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

Viisanen T
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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

Tyyne Viisanen,1 Emmi-Leena Ihantola,1 Kirsti Näntö-Salonen,2 Heikki Hyöty,3 Noora Nurminen,3 Jenni Selvenius,4 Auni Juutilainen,5,6 Leena Moilanen,5 Jussi Pihlajamäki,7 Riitta Veijola,8 Jorma Toppari,9 Mikael Knip,4,10,11,12 Jorma Ilonen13 and Tuure Kinnunen1

1Department of Clinical Microbiology, Institute of Clinical Medicine, University of Eastern Finland, Kuopio, Finland; 2Department of Pediatrics, Turku University Hospital and University of Turku, Turku, Finland; 3School of Medicine, University of Tampere and Fimlab Laboratories, Pirkanmaa Hospital District, Tampere, Finland; 4Tampere Center for Child Health Research, Tampere University Hospital, Tampere, Finland; 5Department of Medicine, Kuopio University Hospital, Kuopio, Finland; 6Institute of Clinical Medicine, University of Eastern Finland, Kuopio, Finland; 7Institute of Public Health and Clinical Nutrition, University of Eastern Finland and Clinical Nutrition and Obesity Center, Kuopio University Hospital, Kuopio, Finland; 8Department of Pediatrics, Medical Research Center, PEDEGO Research Unit, Oulu University Hospital and University of Oulu, Oulu, Finland; 9Department of Physiology, Institute of Biomedicine, University of Turku and Department of
Pediatrics, Turku University Hospital, Turku, Finland; \textsuperscript{10}Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland; \textsuperscript{11}Research Programs Unit. Diabetes and Obesity, University of Helsinki; \textsuperscript{12}Folkhälsan Research Center, Helsinki, Finland; \textsuperscript{13}Immunogenetics Laboratory, University of Turku, Turku, Finland

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\textbf{Corresponding author:} Tuure Kinnunen, Department of Clinical Microbiology, University of Eastern Finland, Yliopistonranta 1 C, FIN-70210 Kuopio, Finland

Tel: +358 50 5629349; Email: tuure.kinnunen@uef.fi

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ABSTRACT

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 ≥ 2 biochemical autoantibodies. No alterations in the circulating B-cell compartment were observed in either prediabetic or diabetic children. 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.
INTRODUCTION

Type 1 diabetes (T1D) is caused by an autoimmune process that leads to the destruction of the insulin-producing \( \beta \)-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 \( \beta \)-cell autoimmunity. Subjects with multiple islet autoantibodies have an extremely high risk of developing T1D, with a 5-year risk of around 50% and 15-year risk of more than 80% (4), and this risk is further increased to almost 90% within 2 years in individuals who also develop impaired glucose tolerance (IGT) (5). The partial preservation of \( \beta \)-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 support 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 helper T 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 comprise a heterogeneous population and subsets of CXCR5+ T cells with variable Tfh functionality \textit{ex vivo} have been identified. The most consistent finding has been that the expression of PD-1 and ICOS delineate 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 that 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 that 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 (AAb+; mean age 8.3 years +/- SD 4.6, age range 2-17 years), 15 autoantibody-positive at-risk children with impaired glucose tolerance (IGT; mean age 10.7 years +/- SD 4.0, age range 2-18 years) and 149 autoantibody-negative healthy children (controls; 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 (AAb+) 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-hour 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.

PBMC sample preparation
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-positive 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 approximately 10⁶ 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 IL-21 production, PBMCs were first stimulated for 5 hours with 50ng/ml of PMA (Sigma Aldrich), 1 μg/ml of ionomycin (Sigma Aldrich) and 3 μg/ml of Brefeldin A (Ebioscience) and stained with antibodies to surface receptors (see 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 was 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 FACS Aria III (BD Biosciences). 2 x 10⁴ T cells were cultured together with 2 x 10⁴ 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’s post-test was used. Paired t-tests were used when analyzing paired samples. Relationships between different results were examined using Pearson’s 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 on 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+CD25low/- T cells (Fig. 1A and Supplementary Figure 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. 1B). CXCR5+PD-1+ICOS+ cells express high levels of the proliferation marker Ki67 (Fig. 1C), 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 CXCR5+PD-1-ICOS- subset, or the corresponding subsets sorted from CXCR5- memory CD4+ T cells (Fig. 1D). 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 1E and F, and Supplementary Figure 2). Taken together, our current results corroborate previous findings in that the function and phenotype of peripheral blood CXCR5+PD-1+ T cells most closely resembles that of bona fide Tfh cells in lymphoid tissues (12,13). Moreover, ICOS expression identifies an activated CXCR5+PD-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 impaired glucose tolerance

