 B cell–depleting rituximab therapy (6), as well as the demonstration of B-cell tolerance defects in developing naive B cells in patients with T1D (7,8), further supports an important role for B cells in the pathogenesis of human T1D.

Antibody production by B cells is largely dependent on the help provided by $CD4^+$ helper T cells, most particularly by a $CD4^+$ T-cell subset expressing CXCR5, follicular T helper cells (Tfh) (9,10). A subset of peripheral blood memory $CD4^+$ T cells in humans also expresses CXCR5, and recent studies suggest that they represent the circulating memory counterpart of Tfh cells (11). $CXCR5^+$ memory $CD4^+$ T cells in humans compose a heterogeneous population, and subsets of $CXCR5^+$ T cells with variable Tfh functionality ex vivo have been identified. The most consistent finding has been that the expression of PD-1 and ICOS delineates $CXCR5^+$ T cells with a Tfh-like phenotype. More specifically, PD-$1^+ICOS^-CXCR5^+$ T cells seem to represent quiescent and PD-$1^+ICOS^+CXCR5^+$ T cells recently activated memory Tfh cells (12,13).

In recent years, alterations in the circulating Tfh compartment have been observed in multiple autoimmune diseases, such as systemic lupus erythematosus (14), rheumatoid arthritis (15–17), multiple sclerosis (18), and myasthenia gravis (19). Three recent studies have suggested that circulating Tfh cells are also increased in patients with established T1D (20–22). However, the important questions of whether changes in the circulating Tfh compartment occur before the manifestation of clinical T1D and whether they associate with changes in the B-cell compartment and the emergence of serum islet autoantibodies remain unanswered.

In the current study, we used samples from a large follow-up study of children at increased genetic risk for T1D to analyze changes in the Tfh- and B-cell compartments during the development of the disease. We observed an increased frequency of activated circulating Tfh cells near the presentation of clinical T1D but only in individuals who tested positive for multiple autoantibodies. Our current results demonstrate that circulating Tfh cells are increased as a marker of disease progression in T1D and that positivity for multiple autoantibodies can be used to distinguish individuals who could potentially benefit more from immune therapies targeting either Tfh cells or B cells.

RESEARCH DESIGN AND METHODS

Study Subjects

The study cohort comprised 54 children with newly diagnosed T1D (time after clinical diagnosis 0–7 days; mean age 8.3 years ± SD 3.6, age range 2–17 years), 58 autoantibody-positive at-risk children (mean age 8.3 years ± SD 4.6, age range 2–17 years), 15 autoantibody-positive at-risk children with IGT (mean age 10.7 years ± SD 4.0, age range 2–18 years), and 149 autoantibody-negative healthy children (control subjects) (mean age 8.8 years ± SD 4.0, age range 2–16 years). Blood samples for the study were collected over a period of 30 months (August 2013 to January 2016). With the exception of children with newly diagnosed T1D, all study subjects, including the autoantibody-negative healthy control children, participated in the Finnish Type 1 Diabetes Prediction and Prevention Project (DIPP) follow-up study and had HLA types associated with increased risk for T1D (23). Autoantibody positivity was analyzed in the subjects at sampling, as previously described (24). Autoantibody-positive at-risk subjects were defined based on positivity for one or more biochemical autoantibodies (IAA, IA-2A, and/or GADA). Subjects positive for GADA only were excluded from the analyses, since these individuals appear not to have an increased risk for the development of T1D (24). IGT was determined as an elevated (7.8–11.0 mmol/L) plasma glucose level after a standard 2-h oral glucose tolerance test (5). The study has been approved by local ethics committees in the participating university hospitals. All families participating in the study have provided written informed consent.

Peripheral Blood Mononuclear Cells Sample Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by Ficoll gradient centrifugation, resuspended in RPMI 1640 complete medium + 5% human AB serum, and shipped overnight at +4°C from the DIPP study centers in Turku and Tampere to the University of Eastern Finland in Kuopio. Blood samples from healthy age-matched control children were in most cases drawn on the same day and processed in parallel with those from children with newly diagnosed T1D and autoantibody-positive2 children to control for spurious results caused by differential sample preparation. The viability of the PBMCs before flow cytometric assays was routinely >97%, as assessed by viability staining.

Flow Cytometric Analyses

Surface immunostaining was performed on $\sim 10^6$ PBMCs per staining by incubating the cells with a panel of fluorochrome-labeled antibodies for 20 min at +4°C (see Supplementary Table 1 for antibody panels used). For the determination of interleukin (IL)-21 production, PBMCs were first stimulated for 5 h with 50 ng/mL phorbol myristic acid (PMA; Sigma-Aldrich), 1 μg/mL ionomycin (Sigma-Aldrich), and 3 μg/mL brefeldin A (Ebioscience) and stained with antibodies to surface receptors (Supplementary Table 1). Fixation and permeabilization was performed using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience), followed by staining for intracellular IL-21. All the samples were acquired on a FACSCanto II flow cytometer (BD Biosciences), and the flow cytometry data were analyzed using FlowJo software (FlowJo). Coded samples were used throughout, and the flow cytometric analyses were performed blinded to the clinical classification of the sample.

T-Cell and B-Cell Cocultures

Different T-cell subsets as well as naive B cells ($CD20^+IgD^+CD27^-$) were flow cytometrically sorted from PBMCs of healthy donors by FACSAria III (BD Biosciences).

T cells (2×10^4) were cultured together with 2×10^4 naive B cells in the presence of 1 μg/mL Staphylococcal enterotoxin B (SEB; Sigma-Aldrich) in RPMI 1640 complete medium + 10% FCS in a 96-well plate for 7 days. The cells were harvested and stained with CD20 APC, CD38 BV421, CD3 PerCP-cy5.5, and CD4 FITC antibodies (all from Biolegend). AccuCount counting beads (Spherotech) were added to the samples before acquisition by the flow cytometer to allow absolute cell counts. IgM-class antibody concentrations were measured in the culture supernatants by using the Human IgM ELISA Ready-SET-Go! kit (eBioscience).

Statistical Analyses

Statistical analyses were performed using Prism software (GraphPad). When comparing differences between multiple groups, one-way ANOVA with Dunnett posttest was used. Paired Student *t* tests were used when analyzing paired samples. Relationships between different results were examined using Pearson correlation coefficient. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Peripheral Blood CXCR5$^+$PD-1$^+$ICOS$^+$ CD4$^+$ T Cells Are Activated In Vivo, Produce IL-21, and Are Highly Efficient in Activating Naive B Cells

CXCR5 is expressed in 5–15% of human CD4$^+$ T cells. All CD4$^+$CXCR5$^+$ cells express memory markers (i.e., they are negative for CD45RA) and represent 20–40% of total memory CD4$^+$CD25$^{low/-}$ T cells (Fig. 1*A* and Supplementary Fig. 1). Around 30–40% of CD4$^+$CXCR5$^+$ T cells express the activation marker PD-1, and a small subset of these cells also expresses ICOS (Fig. 1*B*). CXCR5$^+$PD-1$^+$ICOS$^+$ cells express high levels of the proliferation marker Ki67 (Fig. 1*C*), suggesting that they have been recently activated in vivo. In contrast, the CXCR5$^+$PD-1$^+$ICOS$^-$ T cells do not express Ki67, and they have been previously suggested to represent resting memory Tfh cells (12,13). To further functionally analyze these T-cell subsets, we sorted peripheral blood CD4$^+$ T cells based on their expression of CXCR5, PD-1, and ICOS. These experiments demonstrated that both the CXCR5$^+$PD-1$^+$ICOS$^-$ and CXCR5$^+$PD-1$^+$ICOS$^+$ subsets produced higher levels of the Tfh signature cytokine IL-21 ex vivo upon PMA and ionomycin stimulation than the

