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ORIGINAL RESEARCH

Helios Expression in T-regulatory Cells in Patients with di George Syndrome

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Abstract
Purpose Syndrome diGeorge is associated amongst other clinical signs with various degrees of thymic dysplasia, related immunodeficiency and autoimmune disorders. Helios, a transcription factor from Ikaros family, has been proposed as a marker for thymus derived Tregs. We therefore examined Helios+Tregs in a cohort of patients with genetically proven diGeorge syndrome with typical T cell lymphopenia due to the thymic pathology.
Methods T cells, FoxP3+ Tregs and Helios+FoxP3+ Tregs were examined in 52 samples from 37 patients. One patient with diGeorge/CHARGE syndrome with total thymic aplasia was also included. Statistical analysis was performed using a linear regression comparison.
Results Total absolute Tregs were significantly lower in diGeorge patients as compared to controls in all age groups (0–20 years) (p=0.0016). The difference was more expressed in the first four years of age. Relative Treg numbers expressed as the percentage of Tregs in CD4+ T-cells, however, were not different in patients and controls in all age groups (p=0.661), neither could we find any significant difference in the percentage of Helios+Tregs between patients and controls (p=0.238). Helios+Tregs were still present in a patient with diGeorge/CHARGE syndrome with complete athymia 7 years after partially matched unrelated repeated T lymphocytes infusions.
Conclusion Our findings show that while there was a significant decrease in absolute numbers of Tregs in patients with diGeorge syndrome, the relative percentage of this population did not differ between patients and controls. Low absolute Tregs thus reflected typical T cells lymphopenia in patients. Helios expression was not affected in diGeorge syndrome.

Keywords T-lymphocytes · Tregs · Immunodeficiency · Digeorge · Helios · Charge

Introduction

T regulatory (Treg) cells form an important subset of CD4+T cells with a crucial role in immune tolerance. Since their description in 1995 [1] Tregs were thoroughly investigated and are now well characterized. So far several subsets of Tregs are described, comprising nTregs and iTregs, complemented by Tr1 cells and Th3 cells [2]. nTregs and iTregs represent major subpopulations of CD25+Foxp3+ cells [2]. nTregs are thymus derived regulatory cells with a proposed role in central tolerance, while iTregs are induced in the periphery [3]. As the field of Tregs is still evolving, there are some subtle differences in nomenclature, for example Tregs derived in periphery are also named pTregs (for review see [4]). So far there are no reliable markers how to distinguish these subpopulations of Treg cells. Helios, a protein encoded by Ikaros gene [5] was recently indicated as a promising marker typical for nTreg cells [6, 7].

Syndrome diGeorge is an embryopathy resulting typically from 22q11 deletion. First publication on the syndrome comes from 1967 [8], describing children presenting with congenital heart malformation, hypoparathyreosis, thymus hypo/dysplasia or aplasia in rare cases of complete diGeorge syndrome, and other phenotypic features. The syndrome was later connected with deletion on chromosome 22 [9]. DiGeorge syndrome causes immunodeficiency characterized mainly by impaired T cell mediated immune reactions due to a

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thymic hypoplasia. Overlapping CHARGE syndrome is, besides classical diGeorge phenotype, characterized by additional features such as of coloboma of the eye, choanal atresia, and deafness [10]. Patients suffering from CHARGE syndrome [11] also present with immunodeficiencies associated with thymic pathology [12].

Besides repeated infections some children with diGeorge syndrome also develop autoimmune and allergic complications, suggesting a brakeage of mechanisms of tolerance [13]. T regulatory cells were therefore previously studied in this condition. While the percentage of Tregs within CD4 population was comparable to controls, absolute numbers of Foxp3+ T regulatory cells were low in diGeorge patients particularly in the first 3 years of age [14–16]. These published results suggested that observed decrease in Tregs in diGeorge syndrome is due to thymic hypoplasia and associated impairment of T cell development is mostly expressed in children during their first years of life.

We follow, on a long time basis, a cohort of patients with diGeorge syndrome. In presented study we extended longitudinal follow-up of T regulatory cells by using Helios as a suggested marker for nTreg population arising in thymus. We have included also one patient with diGeorge/CHARGE syndrome who presented with complete athymia and a lack of T cells and was transplanted in age of 6 months with unrelated partially matched lymphocytes infusions [17]. Based on thymic hypoplasia and low T cells numbers in diGeorge syndrome, and thymic aplasia in patient with diGeorge/CHARGE syndrome we hypothesized that thymic output would be impaired in patients and the number of potentially thymus derived Helios expressing Foxp3+ Tregs would be low. We were interested whether this hypothesis would prove true, particularly in the light of several recent publications that challenged the correctness of Helios as a marker of nTregs [7, 18, 19].

Patients and Methods

Patients and Controls

This study follows a cohort of 37 patients, 13 male and 24 female, for a total of 52 samp les from patients aged 6 months to 19 years (7.3, 0.75–18.41, median, 5., 95. percentile). Diagnosis of DiGeorge syndrome was verified in all patients as a present del22q11.2 microdeletion by FISH (fluorescent in-situ hybridization) using DiGeorge/VCFS TUPLE 1/22q Deletion Syndrome LPU004 probe (Cytocell, Cambridge, UK). One patient was diagnosed with diGeorge/CHARGE syndrome based on complex clinical manifestation and laboratory findings [17].

T-cell counts were compared with referential age-related values [20]. For the examination of Tregs and Helios+Tregs, a control group comprised of 34 healthy age matched children, 23 male and 11 female, age 1 to 18 years (8, 1.59–17.94). TRECs were measured in 26 of the patients, 8 male and 18 female, age 8 months to 18 years (7.3, 0.7–18.5).

Informed consent was obtained prior to inclusion in the study from the patients, patients' parents/guardians and from healthy children.

Sample Collection

In all instances blood was drawn from peripheral venepuncture into EDTA-coated tubes and then processed further according to respective protocols as described below.

Immunophenotyping

T-cells

Relative T-lymphocyte count was repeatedly measured in all patients. Comparison was drawn to published age-matched values [20]. Full blood was stained with a CD3-FITC, CD4-APC, CD8-PE and CD45-PE-Dy747 antibody-fluorochrome conjugate mix (Exbio Praha a.s., Vestec, Czech Republic) and measured on a Beckman Coulter Cytomics FC500 flow cytometer. This measurement was recalculated into an absolute number of T-lymphocytes in blood.

