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# Increased Circulating T Follicular Helper Cells in Iranian Children with Type I Diabetes

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## ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease resulting from the damage of pancreatic $\beta$-cells mediated by autoreactive $CD4^+$ and $CD8^+$ T cells. In recent years, follicular T helper (Tfh) cells have been recognized as a subpopulation of $CD4^+$ T cells providing help for B cells differentiation and antibody production. In this study, we examined the frequency of circulating $CD4^+CXCR5^+$ and $CD4^+CXCR5^+ICOS^+$ (representing Tfh) cells as well as serum levels of anti-glutamic acid decarboxylase 65 (GAD65) and islet cell autoantibodies (ICA) in children with type I diabetes.

We analyzed the percentage of Tfh cells within peripheral blood mononuclear cells in 20 children with T1D (≤300 days from disease onset; Mean age 6.8±4.6 years) and 18 healthy individuals (Mean age 8.8±2.2 years) using flow cytometry. Anti-glutamic acid decarboxylase (GAD) and islet-cell cytoplasmic autoantibodies (ICA) levels were determined by ELISA and indirect immunofluorescence respectively.

We found that the frequency of $CD4^+CXCR5^+$ and $CD4^+CXCR5^+ICOS^+$ (Tfh) cells were significantly increased in the peripheral blood of patients compared with healthy controls ($p<0.001$). Furthermore, elevated levels of anti-GAD and ICA antibodies were detected in children with T1D ($p=0.001$ and $p=0.02$ respectively). There was no correlation between Tfh cells frequency and the autoantibody levels.

The results of our study indicate an increased frequency of Tfh cells in children With T1D that could suggest a possible role of these cells in the disease pathogenesis.

**Keywords:** Anti-glutamic acid decarboxylase antibody; Islet-cell antibodies; Type 1 diabetes; T follicular helper cell

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## INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease occurs following the destruction of pancreatic $\beta$-cells mediated by T cells in genetically susceptible individuals.[1-3] The incidence of type 1 diabetes in children differs between countries ranging from 0.1 to 40.9 per 100,000 per year. Type1 diabetes can be diagnosed at any age, but it is often happened between birth and 14 years old.[4] Unlike most autoimmune disorders, T1D affects females and males equally.[5]

Although autoreactive CD4+ and CD8+ T cells are main factors of β-cell destruction, B cells and autoantibodies may also play a critical role in T1D development and progression.[2] Bruton's tyrosine kinase deficient non-obese diabetic (Btk-NOD) mice showing antibody deficiency are protected from T1D.[6] In human, depletion of B-lymphocytes by anti-CD20 (rituximab) resulted in significant preservation of β-cell function in new onset of T1D.[7] Moreover, autoantibodies against islet antigens such as islet-cell cytoplasmic autoantibodies (ICA), glutamic acid decarboxylase antibodies (GADA) and zinc transporter 8 protein autoantibodies (ZnT8A), are strongly associated with development of the disease. These autoantibodies are also strong predictors of the later development of the disease.[8]

In recent years, T follicular helper (Tfh) cells have been recognized as a subpopulation of $CD4^+$ T cells that required for germinal center formation, B cells differentiation and antibody production.[9] Tfh cells express molecules such as B cell lymphoma 6 (Bcl-6) transcription factor, CXC-chemokine receptor 5 (CXCR5), programmed death-1 (PD-1), inducible costimulatory (ICOS), and interleukin 21 (IL-21) which are functionally important.[10] For example, high levels of CXCR5 expression facilitate the homing of these cells to B cell follicles. Circulating $CXCR5^+CD4^+$ T cells were shown to be potent inducer of naive B cells to produce IgM, IgG and IgA through IL-21 production.[3,11,12] $CD4^+$ T cells that highly express CXCR5 and ICOS are assumed to be the major stimulator of IgG production.[13] Activated Tfh cells also induce immune response against auto-antigens, and finally could cause autoimmune diseases.[10]