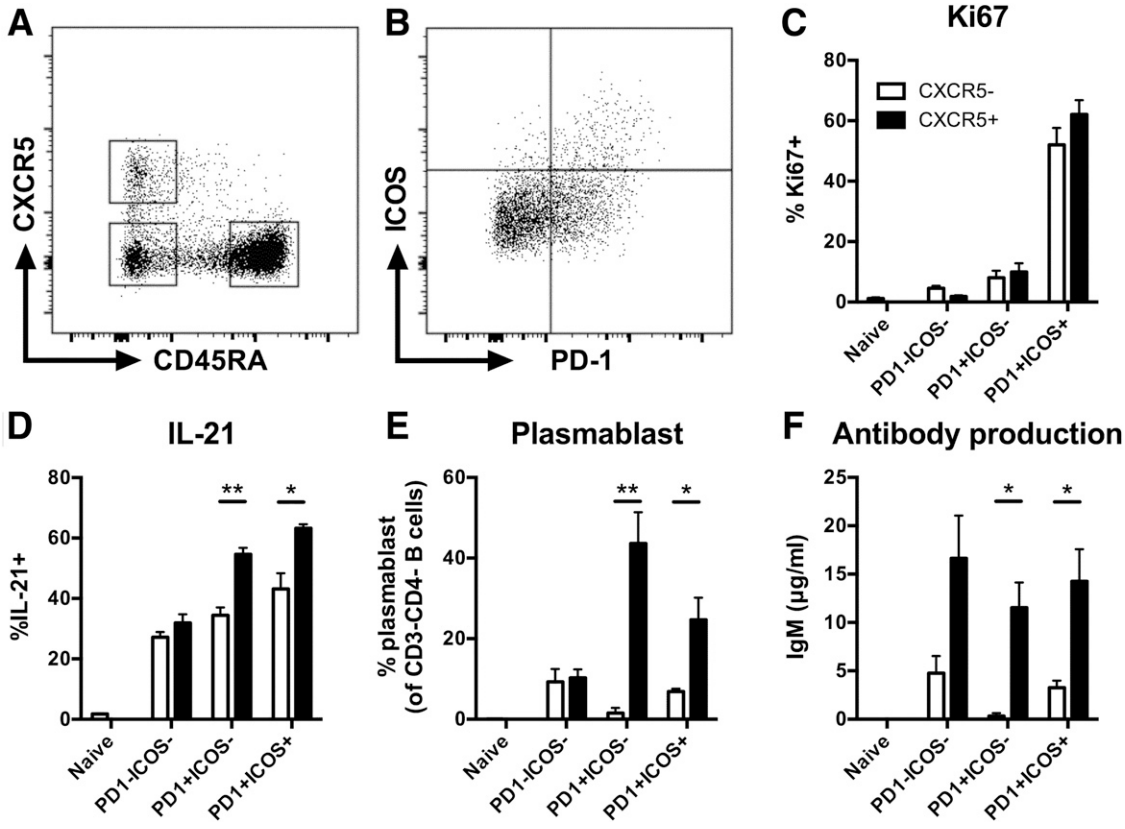

Figure 1—Characterization of peripheral blood CD4$^+$CXCR5$^+$ T-cell subsets. CD4$^+$CD25$^{low/-}$ T cells can be divided into naive (CD45RA$^+$) and memory (CD45RA$^-$) CXCR5$^-$ and CXCR5$^+$ subsets (*A*). CXCR5$^-$ and CXCR5$^+$ memory CD4$^+$ T cells can be further divided into PD-1$^-$ICOS$^-$, PD-1$^+$ICOS$^-$, and PD-1$^+$ICOS$^+$ subsets (*B*). The expression of the proliferation marker Ki67 within different CD4$^+$ T-cell subsets (*C*). Sorted CD4$^+$ T-cell subsets were stimulated ex vivo and intracellular IL-21 production was analyzed (*D*) or cocultured with autologous naive B cells for 7 days after which the proportion of plasmablasts (*E*) and the concentration of secreted antibody (*F*) were measured in the cultures. The results are expressed as mean ± SEM of three to four separate experiments with cells from different individuals. *$P < 0.05$; **$P < 0.01$ (paired Student *t* test).

$CXCR5^+PD\text{-}1^-ICOS^-$ subset, or the corresponding subsets sorted from $CXCR5^-$ memory $CD4^+$ T cells (Fig. 1*D*). Moreover, when cocultured with autologous naive B cells in vitro, these subsets were superior in promoting plasmablast differentiation and antibody production by the B cells (Fig. 1*E* and *F* and Supplementary Fig. 2). Taken together, our current results corroborate previous findings in that the function and phenotype of peripheral blood $CXCR5^+PD\text{-}1^+$ T cells most closely resemble those of bona fide Tfh cells in lymphoid tissues (12,13). Moreover, ICOS expression identifies an activated $CXCR5^+PD\text{-}1^+ICOS^+$ Tfh subset in peripheral blood (13).

Activated Circulating Tfh Cells Are Increased in Children With Newly Diagnosed T1D and in At-Risk Children With IGT

We analyzed the circulating Tfh compartment in a total of 54 children with newly diagnosed T1D (within 1 week of diagnosis), 58 at-risk children positive for islet autoantibodies, and 15 autoantibody-positive children with IGT, as well as in 149 age- and HLA-matched autoantibody-negative healthy control children. No differences in the frequency of $CD4^+CXCR5^+$ T cells within the total $CD4^+$ or memory $CD4^+$ T-cell compartment were observed (Fig. 2*A*), even when the results were stratified by age (Supplementary Fig. 3). Moreover, the frequency of $CXCR5^+PD\text{-}1^+ICOS^-$ Tfh cells was comparable between the groups (Fig. 2*B* and Supplementary Fig. 3). However, the frequency of $CD4^+CXCR5^+PD\text{-}1^+ICOS^+$–activated Tfh cells was markedly increased in children with newly diagnosed T1D and in at-risk children with IGT (Fig. 2*C*). We noted that the frequency of $CXCR5^+PD\text{-}1^+ICOS^+$ cells within the memory $CD4^+$ T-cell compartment strongly decreased with age (Supplementary Fig. 3). However, a strict pairwise comparison with samples from age-matched healthy children processed and analyzed on the same day confirmed the increase in $CXCR5^+PD\text{-}1^+ICOS^+$ Tfh cells in both children with T1D or IGT (Supplementary Fig. 4). Importantly, the increase in $PD\text{-}1^+ICOS^+$ cells appeared to be specific for the $CXCR5^+$ compartment as the frequency of CXCR5-$PD\text{-}1^+ICOS^+$ memory $CD4^+$ T cells did not differ between the study groups (Supplementary Fig. 5). Thus, our results demonstrate that the frequency of $CXCR5^+PD\text{-}1^+ICOS^+$–activated circulating Tfh cells is specifically increased in both children with newly diagnosed T1D as well as in at-risk children with IGT. We also analyzed peripheral blood Tfh frequencies in a separate cohort of adults with long-standing T1D but could not detect differences in the frequency of activated $CXCR5^+PD\text{-}1^+ICOS^+$ Tfh cells in these patients (Supplementary Fig. 6).