Tregs

Peripheral blood monocytic cells (PBMC) were stained with CD3-Alexa700 (Exbio Praha a.s.), CD4-PC7 (eBioscience, San Diego, CA, USA), CD8-PE-Dy590 (Exbio Praha a.s.), CD25-PerCP-Cy5.5 (BioLegend, San Diego, CA, USA) and CD127-Alexa647 (Exbio Praha a.s.) fluorochrome conjugates and further processed using the eBioscience Fixation/Permeabilization solution (eBioscience) and the AntiFoxP3-Alexa488 (eBioscience, clone PCH101) intracellular antibody-fluorochrome conjugate, according to the provided eBioscience intracellular staining protocol. The samples were measured on a BD FACSAria IIu flow cytometer (BD Biosciences, San Jose, CA, USA).

Gating strategy was as shown in Fig. 2a and b, PBMC into lymphocytes based on FSC and SSC, lymphocytes into $CD4^{+}CD8^{-}$ lymphocytes based on CD4 and CD8 expression, $CD4^{+}CD8^{-}$ lymphocytes into Tregs based on FoxP3 and CD25 expression. For more information on CD25, CD127 and FoxP3 expression, please see Online Resource 2 and 3.

Helios+ Tregs

PBMC were stained with CD3-Alexa700 (Exbio Praha a.s.), CD8-PE-Dy590 (Exbio Praha a.s.), CD4 PC7 (eBioscience), CD25 PerCP-Cy5.5 (BioLegend), CD127 Alexa647 (Exbio Praha a.s.) and further processed using

the eBioscience Fixation/Permeabilization solution (eBioscience) and the AntiFoxP3-Alexa488 (eBioscience, clone PCH101) and antiHelios-PE (BioLegend) according to the provided eBioscience intracellular staining protocol. The samples were measured on a BD FACSAria IIu flow cytometer (BD Biosciences).

Gating strategy was as with Tregs (see above and Fig. 2a and b). For discrimination between Tregs and the Helios+Treg subpopulation a Helios isotype control sample was used for each patient and control, determining the level of unspecific fluorescence and allowing for gating of specific Helios+Treg cells (see Fig. 3a). For more information on Helios expression, please see Online Resource 4 and 5.

DNA Isolation and TREC Detection

DNA was isolated from 200 μl of peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). From DBS, a circle with a diameter of 3.2 μm was cut and DNA was eluted at 99 °C for 1 h in a shaker (500 rpm) using 100 μl of Generation DNA Elution Solution (Qiagen GmbH, Hilden, Germany) supplemented with 100 μg/ml of yeast tRNA (Life Technologies, Carlsbad, CA, USA).

The albumin gene level was quantitatively detected in isolated samples using qPCR [21] and standard dilution series derived from Human Genomic DNA with a known starting concentration of 200 ng/μl (Roche, Basel, Switzerland). TREC and KREC levels were assessed separately using cloned plasmid standards as previously described [22, 23]. The results were expressed as the number of TREC (KREC) copies per one microgram of DNA.

Statistical Analysis and Figures

Statistical analysis was performed in R statistical software (The R Foundation for Statistical Computing, Vienna, Austria) and MS Excel (Microsoft, Redmond, WA, USA). Comparison was drawn using linear regression curves with two independent variables, age and diagnosis. Significance cut-off point was set at $p=0.05$. Figures were created in GraphPad Prism software (GraphPad Software, San Diego, CA, USA) and Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA). Trendlines shown in figures are best-fit linear trendlines calculated in GraphPad Prism.

Results

T cells Counts

In order to ascertain that our patients had basic immunologic characteristics tied to the diGeorge syndrome, we measured absolute number of CD3+ T-cells in blood (see Fig. 1). T-cell numbers were below published healthy values median in 48 out of 53 samples. T-cell lymphopenia was particularly expressed during the first 4 years of age, when 18 out of 18 samples (100 %) were below median and 4 samples (22.2 %) were below 5th percentile. For more information on CD4+ and CD8+ T-lymphocyte populations please see Online Resource 1.

Absolute and Relative Numbers of Tregs

Based on previously published results [24], we compared the absolute number of Tregs in peripheral blood of patients to that of controls (see Fig. 2c). On average patients have

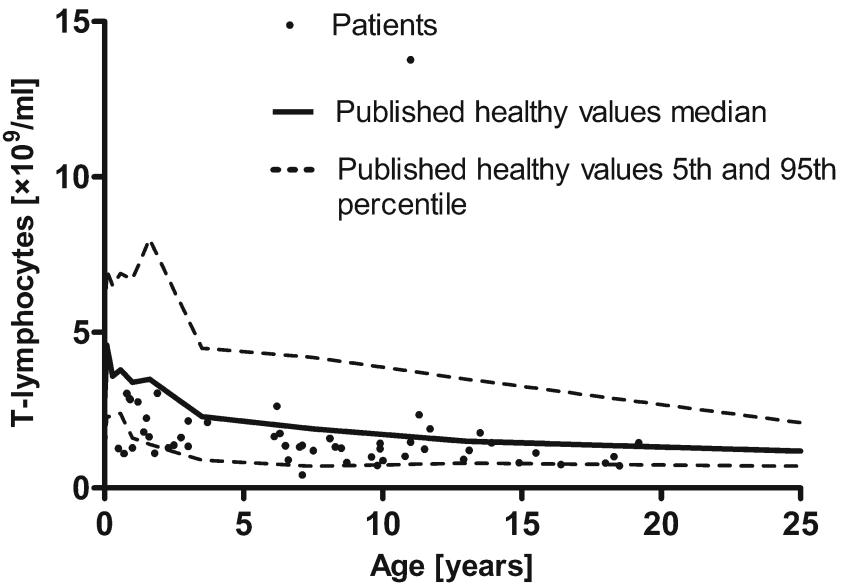

Fig. 1 T-lymphocytes. Absolute number of T-lymphocytes in blood of patients and controls

14.9cells/μl less Tregs than controls and the difference is highly significant (p=0.0016).