Altered Tfh cells frequency and function has been reported in a variety of autoimmune diseases including Sjogren's syndrome,[14] systemic lupus erythematosus (SLE),[15] rheumatoid arthritis (RA),[16,17] ankylosing spondylitis (AS),[18] myasthenia gravis, autoimmune thyroid disease (AITD),[19] Idiopathic thrombocytopenic purpura (ITP),[20] juvenile dermatomyositis and multiple sclerosis (MS).[21] Moreover, a number of studies demonstrated higher level of Tfh cells in blood of adult T1D patients and its correlation with autoantibody production.[22-24] In the present research, we sought to investigate the frequency of Tfh cells and Anti-GAD65 autoantibodies and ICA in children with new-onset T1D.

## MATERIALS AND METHODS

### Patients and Controls

A total of 20 patients with T1D (11 girls and 9 boys; age: 7.5±0.9 years; range: 1-15 years) were enrolled in this study. The duration of the disease was 186.76±95.8 days (disease duration≤300 days from the onset). The diagnosis was based on the criteria of World Health Organization[25] and American Diabetes Association.[26] All the patients were referred to Shariati General Hospital, Tehran University of Medical Sciences, Tehran, Iran. Eighteen age- and sex-matched healthy individuals who had no history of T1D or other autoimmune diseases were recruited as controls (12 girls and 6 boys; age: 8.8±2.2 years). We obtained informed consent from all the guardians of participants. This study was approved by Tehran University of Medical Sciences Research Ethics Committee and conducted in accordance with the Declaration of Helsinki.

### Evaluation of Tfh Cells using Flow Cytometry

To assess circulating $CD4^+CXCR5^+$ T and $CD4^+$ $CXCR5^+$ $ICOS^+$ (Tfh) cells using flowcytometry, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Hypaque (Lymphodex, inno-train, Germany). One million cells per tube were stained with appropriate amount of ) anti-ICOS-FITC, CXCR5-PE and CD4-PE-Cy5 monoclonal antibodies according to the manufacture instructions (eBioscience; USA) for 30 minutes in the dark at 4°C. The stained cells were analyzed using BD FACScalibur instrument (DB; USA). At least 50,000 events per sample were counted.

### Measurement of ICA and Anti-GAD65 Autoantibodies

For measurement of autoantibodies levels, sera of

patients and controls were separated and stored at -80°C until the day of test. Islet cells autoantibody (ICA) in sera of studied subjects were detected by indirect immunofluorescence assay according to the manufacture instruction (EUROIMMUNE, Germany). Briefly, frozen sections of monkey pancreas were incubated with diluted serum samples. In the next step, the attached antibodies were stained with FITC conjugated anti-human antibodies. The glass slides were analyzed by the fluorescence microscope and considered positive at a titer of ≥1:10.

Sera from T1D patients and healthy controls were tested for the presence of anti-GAD (glutamic acid decarboxylase) antibody using ELISA, according to the manufacturers' instructions (EUROIMMUNE, Germany). Microplate was coated by human recombinant glutamic acid decarboxylase, isoform GAD65. The lower detection limit of the anti-GAD ELISA was 0.2 IU/mL. Results were interpreted by a cut-off value recommended by EUROIMMUN as follows: < 10 IU/mL: Negative; ≥ 10 IU/mL: Positive.

### Statistical Analyses

Statistical analyses were performed using SPSS Statistics, version 21 (SPSS Inc., Chicago, Ill., USA). Values were expressed as means±SD. The Shapiro–Wilk test was used as a test of normality. Statistical significance of Tfh frequency between groups was calculated by independent T test. Chi-square test was used for analysis of GAD and Islet cells autoantibodies in T1D patients and healthy controls. We applied Pearson correlation test to obtain the association of Tfh frequency, the autoantibodies, disease duration and age. The *p* value less than 0.05 were considered significant.