Longitudinal Analysis Reveals That Activated Circulating Tfh Cells Are Increased Near the Presentation of Clinical Disease

Six autoantibody-positive at-risk children that we had analyzed at one or more time points progressed to clinical T1D later during our sample collection period. When we analyzed the data of these subjects longitudinally, we noted that in four of these six children, the frequency of $CXCR5^+PD\text{-}1^+ICOS^+$–activated circulating Tfh cells increased at the clinical manifestation of the disease (Fig. 3*A*). In contrast, in five autoantibody-positive children analyzed repeatedly who did not progress to T1D during our study, the frequency of $CXCR5^+PD\text{-}1^+ICOS^+$–activated circulating Tfh cells remained stable (Fig. 3*B*). These findings further support the notion that the increase in the frequency of activated circulating Tfh cells is associated with the progression to clinical T1D.

Activated Circulating Tfh Cells Are Increased Only in Children Positive for Multiple Autoantibodies

Next, we wanted to analyze whether the increase in $CXCR5^+PD\text{-}1^+ICOS^+$–activated Tfh cells is associated with the number of distinct islet autoantibodies in the serum. For this, we stratified our study subjects to two groups based on their positivity for one or more biochemical autoantibodies tested (IAA, GADA, and IA-2A). No difference in the frequency of $CXCR5^+PD\text{-}1^+ICOS^+$–activated Tfh cells was observed between autoantibody-positive children positive for one or two or more

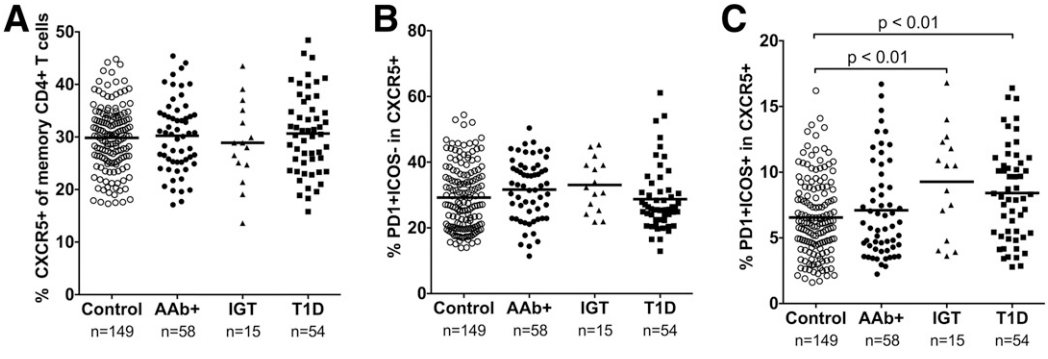

Figure 2—Increased frequency of $CXCR5^+PD\text{-}1^+ICOS^+$–activated circulating Tfh cells in children with IGT and newly diagnosed T1D. The frequencies of $CXCR5^+$ T cells within total memory $CD4^+$ T cells (*A*), as well as the frequencies of $PD\text{-}1^+ICOS^-$ (*B*) and $PD\text{-}1^+ICOS^+$ subsets (*C*) within $CXCR5^+$ T cells, were determined in autoantibody-negative healthy control children, autoantibody-positive (Aab^+) children without and with IGT, and children with newly diagnosed T1D.