When limited to first 4 years of age, the difference in absolute number of Tregs in blood rose to 29cells/μl fewer in patients, a significant difference (p=0.024).

The percentage of Tregs in CD4+ T-cells compartment is shown in Fig. 2d. This relative Treg count decreases with age in patients, whereas it remains mostly constant in controls. Throughout all age groups, no significant difference was found between patients and controls (p=0.991).

Absolute and Relative Numbers of Helios+Tregs

Absolute number of Helios+Tregs in blood (see Fig. 3b) is significantly different (p<0.001) between patients and

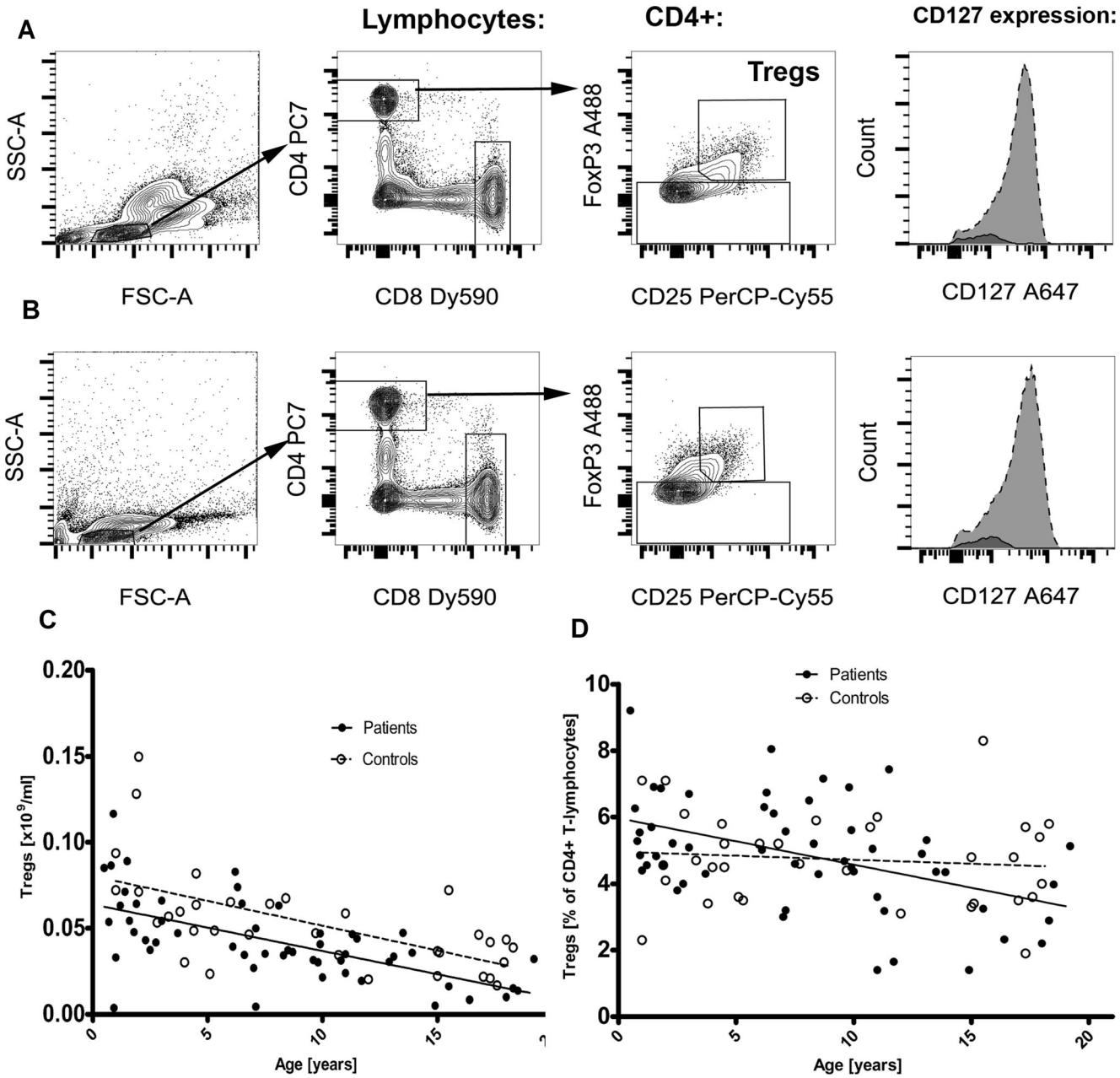

Fig. 2 Tregs. Gating strategy for Tregs out of PBMC, shown for patient **a** and control **b**. CD127 expression column shows expression of CD127 in all CD4+ lymphocytes (dashed line, light background) and in Tregs (solid line, dark background). Absolute **c** and relative **d** number of Tregs in blood of patients and controls

controls in all ages, on average patients have 13.07cells/μl fewer Helios+Tregs than controls.

The difference rises to 27.5cells/μl fewer Helios+Tregs in patients, as compared to controls ($p<0.01$), when limited to the first 4 years of age.

Finally, the percentage of Helios+Tregs was not significantly different between patients and controls in neither all age groups (see Fig. 3c, $p=0.241$), nor the 0–4 year old age group ($p=0.217$).