## RESULTS

### Increased $CD4^+CXCR5^+$ and $CD4^+$ $CXCR5^+$ $ICOS^+$ T Cells in T1D Patients

For analysis of $CD4^+CXCR5^+$ and $CD4^+$ $CXCR5^+$ $ICOS^+$ T cells frequency, $CD4^+$ T cells were first gated by CD4/side scatter features. Then we analyzed $ICOS^+$ $CXCR5^+$ T cells on $CD4^+$ gated cells. We also analyzed $CXCR5^+$ $CD4^+$ T cells in gated lymphocytes and $CD4^+$ cell populations. Figure 1 shows the gating strategy to determine Tfh cells frequency in gated lymphocytes and $CD4^+$ cells. Interestingly, we observed a significant higher percentage of $CXCR5^+$ $CD4^+$ T cells in lymphocytes ($p$=0.002) and $CD4^+$ cells ($p$=0.001) in patients compared to HCs (Figure 2). We also found higher frequency of Tfh cells ($CD4^+$ $ICOS^+$ CXCR5+ T cells) ($p$<0.001) in children with T1D (Figure 2). A positive correlation between age and %$CXCR5^+$ $CD4^+$ T cells in $CD4^+$ cells ($p$=0.002, r=0.6) and Tfh cells ($p$=0.04, r=0.4) of controls were seen. There was a trend to correlate % $CXCR5^+$ $CD4^+$ T cells with age of patients ($p$=0.06) (Figure 3).

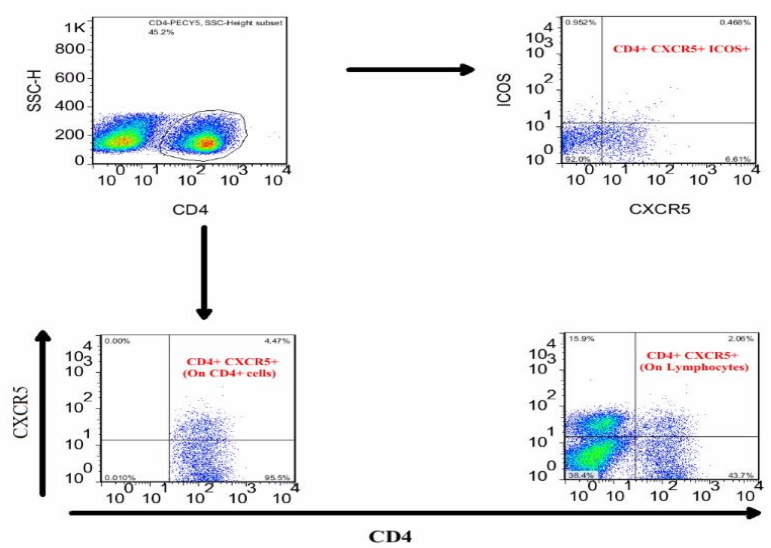


**Figure 1. Representative gating strategy for follicular T helper (Tfh) cells in a sample. $CD4^+$ cells were gated by CD4/side scatter features. $ICOS^+$ $CXCR5^+$ T cells were analyzed within $CD4^+$ gated cells. $CD4^+CXCR5^+$ cells were also analyzed in lymphocytes and $CD4^+$ T cells.**

**Anti-GAD and ICA Autoantibodies in T1D Patients**

In this study, we examined the serum level of these autoantibodies in patients and controls. Our data revealed higher frequency of anti- GAD and ICA positive cases in T1D patients compared to controls (45% vs 0%; $p$=0.001 and 80% vs 38.8%; $p$=0.02 respectively) (Table 1).