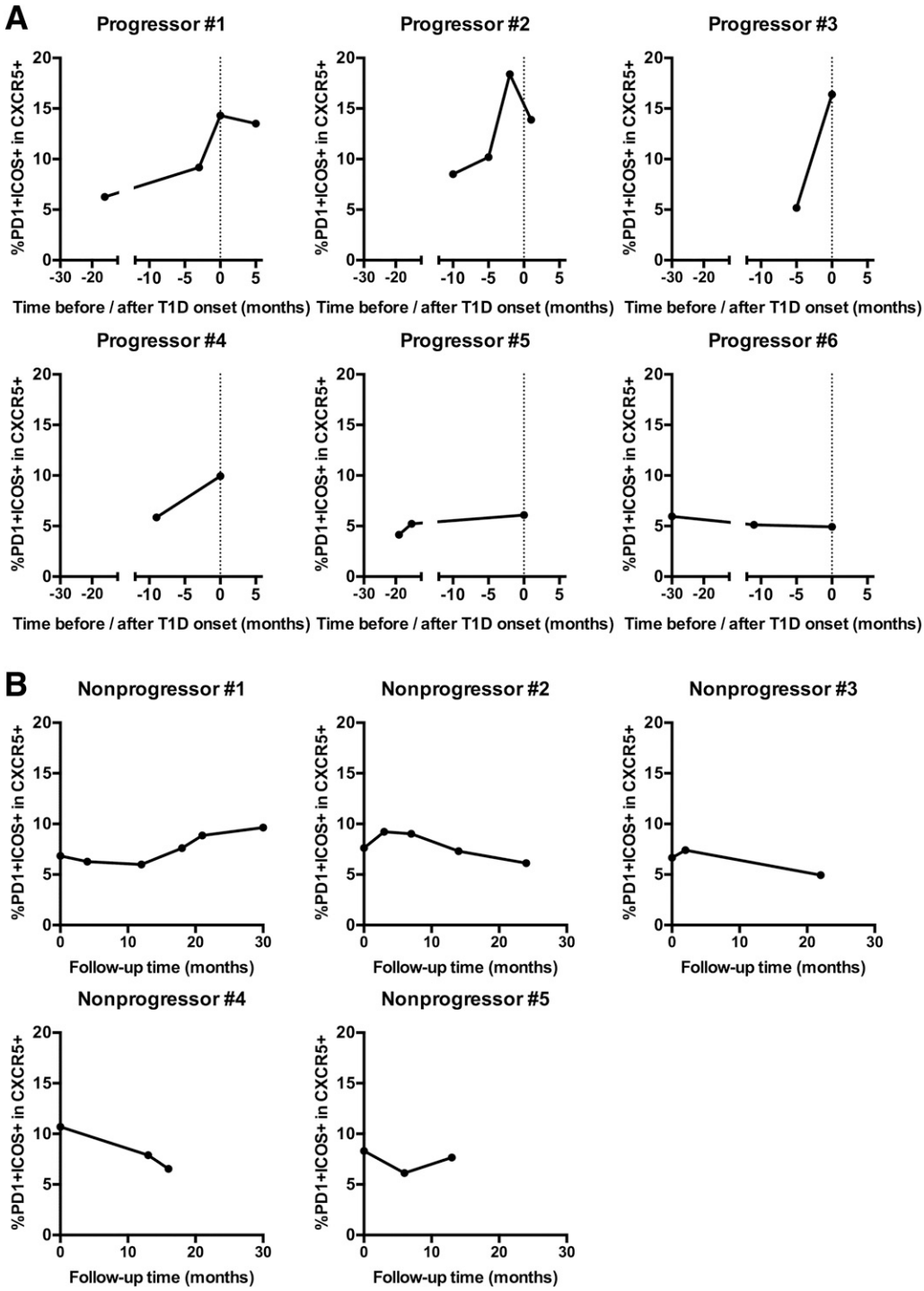

Figure 3—The frequency of $CXCR5^+PD\text{-}1^+ICOS^+$–activated circulating Tfh cells increases at the manifestation of clinical T1D. Six autoantibody-positive children who progressed to T1D were longitudinally analyzed before the diagnosis of the disease (*A*). The vertical line indicates the time point of T1D diagnosis. For progressors #1 and #2, the last prediabetic samples were analyzed at 3 and 2 months before diagnosis, respectively. Five autoantibody-positive children that did not progress to T1D (nonprogressors) were followed longitudinally for up to 30 months (*B*).

autoantibodies (Fig. 4*A*). However, when we analyzed the groups of at-risk children with IGT and those with newly diagnosed T1D, we noticed that the frequency of $CXCR5^+PD\text{-}1^+ICOS^+$–activated Tfh cells was increased only in subjects positive for two or more autoantibodies but not in those positive for one or less autoantibody (Fig. 4*B* and *C*). This striking observation suggests that peripheral blood Tfh cell activation at the manifestation of T1D is observed only in a subset of individuals identifiable by positivity for multiple islet autoantibodies.

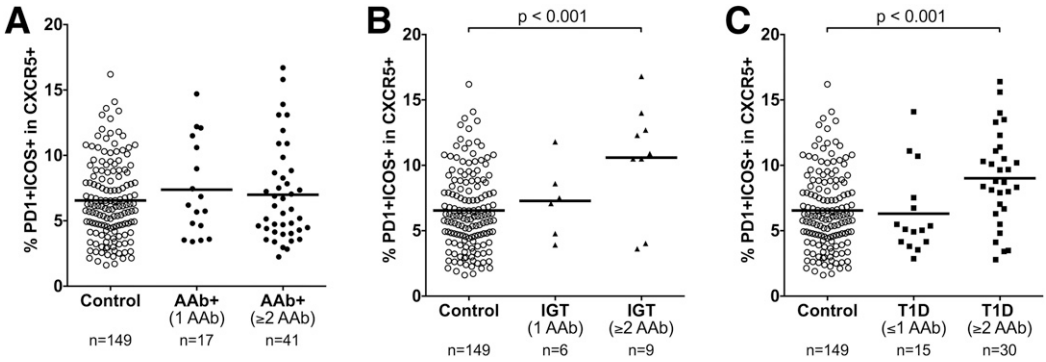

Figure 4—$CXCR5^+PD\text{-}1^+ICOS^+$–activated circulating Tfh cells are increased only in children positive for multiple autoantibodies (Aabs) near the manifestation of T1D. Autoantibody-positive children with normal glucose tolerance (*A*) or with IGT (*B*) and children with newly diagnosed T1D (*C*) were stratified based on the number of biochemical autoantibodies (IAA, GADA, and IA-2A) detected in their blood at the time of sampling.

Additional Phenotyping of Circulating Tfh Cells Does Not Reveal Major Differences Between Children With Newly Diagnosed T1D, Autoantibody-Positive At-Risk Children, and Healthy Control Subjects

We analyzed additional phenotypic markers on $CD4^+CXCR5^+$ T cells that have recently been reported to correlate with Tfh activity. The chemokine markers CCR6 and CXCR3 can be used to classify $CD4^+CXCR5^+$ Tfh cells into Tfh1 ($CCR6^-CXCR3^+$), Tfh2 ($CCR6^-CXCR3^-$), and Tfh17 ($CCR6^+CXCR3^-$) subsets (25). Using this approach (Supplementary Fig. 1), we detected a slight increase of Tfh1-type $CXCR5^+$ T cells in children with newly diagnosed T1D but not in autoantibody-positive at-risk children without or with IGT (Fig. 5*A*). No differences in the frequencies of Tfh17- or Tfh2-type $CXCR5^+$ T cells were observed (Fig. 5*B* and *C*). Interestingly, the increase in $CXCR3^+CCR6^-$ T cells appears not to be restricted to the $CXCR5^+$ compartment since a similar phenomenon was also observed in $CXCR5^-$ memory T cells (Supplementary Fig. 5), suggesting a more global shift toward Th1 differentiation in children with newly diagnosed T1D.

The combinations of the CCR7 and PD-1 markers (12) and $CXCR3^+$ and PD-1 markers (13) have also recently been suggested to identify the most Tfh-like population within $CD4^+CXCR5^+$ T cells. When we looked at the expression of these markers, we did not observe any differences in the frequency of either $CCR7^-PD\text{-}1^+$ or $CXCR3^-PD\text{-}1^+$ Tfh cells between the study groups (Fig. 5*D* and *E*). Taken together, additional phenotyping of the peripheral blood $CD4^+CXCR5^+$ compartment did not reveal major alterations in children with T1D-associated autoimmunity.

Production of IL-21 by $CD4^+CXCR5^+$ Memory T Cells From Children With Newly Diagnosed T1D, Autoantibody-Positive At-Risk Children, and Healthy Control Subjects

Since $CD4^+CXCR5^+PD\text{-}1^+ICOS^+$–activated Tfh cells produce higher amounts of IL-21 (Fig. 1) and the frequency of this subset is increased in children with newly diagnosed T1D (Fig. 2), we wanted to also analyze the production of IL-21 by T cells ex vivo in our study cohort. For this, we stimulated fresh PBMC samples with PMA and ionomycin and analyzed the production of intracellular IL-21. Using flow cytometrically sorted $CD4^+CXCR5^+$ and $CD4^+CXCR5^-$ memory T cells, we first demonstrated that the expression of CXCR5 is not significantly altered by PMA and ionomycin stimulation (Supplementary Fig. 7), allowing us to use CXCR5 to distinguish between $CXCR5^+$ and $CXCR5^-$ $CD4^+$ T cells directly ex vivo. In line with our results with sorted T-cell subsets (Fig. 1), we observed that IL-21 production was consistently higher within the $CD4^+CD45RA^-CXCR5^+$ Tfh subset than within the $CD4^+CD45RA^-CXCR5^-$ memory T-cell subset ($16.8 \pm 0.6\%$ vs. $12.5 \pm 0.3\%$, $P < 0.0001$, paired Student t test, $n = 103$) (Fig. 6*A*). Similarly, the frequency of $IL\text{-}21^+$ cells correlated well with the frequencies of $PD\text{-}1^+ICOS^-$ and $PD\text{-}1^+ICOS^+$ cells within $CXCR5^+$ T cells in the same sample and displayed a clear inverse correlation with age (Fig. 6*B* and Supplementary Fig. 7). No differences in the frequency of IL-21–producing T cells within total memory $CD4^+$ T cells or $CXCR5^-$ memory T cells could be observed between the study groups (Fig. 6*C* and *D*). There was a tendency for a higher frequency of IL-21–producing T cells within $CXCR5^+CD4^+$ T cells in children with T1D, but this did not reach statistical significance ($P = 0.08$, Dunnett posttest) (Fig. 6*E* and Supplementary Fig. 7). We also analyzed the production of IL-21 within the $CXCR5^+CXCR3^+$ (Tfh1) and $CXCR5^+CXCR3^-$ (Tfh2/17) subsets. In general, the frequency of IL-21–producing T cells within the $CXCR5^+CXCR3^+$ fraction was slightly higher than in the $CXCR5^+CXCR3^-$ fraction ($17.9 \pm 0.6\%$ vs. $16.4 \pm 0.6\%$, $P < 0.0001$, paired Student t test, $n = 103$), but no differences between the study groups could be observed (Supplementary Fig. 7).