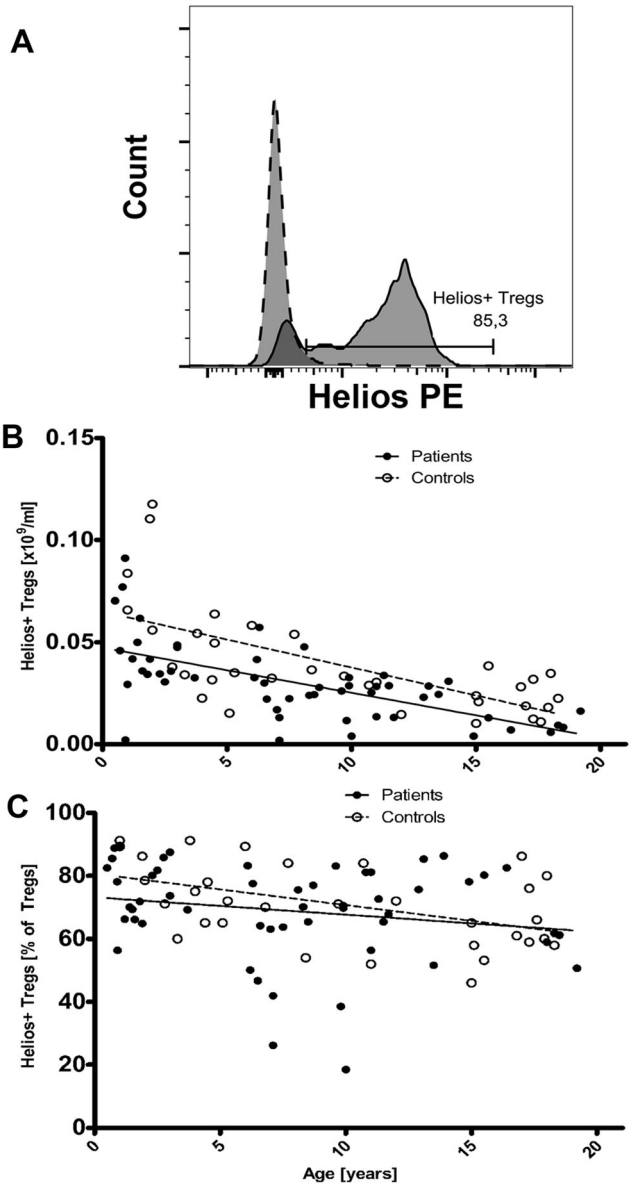

Fig. 3 Helios Tregs. Gating strategy for Helios Tregs **a** using isotype control sample (dashed line) and full panel sample (solid line). Absolute **b** and relative **c** number of HeliosTregs in blood of patients and controls

Tregs vs TRECs

There is a clear correlation between the absolute number of both Tregs (see Fig. 4a) and Helios+Tregs (see Fig. 4b) in peripheral blood and the number of TRECs isolated from lymphocytes in diGeorge patients.

Tregs and Helios+Tregs in di George/CHARGE Syndrome

One of our patients, aged 7 years in the time of study, with diGeorge/CHARGE syndrome presented with thymic aplasia and almost complete lack of T cells in neonatal period (2 cells/μl T-lymphocytes, no proliferative response to PHA, maternal cell engraftment was excluded). He was treated with repeated partially matched unrelated lymphocyte infusions in his 6 months of age [17].

His current finding shows low both Tregs and Helios+Tregs (4.4cells/μl Tregs (mean of other diGeorge 6–8 year olds is 49.3cells/μl) and 1.85cells/μl Helios+Tregs (mean of other diGeorge 6–8 year olds is 31.5cells/μl)). However, his percentage of Helios+Tregs, while lowered (41.9 % of Tregs, diGeorge 6–8 year olds mean 61 %, controls 6–8 year olds mean 81 %), is not deeply affected. His original lack of T-cells was also documented by 0 TRECs, suggesting that his immunity, including Tregs, is driven completely by the expansion of infused donor T-lymphocytes.

Discussion

Here we show, on a cohort of patients with syndrome diGeorge, lower absolute numbers of Tregs and Helios+Tregs in the patient cohort, which is in accordance to previously published findings [14-16]. However, observed decrease of Tregs populations only reflects total T lymphopenia in diGeorge patients as in relative numbers, expressed as percentage of CD4+ T cells, proportion of Tregs does not differ significantly between patients and controls. The same is true for Helios+Tregs. Simultaneously measured Helios expression shows approximately 70 % Helios positive cells among Foxp3+ population both in diGeorge patients and controls. Cohorts of healthy controls were already previously tested for Helios expression which was also about 70 %, similar to our observation in control group [2]. The proportion of Helios positive cells in diGeorge patients is thus comparable to controls, even if their absolute numbers are lower. We could not, therefore, confirm our initial working hypothesis that, based on thymic pathology associated with diGeorge syndrome, the generation of Helios+Tregs subset would be impaired.

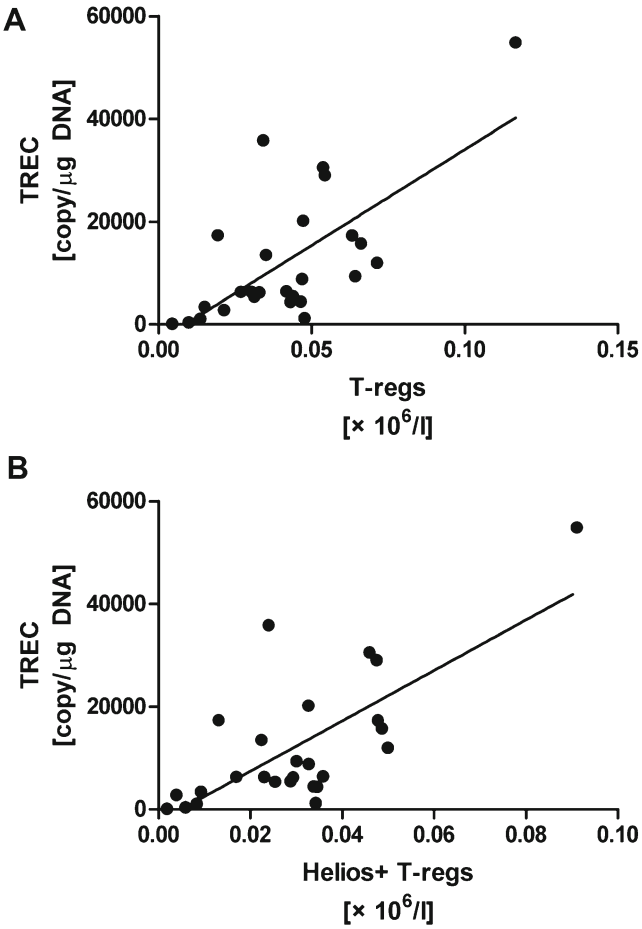

Fig. 4 Tregs vs TRECs. The relation between number of Tregs **a** and HeliosTregs **b** in blood and TRECs isolated from lymphocytes

Thymic function is a very delicate parameter and so far there are no reliable markers to objectively measure functional potential of thymus. Also in diGeorge syndrome their thymic pathology is described more in details on a structural basis, and their functional thymic impairment is mostly expressed as low number of T cells. Recently introduced TREC assay (T cell excision circle) is one of rare possibilities how to objectify thymic output. A subset of patients with diGeorge syndrome is identified by TREC assay in recently established newborn screening programs in some countries [25]. When applied to diGeorge cohort, TREC assay indetified approximatelly one third of patients with TRECs bellow treshold in one study [26] and showed significantly lower TRECs in a small cohort of patients with 22q11 deletion in another study [16], results very similar to our cohort (unpublished results), thus objectively showing low thymic output in diGeorge patients.