We analyzed the circulating Tfh compartment in a total of 54 children with newly diagnosed T1D (within one week of diagnosis), 58 at-risk children positive for islet autoantibodies (AAb+) and 15 AAb-positive children with impaired glucose tolerance (IGT), as well as in 149 age- and HLA-matched AAb-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. 2A), even when the results were stratified by age (Supplementary Fig. 3). Moreover, the frequency of CXCR5+PD-1+ICOS- Tfh cells was comparable between the groups (Fig. 2B and Supplementary Fig. 3). However, the frequency of CD4+CXCR5+PD-1+ICOS+ activated Tfh cells was markedly increased in children with newly diagnosed T1D and in at-risk children with IGT (Fig. 2C). We noted that the frequency of CXCR5+PD1+ICOS+ cells within the memory CD4+ T-cell compartment strongly decreased with age (Supplementary Fig. 3). However, a strict pairwise comparison to samples from age-matched healthy children processed and analyzed on the same day confirmed the increase in CXCR5+PD1+ICOS+ Tfh cells in both children with T1D or IGT (Supplementary Fig. 4). Importantly, the increase in PD1+ICOS+ cells appeared to be specific for the CXCR5+ compartment as the frequency of CXCR5-PD1+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-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-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+PD1+ICOS+ activated circulating Tfh cells increased at the clinical manifestation of the disease (Fig. 3A). In contrast, in five autoantibody-positive children analyzed repeatedly that did not progress to T1D during our study the frequency of CXCR5+PD1+ICOS+ activated circulating Tfh cells remained stable (Fig.3B) 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-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 AAbs tested (IAA, GADA and IA-2A). No difference in the frequency of CXCR5+PD-1+ICOS+ activated Tfh cells was observed between AAb+ children positive for 1 or ≥2 autoantibodies (Fig. 4A). 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-1+ICOS+ activated Tfh cells was increased only in
subjects positive for ≥2 AAbs but not in those positive for ≤1 AAb (Fig. 4B 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.

**Additional phenotyping of circulating Tfh cells does not reveal major differences between children with newly diagnosed T1D, autoantibody-positive at-risk children and healthy controls**

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 AAb+ at-risk children without or with IGT (Fig. 5A). No differences in the frequencies of Tfh17- or Tfh2-type CXCR5+ T cells were observed (Fig. 5B 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 towards 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-1+ or CXCR3-PD-1+ Tfh cells between the study groups (Fig. 5D 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 controls**

Since CD4+CXCR5+PD-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 (Fig. 6A; 16.8+/−0.6 % vs. 12.5+/−0.3%, *P* < 0.0001, paired t-test, *n*=103). Similarly, the frequency of IL-21+ cells correlated well with the frequencies of PD1+ICOS- and PD1+ICOS+ cells within CXCR5+ T cells in the same sample and displayed a clear inverse correlation with age (Fig. 6B 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. 6C 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 (Fig. 6E; *P* = 0.08, Dunnett’s post-test, 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+/−0.6% vs. 16.4+/−0.6%, \( P < 0.0001 \), paired 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 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 CD21low 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 to healthy controls, 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 as well as in children at late stages of preclinical T1D characterized by impaired glucose tolerance. 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 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 long-standing T1D, although the small number of patients analyzed in the latter cohort precludes definitive conclusion (Fig. 2C 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 towards 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 ≥2 biochemical autoantibodies at diagnosis of T1D had a clearly increased frequency of activated circulating Tfh cells whereas in the subgroup with ≤1
autoantibody this frequency did not differ from that observed in healthy age-matched controls. 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 type 1 diabetes 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 T1D patients with a more proinflammatory in vitro 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 ≥2 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 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 T1D patients, 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 (Fig. 1 and Fig. 6). In line with this, in the study by Kenefeck et al. the CD4+ T cells that produced higher levels of IL-21 in T1D patients commonly coproduced TNF-α and IFN-γ (21), while a more recent study demonstrated that IL-21 and IFN-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 cell 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 prediabetic children 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 T1D patients (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-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 \textit{in vivo}. Taken together, our data supports 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.
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No potential conflicts of interest relevant to this article were reported.

Author contributions: T.V., E.-L.I and T.K. 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. 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 of 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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REFERENCES


FIGURES

Figure 1 Characterization of peripheral blood CD4+CXCR5+ T cell subsets. CD4+CD25low/- 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 3 to 4 separate experiments with cells from different individuals. * P < 0.05, **P < 0.01 (paired t-test).
Figure 2 Increased frequency of CXCR5+PD-1+ICOS+ activated circulating Tfh cells in children with impaired glucose tolerance (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-1+ICOS- (B) and PD-1+ICOS+ subsets (C) within CXCR5+ T cells were determined in autoantibody-negative healthy control children, autoantibody-positive children without and with IGT, and children with newly diagnosed T1D.
Figure 3 The frequency of CXCR5+PD-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).
Figure 4 CXCR5+PD-1+ICOS+ activated circulating Tfh cells are increased only in children positive for multiple autoantibodies 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.
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 children without or with IGT and in children with newly diagnosed T1D.
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 children (AAb+) and children with newly diagnosed T1D.
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 B cells (C), switched B cells (D), IgG+ (E) and IgA+ (F) memory B cells, plasmablasts (G), IgD-CD27- memory B cells (H) and CD21low anergic B cells (I) within total CD19+ B cells were analyzed in autoantibody-negative healthy control children, in autoantibody-positive children without or with IGT and in children with newly diagnosed T1D.