No Changes in the B-Cell Compartment Are Observed in Children With Newly Diagnosed T1D or in Autoantibody-Positive At-Risk Children

Since Tfh cells are major activators of B-cell responses in germinal centers, we also analyzed the composition of the

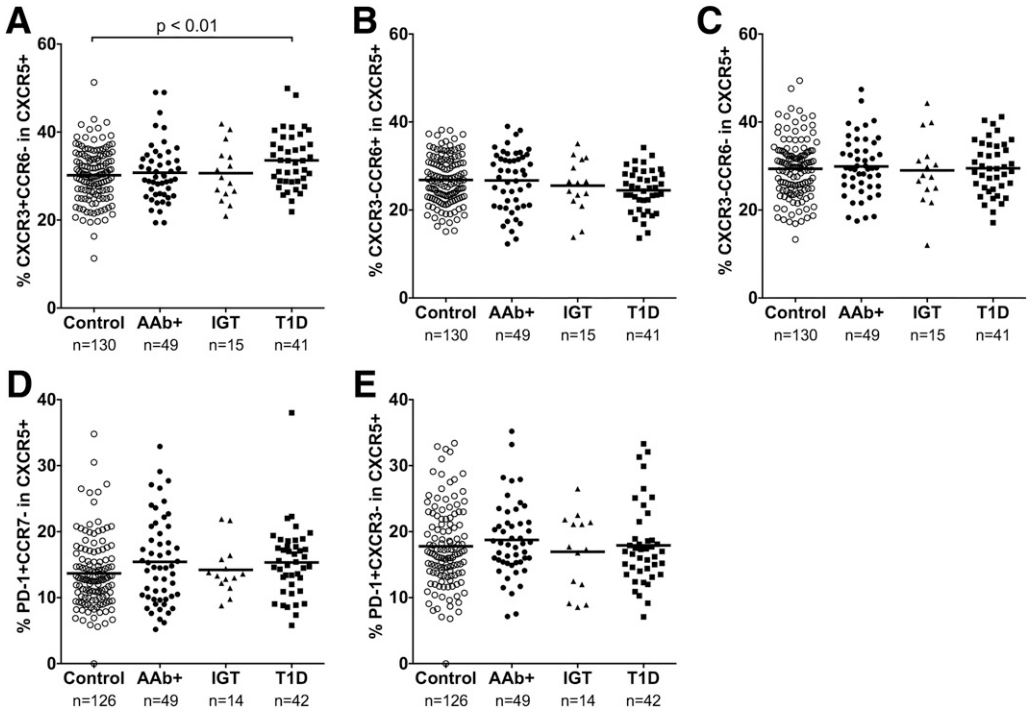

Figure 5—Additional phenotyping of the $CD4^+CXCR5^+$ Tfh compartment in children at risk for T1D or with newly diagnosed T1D. The frequencies of Tfh1 (*A*), Tfh17 (*B*), and Tfh2 (*C*) cells as well as the frequencies of PD-1^+CCR7^- (*D*) and PD-1^+CXCR3^- (*E*) cells within $CD4^+CXCR5^+$ T cells were analyzed in autoantibody-negative healthy control children, in autoantibody-positive (Aab^+) children without or with IGT, and in children with newly diagnosed T1D.

peripheral blood B-cell compartment in our study cohort (Fig. 7 and Supplementary Fig. 8). Specifically, the frequencies of naive B cells ($CD19^+CD27^-IgD^+$) and transitional B cells ($CD19^+CD27^-IgD^-CD10^+CD38^+$) as well as unswitched memory B cells ($CD19^+CD27^+IgD^+$), class-switched memory B cells ($CD19^+CD27^+IgD^-$), and plasmablasts ($CD19^+CD27^{++}CD38^{++}$) were determined. Class-switched memory B cells were further divided into IgA^+ memory B cells and IgG^+ memory B cells (defined as $CD19^+CD27^+IgD^-IgA^-$). We also analyzed the frequency of $CD27^-IgD^-$ double-negative memory cells ($CD19^+CD27^-IgD^-$) and $CD21^{low}$ anergic B cells ($CD19^+CD27^-CD21^-CD10^-CD38^-$), two subsets that have been previously associated with autoimmunity (26,27). Importantly, no differences were observed in the frequencies of these B-cell subsets in children with newly diagnosed T1D or in autoantibody-positive at-risk children compared with healthy control subjects, even when stratified by age (Fig. 7 and Supplementary Fig. 9). Of note, a positive correlation was detected between the frequency of $CXCR5^+$PD-1^+ICOS^+–activated circulating Tfh cells and the frequency of plasmablasts (Supplementary Fig. 10). In conclusion, no apparent alterations in major peripheral blood B-cell subsets were observed in children with T1D-associated autoimmunity.

DISCUSSION

Autoantibodies produced by B cells are the earliest marker of β-cell autoimmunity in humans. Since the activation of autoreactive B cells is largely dependent on Tfh function, there is a strong rationale for a central role of Tfh cells in T1D pathogenesis.

In this study, we examined the circulating Tfh compartment in children during different stages of T1D progression. We were able to demonstrate that $CXCR5^+$PD-1^+ICOS^+–activated circulating Tfh cells were increased in both children with newly diagnosed T1D and in children at late stages of preclinical T1D characterized by IGT. However, no increase of this subset was observed in children at earlier stages of preclinical T1D, characterized by positivity for islet autoantibodies but without impairment of glucose tolerance. Longitudinal follow-up data obtained from six children who progressed to T1D further supported the view that the increase in activated circulating Tfh appears close to the clinical manifestation of T1D. Importantly, since the increase in activated circulating Tfh cells could also be observed in children with IGT, we can exclude the possibility that the introduction of insulin therapy at the onset of T1D explains the phenomenon.