**Table 1. Anti- glutamic acid decarboxylase (GAD) and islet-cell cytoplasmic autoantibodies (ICA) in type 1 diabetes (T1D) patients and controls. Eighty percent of patients were ICA positive, while 45% were anti-GAD positive.**

| Samples (n) | Gender (F/M) | Anti-GAD[a] n (%) | | ICA[b] n (%) | |
|---|---|---|---|---|---|
| | | Positive | Negative | Positive | Negative |
| Patients (n=20) | 11/9 | 9 (45) | 11 (55) | 16 (80) | 4 (20) |
| Controls (n=18) | 12/6 | 0 (0) | 18 (100) | 7 (38.8) | 11 (61.2) |
| *p* value | | 0.001 | | 0.02 | |

[a] Anti-Glutamic acid decarboxylase [b] Islet-Cell Cytoplasmic Autoantibodies

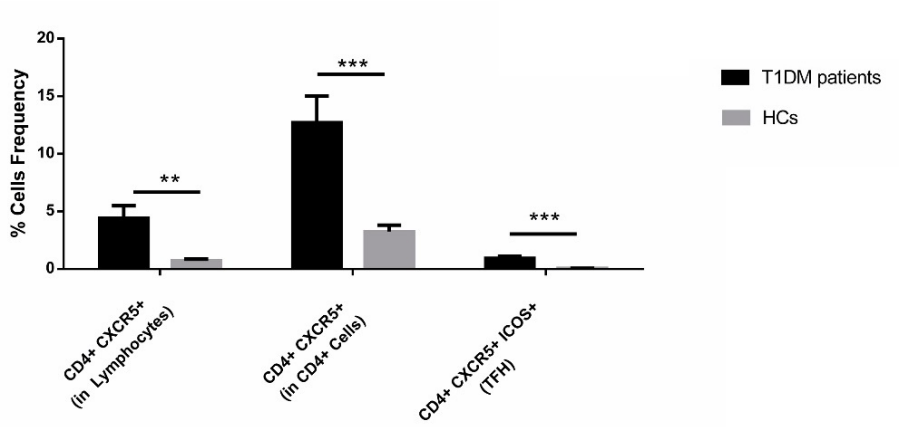


**Figure 2. $CD4^+CXCR5^+$ and $CD4^+$ $CXCR5^+$ $ICOS^+$ follicular T helper (Tfh) cells in children with type 1 diabetes (T1D) and healthy controls. Frequency of $CD4^+CXCR5^+$ among CD4+ T cells and lymphocytes and also $CD4^+$ $CXCR5^+$ $ICOS^+$ T (Tfh) cells increased in T1D patients. To examine the differences between the groups Independent T-test was used. Results are shown as bar graph. ***p≤0.01; ***p≤0.001.***

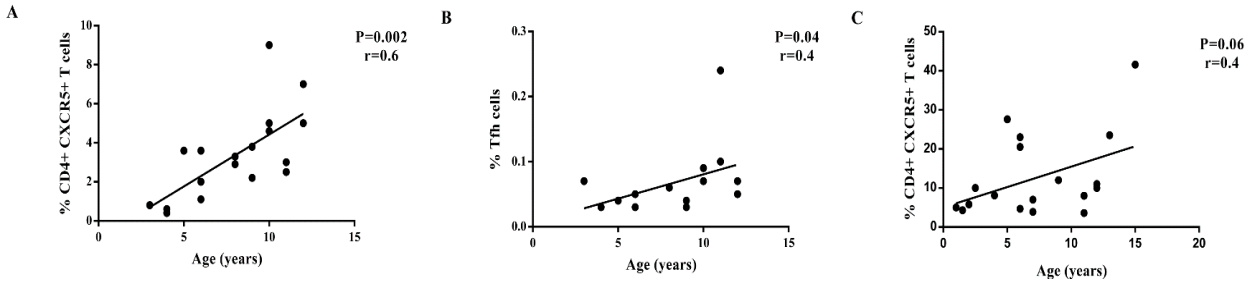


**Figure 3. Pearson's correlation between age and CD4+ CXCR5+ and follicular T helper (Tfh) cells in patients and controls. $CD4^+CXCR5^+$ and $CD4^+CXCR5^+ICOS^+$ cells frequency have a positive correlation with age in healthy children. A & B: control group; C: patients with type 1 diabetes (T1D).**

**Circulating Tfh Cells Is not Associated with Autoantibodies Levels and Disease Duration**

In this study, we also investigated potential correlation between $CD4^+$ $CXCR5^+$ $ICOS^+$ T cells frequency and anti-GAD and ICA in T1D patients. Our results indicated that there was no relationship between Tfh cells percentage and the level of anti-GAD autoantibodies, ICA and disease duration ($p > 0.05$). We also compared Tfh cells between anti-GAD and ICA positive and negative patients. There was no significant difference between the groups.