Regarding T regulatory cells no such direct marker is available. Considering the importance of T regulatory cells and distinct role of their subsets substantial effort was made to detect and analyze thymus derived and peripheral Tregs. Several potential discriminating markers were identified, among them Helios was most thoroughly investigated [4]. History of Helios as a marker of nTregs is formed by an interesting series of consequent findings. First described by Sugimoto in 2006, Helios was later suggested to be a marker of nTregs [6]. The authors of this study conclude that Helios is expressed exclusively on FoxP3$^+$ Tregs and decreases with age and thus suggest Helios as a marker of nTregs. This conclusion was, however, challenged next year [18]. This group proved Helios to be a marker of activation, proliferation and cell division of T cells, both for murine and human T cell populations. In a detailed study they found that Helios can be induced within Tregs, but also within CD4$^+$ T cells and CD8$^+$ T cells in response to cellular activation. Helios thus does not seem to be a good marker distinguishing nTregs and iTregs, but rather a marker of actual cellular processes. Such view was later supported by a recent publication showing Helios positive and negative cell coexisting within nTregs compartment [19]. We do not find proportionally lower Helios population in our genetically confirmed cohort of diGeorge patients suggesting that it is unlikely that Helios would be a marker for thymic output of natural Treg cells. To further question this situation we compared the relation between TRECs and Tregs and Helios Tregs in our patients (see Fig. 4). There is a clear trend of correlation between TRECs and Helios Tregs which suggests that these T-lymphocytes correlate with thymic output, rather than peripheral expansion. However, the same trend is observed for all total Tregs and prevents us thus to make a clear conclusion on Helios as a marker of T derived regulatory cells.

We also present an interesting case of a patient with diGeorge/CHARGE syndrome who was born with complete athymia and a lack of T cells [17]. This patient was treated with repeated partially matched lymphocytes infusions 6.5 years ago. His current findings still demonstrate the presence of regulatory T cells populations, even if lower than in other diGeorge patients and in controls. We detect Helios+ Tregs present almost 7 years after donor lymphocyte infusions. His number of recent thymic emigrants, expressed as TRECs (T-cell receptor excision circles), was zero both at the birth and in current samples, suggesting that Helios+Tregs must still be of donor origin. Whether these Helios+Tregs were produced by expansion of donor FoxP3+ Helios+Tregs or by expansion of donor FoxP3- Helios- CD4+ lymphocytes is unclear, however.

Conclusion

The question about Helios as a marker of thymic derived Tregs is not fully resolved. Based on our investigation of Tregs and Helios+Tregs on a cohort of patients with diGeorge syndrome and in one patient with diGeorge/CHARGE syndrome with thymic aplasia, we could not confirm our initial hypothesis expecting lower proportion of Helios+Tregs in

diGeorge syndrome patients, but we also could not rule out Helios as a marker of thymic Tregs. Further more detailed and specific studies are needed to resolve the role of Helios and other potential markers in a generation of Tregs. For that, diGeorge patients with thymic pathology represent an interesting cohort.

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Low marginal zone-like B lymphocytes and natural antibodies characterize skewed B-lymphocyte subpopulations in del22q11 DiGeorge patients

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ABSTRACT

Purpose: Patients with DiGeorge syndrome suffer from T-lymphopenia. T-cells are important for the maturation and regulation of B-cell function. Our aim was to characterize the B-cell compartment in DiGeorge syndrome patients.
Methods: B-cell subset phenotypization using flow cytometry. Serum BAFF (B-cell activating factor) and serum anti-alpha-galactosyl IgM measurement using ELISA. Serum IgG measurement using nephelometry.
Results: We observed a significantly increased number of naïve B-cells and decreased number of switched memory B-cells in DiGeorge patients. Furthermore, we observed increased BAFF levels and a trend toward hypergammaglobulinemia later in life. Surprisingly, we detected a decrease in marginal zone-like (MZ-like) B-cells and natural antibodies in DiGeorge patients.
Conclusion: The maturation of B-cells is impaired in DiGeorge patients, with high naïve and low switched memory B-cell numbers being observed. There is a clear trend toward hypergammaglobulinemia later in life, coupled with increased serum BAFF levels. Surprisingly, the T-independent humoral response is also impaired, with low numbers of MZ-like B-cell and low levels of anti-alpha-galactosyl IgM natural antibodies being detected.

1. Introduction

DiGeorge syndrome is an embryopathy typically resulting from a 22q11.2 deletion [1]. The first publication defining the syndrome comes from 1967, describing children presenting with congenital heart malformation, hypoparathyreosis, thymus aplasia and other phenotypic features [2]. White pulp atrophy of the spleen has also been described in these patients [3]. DiGeorge syndrome causes immunodeficiency characterized mainly by impaired T-cell-mediated immune reactions due to thymic hypoplasia and T-cell lymphopenia [2,3,4].

Most studies on patients with DiGeorge syndrome performed to date have focused on disturbances in the T-lymphocyte compartment. The B-lymphocyte population and humoral immune response of these patients have been the focus of only a handful of studies. The chief finding among these studies has been a decreased population of memory ($CD19^+CD27^+$) B-lymphocytes [5–7], which is also observed in other T-lymphopenias [8,9] and is usually explained by impaired T-lymphocyte help in B-cell maturation. Decreased levels of serum immunoglobulins (IgG, IgG subclasses, IgM, IgA) [5,6,10] and a weaker response to vaccination [11] have also been reported.