Our current results are supported by the findings of three recent studies that similarly demonstrate an increase in circulating Tfh cells in patients with established T1D (20–22). Although different gating strategies to define Tfh cells as well as patient cohorts differing in age distribution and duration of the disease were used in these studies, two of these studies clearly identified an

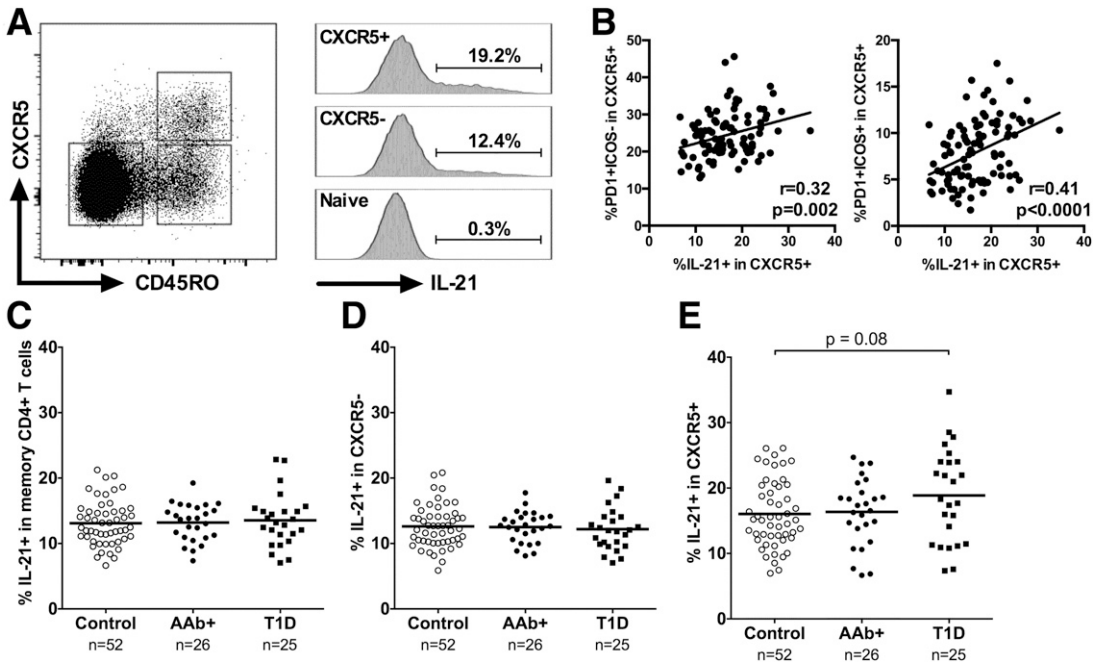

Figure 6—IL-21 production by $CD4^+$ memory T-cell subsets in autoantibody-positive children and children with newly diagnosed T1D. After stimulation with PMA and ionomycin, IL-21 production was analyzed within naive ($CD45RO^-$) and memory ($CD45RO^+$) $CXCR5^-$ and $CXCR5^+CD4^+$ T-cell subsets (*A*). The frequency of IL-21^+ cells correlated with both the frequencies of PD-1^+ICOS^- and PD-1^+ICOS^+ cells within $CD4^+CXCR5^+$ T cells in the same samples (*B*). The frequency of IL-21–producing cells within total (*C*), $CXCR5^-$ (*D*), and $CXCR5^+$ (*E*) memory $CD4^+$ T-cell subsets in autoantibody-negative healthy control children, autoantibody-positive (Aab^+) children, and children with newly diagnosed T1D.

increase in $ICOS^+$ Tfh cells, as also described here (20,21). The increase of $ICOS^+$–activated circulating Tfh cells has also been consistently observed in other autoimmune diseases, such as systemic lupus erythematosus (14), rheumatoid arthritis (15–17), multiple sclerosis (18), and myasthenia gravis (19). Moreover, in most of these studies, this increase has been shown to correlate with the activity of the disease. Our data suggest that the same may also be true in T1D as we did not see any alterations in children with β-cell autoimmunity without impairment of glucose tolerance or in a separate adult cohort of patients with longstanding T1D, although the small number of patients analyzed in the latter cohort precludes definitive conclusion (Fig. 2*C* and Supplementary Fig. 6).

We also looked at other markers that have been described to differentiate circulating Tfh cells. The combination of CCR6 and CXCR3 separates $CXCR5^+$ Tfh cells into Tfh1 ($CCR6^-CXCR3^+$), Tfh17 ($CCR6^+CXCR3^-$), and Tfh2 ($CCR6^-CXCR3^-$) subsets, of which the Tfh2 and Tfh17 subsets have shown to be more efficient than Tfh1 cells in activating naive B cells in vitro (13,25) and have been shown to be expanded in some autoimmune diseases (25,28,29). Moreover, the PD-1^+CCR7^- and PD-1^+CXCR3^- subsets of $CXCR5^+$ T cells have recently been suggested to contain T cells most closely associated with a true Tfh phenotype (12,13). In the current study, we did not see major alterations in the circulating $CXCR5^+$ Tfh cells based on these markers. We observed a slightly elevated frequency of Tfh1 ($CCR6^-CXCR3^+$) cells in children with newly diagnosed T1D. However, a similar phenomenon was also observed in the $CXCR5^-$ memory $CD4^+$ T-cell subset, suggesting that this finding is not a specific alteration in the Tfh compartment but rather represents a more generalized deviation toward Th1 immunity in children with newly diagnosed T1D.

One of the most prominent findings in the current study is that both in children with newly diagnosed T1D and at-risk children with IGT, the increase in activated circulating Tfh cells strongly associated with positivity for multiple autoantibodies. The subgroup of children positive for two or more biochemical autoantibodies at diagnosis of T1D had a clearly increased frequency of activated circulating Tfh cells, whereas in the subgroup with one or less autoantibody, this frequency did not differ from that observed in healthy age-matched control subjects. A similar dichotomy was not observed in autoantibody-positive at-risk children without IGT, suggesting that multiple autoantibody positivity is not associated with the increase in circulating Tfh cells per se but rather identifies a subgroup of individuals with increased Tfh activation at disease presentation. Our current findings lend further support to the concept that immunological heterogeneity exists in human T1D and that it may be possible to identify patients with different endotypes of the disease (30). Analogous to our results, Arif et al. (31) recently demonstrated that positivity for multiple autoantibodies could identify a subgroup of patients with T1D with a more proinflammatory in vitro