## DISCUSSION

In the present study, we showed that circulating $CXCR5^+CD4^+$ and $CD4^+CXCR5^+ICOS^+$ Tfh cells significantly increased in children with T1D compared with healthy controls. There was a positive correlation between age and frequency of these cells in controls ($p$=0.002). According to our result and results reported by Morita and Xu,[3,22] the percentage of $CXCR5^+CD4^+$ and $CD4^+CXCR5^+ICOS^+$ Tfh cells in children are less than adults.

There are few studies looking at the frequency of Tfh cells in T1D. Increased frequency of Tfh cells was reported in all of the previous studies. Ferreira et al reported increased frequency of circulating $CD4^+$ $CXCR5^+PD\text{-}1^+$ cells in T1D patients.[24] In two other distinct studies, increased frequency of circulating $CD4^+CXCR5^+$ and $CD4^+CXCR5^+ICOS^+$T cells has been also reported in T1D.[22,23] Xu et al also showed that $CD4^+CXCR5^+ICOS^+$T cells decreased after Rituximab therapy.[22] To our knowledge, this is the second report working on children with new-onset T1D and duration of the disease less than 300 days. There is a study in 2017 which reported increased percentage of circulating Tfh cells in children between 2-17 years old with new onset of T1D.[27]

Circulating Tfh cells are investigated by different markers in different studies. For example, $CD4^+CXCR5^+$, $CD4^+CXCR5^+ICOS^+$, $CD4^+CXCR5^+$ICOShi, $CD4^+CXCR5^+PD\text{-}1^+$, $CD4^+CXCR5^+PD\text{-}1^{hi}$, and $CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ T cells have been used to define Tfh cells in different diseases. Hennerici et al showed increased $CD4^+$ $CXCR5^+$ T cells in the peripheral blood of pemphigus patients.[28] High frequency of circulating $CD4^+CXCR5^+ICOS^+$ T cells was found in patients with rheumatoid arthritis in remission and active phase. Interestingly, there was a positive correlation between the frequency of circulating plasmablasts and Tfh cells.[16] In another study, increased frequency of circulating $CD4^+CXCR5^+$ and $CD4^+CXCR5^+ICOS^{high}$ Tfh cells was detected in RA patients.[17] Le Coz et al also showed increased frequency of Tfh17 ($CD4^+CD45RA^-CXCR5^+$ $CXCR3^-$ $CCR6^+$) cells in SLE patients while, the Tfh2 ($CD4^+CD45RA^-CXCR5^+$ $CXCR3^-CCR6^-$) cell frequency was correlated with disease activity.[15]

Type1 diabetes is characterized by the presence of circulating autoantibodies to a variety of islet cell antigens including GAD and ICA.[29] These autoantibodies are strongly associated with development of the disease and serve to diagnose and predict the later development of T1D.[8] However, the antibody titer levels might be decrease as the disease progresses.[29] In our study eighty percent of patients were ICA positive while 45% of patients were anti-GAD positive. Consistent with our result, ICA are observed in 70% to 80% of individuals with new-onset T1D and positivity decrease after the diagnosis of the disease. About 5% of patients are ICA positive after 10 years.[8] Furthermore, approximately 38% of healthy controls were also positive for ICA. Nondiabetic ICA positive individuals are susceptible for the development of T1D and ICA is used for selection of high risk subjects.[30] In agreement with previous studies, we did not observe significant correlations between autoantibody levels and frequencies of circulating Tfh cells.

Our data support the idea that increased frequency of circulating Tfh cells may be associated with the development of T1D in children and manipulation of these cells could be considered as a therapeutic target.

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