B-lymphocytes are a diverse population with a complex ontogenesis, which takes place in both central and peripheral lymphatic organs and involves a host of other cellular populations. The spleen is crucial for the development and delineation of B-lymphocytes. Inside the spleen, the bone-marrow emigrant transitional 1 B-lymphocytes differentiate into transitional 2 (T2) B-lymphocytes, most of which then give rise to naïve, mature recirculating follicular B-cells (henceforth referred to as "naïve B-cells"). Weaker BCR signaling [12,13], along with Notch2 receptor-Notch2 delta ligand 1 interaction [14] and other factors predestine T2 lymphocytes to become marginal zone-like (MZ-like, also called natural effector) B-cells instead, a sub-group that facilitates a rapid T-independent response to conserved non-protein antigens. MZ-like B-cells are long-lived and self-replenishing.

To the contrary, naïve B-cells have a short half-life and are very susceptible to apoptosis due to an inability to engage with their BCR-complementary antigen. However, they can be rescued from apoptosis and their survival can be supported by BAFF, a cytokine produced by follicular dendritic cells and other stromal cells as well as various cells of myeloid origin [15–17]. Increased BAFF levels are frequently found in autoimmune disorders with a dysregulated humoral compartment, such as SLE, Sjögren's syndrome, RA or CGD [18–20].

Naïve circulating follicular B-cells can undergo somatic hypermutation, increasing the affinity of the antibodies that are produced and immunoglobulin class-switching, allowing the production of IgG, A and E in germinal centers of the lymph nodes and spleen.

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This process is mediated by follicular helper T-lymphocytes, CD40–CD40L interaction and various soluble cytokines [21] produced chiefly by the T-lymphocytes.

2. Theory

In this study, we follow a cohort of patients with DiGeorge syndrome who display various degrees of T-lymphopenia and thymic dysplasia. Based on the importance of T-cells and the proper splenic microenvironment for the development, maturation and function of B-lymphocytes, as described above, we hypothesized that B-cell maturation and the humoral response would be impaired in our patients. To address these hypotheses, we performed flow cytometry on B-lymphocyte subpopulations in peripheral blood and measured serum IgG. We also examined serum BAFF levels to assess the extent of T-independent homeostatic proliferation of B-lymphocytes and its role in creating the autoimmune environment. Finally, because the characteristic T-lymphopenia of DiGeorge patients becomes less pronounced with age, presumably due to extra-thymic generation of T-lymphocytes [22], we focused on capturing the temporal dynamics of all of the measured parameters by stratifying the patients and controls into several age groups.

3. Patients and methods

3.1. Patients and controls

In our department, we follow a cohort of 76 DiGeorge patients, including 43 females and 33 males, who we routinely test for various immunological parameters. All of the patients harbor a del22q11.2 deletion, verified through multicolor fluorescent in-situ hybridization using the DiGeorge/VCFS TUPLE 1/22q Deletion Syndrome LPU004 probe (Cytocell, Cambridge, UK), and at the time of diagnosis, they fulfilled the ESID diagnostic criteria for DiGeorge syndrome. All of the tests described in this article were performed on a subset of these patients, chosen without bias based on the availability of samples. Unique subcohorts are always clearly characterized for each specific method used.

Controls were age-matched healthy donors and patients who were admitted or examined for an unrelated non-immunological reason. For gross lymphocyte numbers, published reference values obtained from a larger control cohort were used [23]. For serum IgG, normal in-house values were used.

Written parental permission was obtained for all tested subjects according to the procedures established by the Ethical Committee of our institution.

3.2. B-cell subset determination via flow cytometry

Peripheral blood mononuclear cells (PBMCs) isolated through Ficoll-Paque (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient centrifugation were incubated with anti-CD27 Pacific Blue, anti-CD38 Alexa-Fluor 700, anti-CD20 PerCP (Exbio Praha a.s., Prague, Czech Republic), anti-human IgM FITC, anti-CD21 APC (BD Biosciences, San Jose, CA, USA), anti-CD24 PE and anti-CD19 PC7 (Beckman Coulter, Brea, CA, USA). A second-generation antibody panel was developed, replacing several fluorochrome conjugates with the brighter alternatives anti-CD27 BV421, anti-IgM BV510 (BioLegend, San Diego, CA, USA), CD38 FITC (BD) and CD24 APC-Ax750 (BC). The performance of both panels was verified in samples processed in parallel. The samples were then measured using a Cyan ADP flow cytometer (Dako, Glostrup, Denmark) or BD FACS Canto II (BD Biosciences), employing the EuroFlow standardized instrument settings [24]. Gating of B-cell subsets was performed as described previously [25] (see Supplementary Fig. 1).

3.3. B-cell activating factor and anti-alpha-galactosyl IgM

Serum was separated from full blood samples drawn into non-coated tubes, stored frozen at −8 °C and subsequently thawed on ice immediately before testing. BAFF was measured in duplicate in each sample using the R&D Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the provided protocol. Anti-alpha-galactosyl IgM was measured in duplicate in each sample using the R&D Anti-Alpha-Galactosyl IgM Human ELISA (R&D Systems, Minneapolis, MN, USA) according to provided protocol.

Optical density was measured on a Dynex MRX II microplate reader (Dynex Technologies, Chantilly, VA, USA), and the concentration was extrapolated from a best-fit curve using Dynex Revelation 4.25 software (Dynex Technologies).

3.4. Statistical analysis and figures

Statistical analysis was performed using R statistical software (The R Foundation for Statistical Computing, Vienna, Austria) and MS Excel (Microsoft, Redmond, WA, USA). The significance cut-off point was set at $p = 0.05$, and the Mann–Whitney *U*-test was used in all comparative calculations. Figures were created with GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) and Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA). The trendlines shown in the figures are the best-fit linear, one-phase decay and one-phase association trendlines calculated in GraphPad Prism.

4. Results

4.1. B-cell subpopulation skewing as a result of T-lymphopenia

To ascertain whether our patients exhibited the basic immunologic characteristics tied to DiGeorge syndrome, we measured both the absolute number of T- and B-lymphocytes in the blood and the percentages of T- and B-lymphocytes among all lymphocytes.