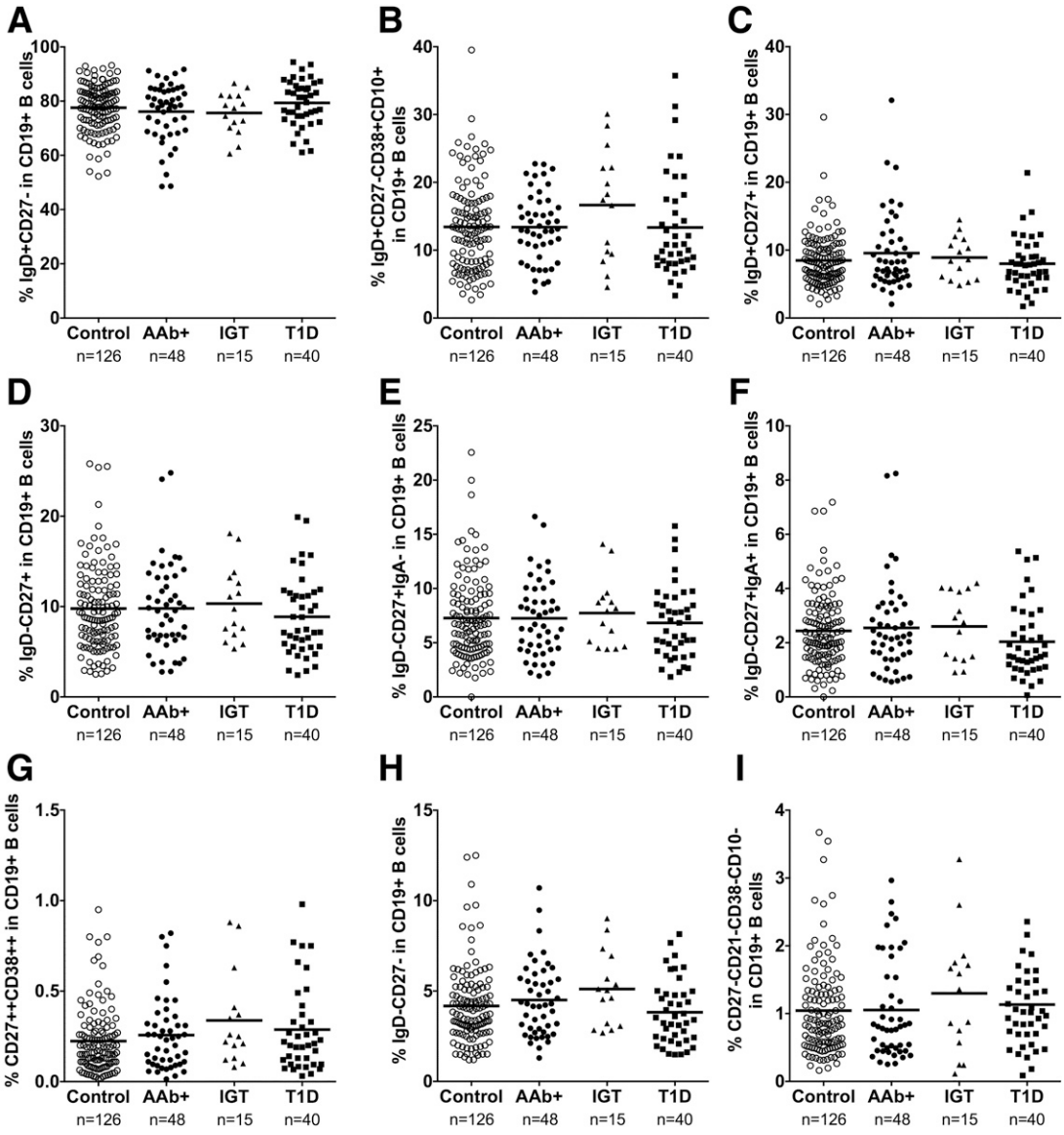

Figure 7— B-cell frequencies in autoantibody-positive children and children with newly diagnosed T1D. The frequencies of naive B cells (*A*), transitional B cells (*B*), unswitched memory B cells (*C*), switched memory B cells (*D*), IgG^+ (*E*) and IgA^+ (*F*) memory B cells, plasmablasts (*G*), IgD-$CD27^-$ memory B cells (*H*), and $CD21^{low}$ anergic B cells (*I*) within total $CD19^+$ B cells were analyzed in autoantibody-negative healthy control children, autoantibody-positive (Aab^+) children without or with IGT, and children with newly diagnosed T1D.

T-cell response to β-cell antigens and that this phenomenon potentially correlated with an increased "hyperimmune" B-cell infiltration of the islets. These findings have important implications for the development of immune therapies for T1D. Based on our data, it seems feasible that multiple autoantibody positivity at the onset of T1D could be used to identify individuals with a pronounced immunopathological activation of autoreactive Tfh cells and B cells. These patients would potentially benefit more from therapies that target Tfh cells, the IL-21 pathway, or B cells than individuals positive for one or no autoantibodies at disease manifestation. In fact, in line with this notion, in the subgroup analysis of the B cell–depleting rituximab trial in T1D, a trend for greater treatment efficacy was observed in subjects positive for two or more autoantibodies (6).

IL-21 is a signature cytokine of Tfh cells, and there is evidence from mouse models for an important role for both IL-21 and Tfh cells in the pathogenesis of autoimmune diabetes (21,32). Two recent studies have also demonstrated an increased frequency of IL-21–producing $CD4^+$ T cells in patients with T1D (21,22). However, we were not able to replicate these findings here, as we did not observe any difference in the frequency of IL-21–producing memory $CD4^+$ T cells between our study groups and only a tendency for higher production of IL-21 within the $CXCR5^+$ memory T-cell subset in children with newly diagnosed T1D. Differences in ex vivo stimulation strategies

and the age range and disease duration of patients with T1D, as well as the lack of statistical power to detect subtle alterations, could explain the discrepancy between our results and previous studies. However, it is important to note that IL-21 is not exclusively produced by $CXCR5^+$ Tfh cells, as also demonstrated by our results here (Figs. 1 and 6). In line with this, in the study by Kenefeck et al. (21), the $CD4^+$ T cells that produced higher levels of IL-21 in patients with T1D commonly coproduced tumor necrosis factor-α and interferon-γ (21), whereas a more recent study demonstrated that IL-21 and interferon-γ–coproducing T cells do not display phenotypic properties consistent with a Tfh function (33). Nevertheless, as we observed an increased frequency of activated circulating Tfh cells in children with newly diagnosed T1D, our data also support IL-21 as a potential pathogenetic factor in the development of T1D and as a feasible therapeutic target in the disease. However, more work needs to be performed in order to identify whether only certain subpopulations of T cells show dysregulated IL-21 production in patients with T1D.

Finally, in this study, we comprehensively characterized the B-cell compartment in our large study cohort. In agreement with recent results from another group (34), we observed no alterations in the B-cell subsets analyzed in children with prediabetes or in children with newly diagnosed T1D. As reported previously (34,35), the frequency of transitional naive B cells decreased sharply with age (Supplementary Fig. 9). An earlier study suggested that the frequency of transitional B cells is increased in patients with T1D (36), but in agreement with the more recent results by Thompson et al. (34), we were unable to corroborate that finding in our cohort. Of note, we observed a positive correlation between the frequency of $CXCR5^+PD\text{-}1^+ICOS^+$–activated circulating Tfh cells and $CD19^+CD27^{++}CD38^{++}$ circulating plasmablasts, as previously also reported by others (17,18,37). This finding is consistent with the idea that the activation of Tfh cells is linked with B-cell activation in vivo. Taken together, our data support the notion that no major alterations in the circulating B-cell compartment can be observed during the development of T1D in humans.

In conclusion, through an analysis of a large cohort of children at different stages of T1D progression, we were able to demonstrate an increase in activated circulating Tfh cells close to the clinical manifestation of the disease. This increase appears to be restricted to a subset of patients that display positivity for multiple autoantibodies. Therefore, our data provide direct evidence for a pathogenetic role for Tfh cells in progression to T1D, and support for Tfh cells and B cells as potential therapeutic targets in T1D, especially in subjects with multiple autoantibody positivity.

Acknowledgments. The skillful technical assistance of Anne Suominen (University of Turku), as well as Virpi Fisk, Hanna Eskelinen, and Matti Laitinen (University of Eastern Finland), is gratefully acknowledged.