Most of the patients (46/53 samples, 87%) displayed below-average T-cell counts, with a sizeable portion (22/53 samples, 42%) presenting a T-cell count below the 10th percentile of healthy reference range, thus showing T-lymphopenia typical for DiGeorge syndrome (see Fig. 1A).

The absolute numbers of B-lymphocytes in peripheral blood were not significantly increased or decreased (66% below the healthy median, 34% above the healthy median) (see Fig. 1B).

The distribution of various B-cell subsets, however, was significantly skewed in DiGeorge patients above 5 years of age, as documented through SPICE analysis ($p = 0.022$) (see Fig. 2).

4.2. A block in B-cell maturation is evident in the increase in naïve and decrease in switched memory B-cells

As we hypothesized, the population of naïve B-cells defined as $CD19^+CD27^-IgD^+$ was significantly increased in DiGeorge patients, presumably due to impaired T-cell help in the formation of germinal centers and associated somatic hypermutation and class switching. The trend appeared only later in life and was statistically significant in the 5–10-years and 10+-years age groups ($p < 0.01$), whereas it was insignificant earlier in life (see Fig. 3).

The picture of impaired B-lymphocyte class-switching and somatic hypermutation was completed by a significantly decreased population of switched memory B-cells, defined as $CD19^+CD27^+IgD^-$ (see Fig. 4). Much like in the population of naïve B-cells mentioned above, the trend became significant later in life, in the age groups of 5-year-old and older patients ($p < 0.01$).

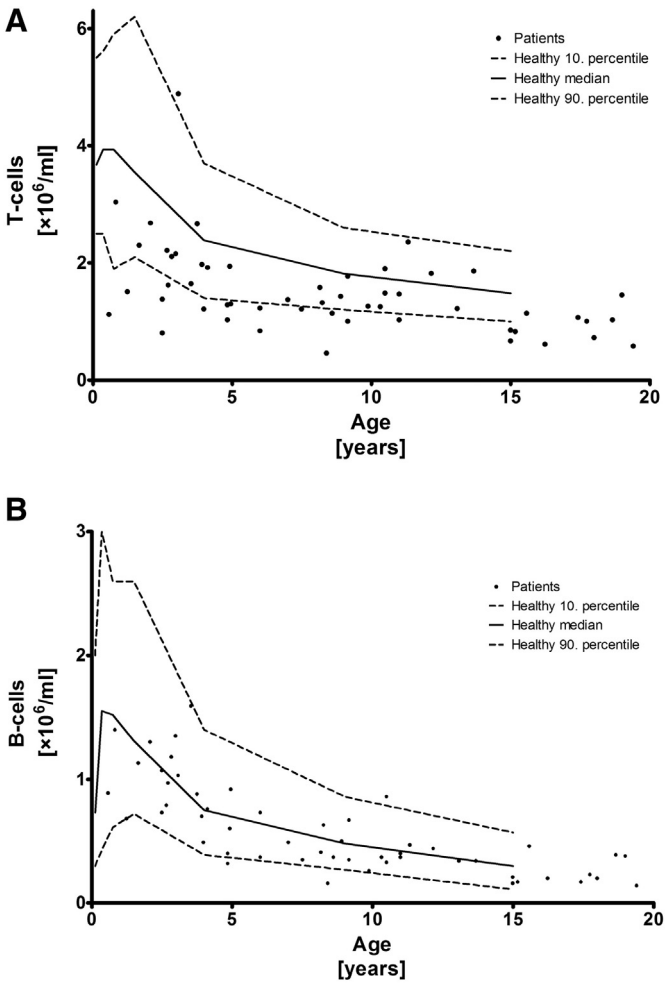

Fig. 1. Lymphocyte counts. The absolute numbers of T-lymphocyte (A) and B-lymphocyte (B) in DiGeorge patients are shown, compared with published healthy reference values. The majority of patients (46/53 samples, 87%) had below-average T-cell counts, with a sizeable portion (22/53 samples, 42%) displaying a T-cell count below the 10th percentile of healthy reference range. However, overall B-lymphocyte counts were unremarkably distributed (66% below the healthy median, 34% above the healthy median). The presented data are for 53 samples from 32 unique patients (14 males, 18 females), including 23 samples from males and 30 samples from females. Healthy values are taken from Shearer, W T et al. [23].

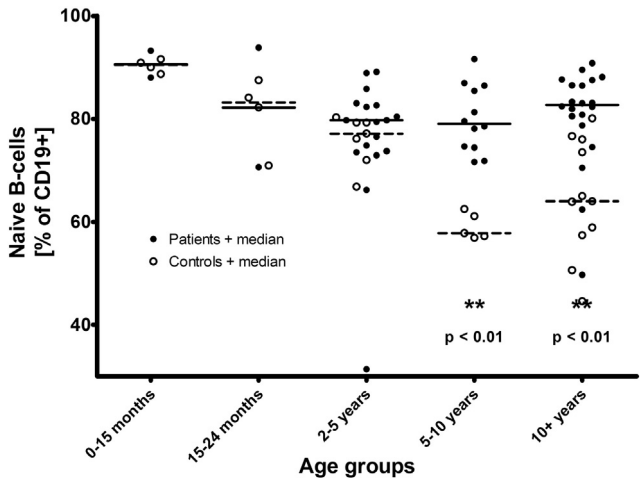

Fig. 3. Naïve B-cells. The percentage of naïve B-cells ($CD19^+CD27^-IgD^+$) among all B-lymphocytes is significantly increased in patients of 5 years and older ($p < 0.01$). For this analysis, 53 samples from 32 patients were compared with 31 healthy controls.

4.3. Increased BAFF and hypergammaglobulinemia are signs of B-cell deregulation

B-cell subpopulation phenotyping revealed a substantial distortion of the B-cell compartment. At the same time, our patients exhibited an increased likelihood of developing signs of autoimmunity surprisingly early in life, which clinically most commonly manifested as autoimmune thrombocytopenias or thyroiditis' and as positivity for ANA or pANCA antibodies (20/44 patients were positive for ANA, ANCA, dsDNA, EMA, anti-Tg or anti-TPO antibodies at some point in their life). Thus, both the phenotype and function of B-cells show dysregulation of the humoral compartment.