Funding. This study was supported by the Sigrid Juselius Foundation, State Research Funding (VTR), the Finnish Diabetes Research Foundation, the Orion-Farmos Research Foundation, the Päivikki and Sakari Sohlberg Foundation, and the Jalmari and Rauha Ahokas Foundation. The DIPP study was supported by the Academy of Finland (Centre of Excellence in Molecular Systems Immunology and Physiology Research 2012–2017, Decision No. 250114), the Sigrid Jusélius Foundation, and JDRF.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. T.V. and E.-L.I. performed the experiments. K.N.-S., H.H., N.N., J.S., A.J., L.M., J.P., and J.T. provided the clinical samples. R.V. and M.K. were responsible for the analyses of diabetes-associated autoantibodies. J.I. was responsible for the HLA screening of the study children. T.K. performed the experiments, analyzed the data, and drafted the manuscript. All authors contributed to the final version of the manuscript. T.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the Immunology of Diabetes Society 14th International Congress, Munich, Germany, 12–16 April 2015, and at the Federation of Clinical Immunology Societies 2016 Meeting, Boston, MA, 22–25 June 2016.

References

1. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 2010;464:1293–1300

2. Serreze DV, Chapman HD, Varnum DS, et al. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. J Exp Med 1996;184:2049–2053

3. Hu C-Y, Rodriguez-Pinto D, Du W, et al. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest 2007; 117:3857–3867

4. Ziegler AG, Rewers M, Simell O, et al. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 2013;309: 2473–2479

5. Helminen O, Aspholm S, Pokka T, et al. OGTT and random plasma glucose in the prediction of type 1 diabetes and time to diagnosis. Diabetologia 2015;58: 1787–1796

6. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, et al.; Type 1 Diabetes TrialNet Anti-CD20 Study Group. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361:2143–2152

7. Menard L, Saadoun D, Isnardi I, et al. The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans. J Clin Invest 2011;121:3635–3644

8. Chamberlain N, Massad C, Oe T, Cantaert T, Herold KC, Meffre E. Rituximab does not reset defective early B cell tolerance checkpoints. J Clin Invest 2016; 126:282–287

9. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity 2014;41:529–542

10. Ueno H, Banchereau J, Vinuesa CG. Pathophysiology of T follicular helper cells in humans and mice. Nat Immunol 2015;16:142–152

11. Schmitt N, Bentebibel S-E, Ueno H. Phenotype and functions of memory Tfh cells in human blood. Trends Immunol 2014;35:436–442

12. He J, Tsai LM, Leong YA, et al. Circulating precursor CCR7(lo)PD-1(hi) $CXCR5^+$ $CD4^+$ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity 2013;39:770–781

13. Locci M, Havenar-Daughton C, Landais E, et al.; International AIDS Vaccine Initiative Protocol C Principal Investigators. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity 2013;39:758–769

14. Simpson N, Gatenby PA, Wilson A, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis Rheum 2010;62:234–244

15. Ma J, Zhu C, Ma B, et al. Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis. Clin Dev Immunol. 2012;2012: 827480
16. Wang J, Shan Y, Jiang Z, et al. High frequencies of activated B cells and T follicular helper cells are correlated with disease activity in patients with new-onset rheumatoid arthritis. Clin Exp Immunol 2013;174:212–220
17. Arroyo-Villa I, Bautista-Caro MB, Balsa A, et al. Constitutively altered frequencies of circulating follicullar helper T cell counterparts and their subsets in rheumatoid arthritis. Arthritis Res Ther 2014;16:500
18. Romme Christensen J, Börnsen L, Ratzer R, et al. Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17- and activated B-cells and correlates with progression. PLoS One 2013;8:e57820
19. Luo C, Li Y, Liu W, et al. Expansion of circulating counterparts of follicular helper T cells in patients with myasthenia gravis. J Neuroimmunol 2013;256: 55–61
20. Xu X, Shi Y, Cai Y, et al. Inhibition of increased circulating Tfh cell by anti-CD20 monoclonal antibody in patients with type 1 diabetes. PLoS One 2013;8: e79858
21. Kenefeck R, Wang CJ, Kapadi T, et al. Follicular helper T cell signature in type 1 diabetes. J Clin Invest 2015;125:292–303
22. Ferreira RC, Simons HZ, Thompson WS, et al. IL-21 production by CD4+ effector T cells and frequency of circulating follicular helper T cells are increased in type 1 diabetes patients. Diabetologia 2015;58:781–790
23. Ilonen J, Hammais A, Laine A-P, et al. Patterns of β-cell autoantibody appearance and genetic associations during the first years of life. Diabetes 2013; 62:3636–3640
24. Siljander HTA, Simell S, Hekkala A, et al. Predictive characteristics of diabetes-associated autoantibodies among children with HLA-conferred disease susceptibility in the general population. Diabetes 2009;58:2835–2842
25. Morita R, Schmitt N, Bentebibel S-E, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity 2011;34:108–121
26. Wei C, Anolik J, Cappione A, et al. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. J Immunol 2007;178:6624–6633
27. Isnardi I, Ng Y-S, Menard L, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood 2010;115: 5026–5036
28. Le Coz C, Joublin A, Pasquali J-L, Korganow A-S, Dumortier H, Monneaux F. Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. PLoS One 2013;8:e75319
29. Che Y, Qiu J, Jin T, Yin F, Li M, Jiang Y. Circulating memory T follicular helper subsets, Tfh2 and Tfh17, participate in the pathogenesis of Guillain-Barré syndrome. Sci Rep 2016;6:20963
30. Gomez-Tourino I, Arif S, Eichmann M, Peakman M. T cells in type 1 diabetes: instructors, regulators and effectors: a comprehensive review. J Autoimmun 2016;66:7–16
31. Arif S, Leete P, Nguyen V, et al. Blood and islet phenotypes indicate immunological heterogeneity in type 1 diabetes. Diabetes 2014;63:3835–3845
32. Sutherland APR, Van Belle T, Wurster AL, et al. Interleukin-21 is required for the development of type 1 diabetes in NOD mice. Diabetes 2009;58:1144–1155
33. Schultz BT, Teigler JE, Pissani F, et al. Circulating HIV-specific interleukin-21(+)CD4(+) T cells represent peripheral Tfh cells with antigen-dependent helper functions. Immunity 2016;44:167–178
34. Thompson WS, Pekalski ML, Simons HZ, et al. Multi-parametric flow cytometric and genetic investigation of the peripheral B cell compartment in human type 1 diabetes. Clin Exp Immunol 2014;177:571–585
35. Morbach H, Eichhorn EM, Liese JG, Girschick HJ. Reference values for B cell subpopulations from infancy to adulthood. Clin Exp Immunol 2010;162:271–279
36. Habib T, Funk A, Rieck M, et al. Altered B cell homeostasis is associated with type I diabetes and carriers of the PTPN22 allelic variant. J Immunol 2012; 188:487–496
37. Chavele K-M, Merry E, Ehrenstein MR. Cutting edge: circulating plasmablasts induce the differentiation of human T follicular helper cells via IL-6 production. J Immunol 2015;194:2482–2485