To gauge the overall functional status of the B-cell compartment, we measured the hallmark of the humoral immune response, serum IgG. The serum IgG levels of the patients were above the 97.5th percentile of healthy reference values in 28/185 (15%) samples and below 2.5th percentile of healthy reference values in 8/185 (4%) samples (see Fig. 5). The trend toward hypergammaglobulinemia was more evident in older patients, with 17/47 (36%) of the samples from patients aged 10 years and older showing levels above the healthy 97.5th percentile. Conversely, all (100%) of the hypogammaglobulinemic samples came from patients under 10 years old. A trend from hypogammaglobulinemia early in life toward hypergammaglobulinemia during adolescence is evident.

To assess the influence of serum cytokine BAFF levels on the expanded compartment of naïve B-cells and hypergammaglobulinemia, we measured BAFF levels in DiGeorge patients. In general, DiGeorge patients exhibit increased serum BAFF levels compared with controls (patient median 2426 pg/ml, SD 653 pg/ml, control median 1788 pg/ml, SD 554 pg/ml, $p = 0.014$) (see Fig. 6). This is in accordance with the fact that BAFF levels are increased in autoimmune conditions such as SLE and RA, among others [18,19], and that patients with DiGeorge syndrome show an increased prevalence of autoimmunity and positivity for

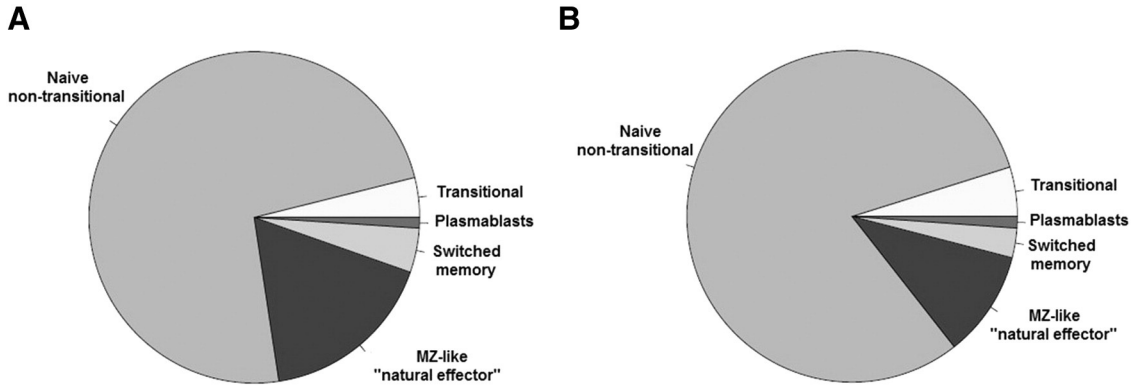

Fig. 2. B-cell subset distribution pattern. The B-cell subset distribution pattern is altered in DiGeorge patients (B) older than 5 years compared with age-matched controls (A), as documented through SPICE analysis ($p = 0.02232$). For this evaluation, 22 DiGeorge patients were compared with 16 healthy controls.

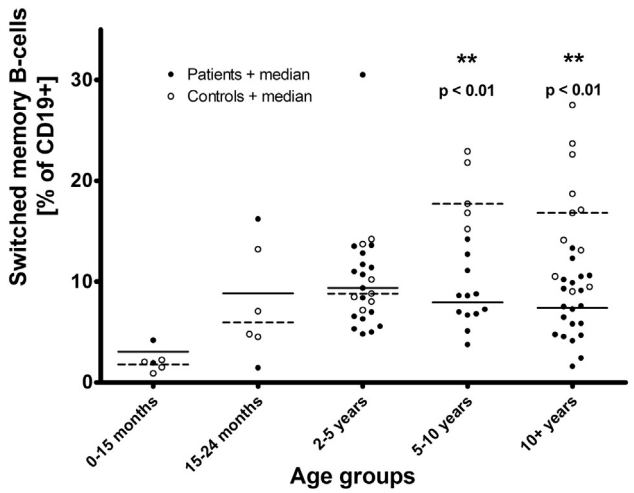

Fig. 4. Switched memory B-cells. The percentage of switched memory B-cells ($CD19^+CD27^+IgD^-$) among all B-lymphocytes is significantly decreased in patients of 5 years and older ($p < 0.01$). For this analysis, 53 samples from 32 patients were compared with 31 healthy controls.

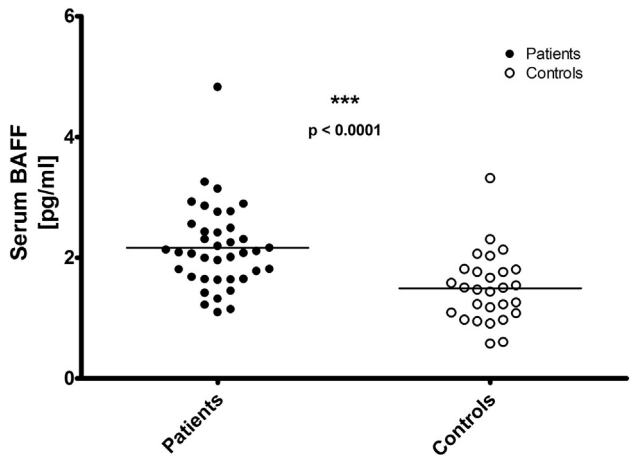

Fig. 6. Serum BAFF. Patients show increased serum BAFF levels compared with the controls (patient median: 2426 pg/ml, SD 653 pg/ml, control median 1788 pg/ml, SD 554 pg/ml). This difference is highly significant ($p = 0.014$). For this analysis, 39 samples from 30 patients were compared with 28 healthy controls.

autoantibodies [11,26–28]. However, we found only an insignificant correlation between serum BAFF levels and the percentage of naïve B-cells in our patients (Spearman $r = 0.477$; $p = 0.166$) (data not shown).

4.4. Decreases in MZ-like B-cells and natural antibodies indicate an impaired T-independent response

A surprising finding revealed by B-cell subset phenotyping was a decrease in marginal zone-like B-cells, defined as $CD19^+CD27^+IgD^+$. These cells provide a T-cell independent response to conserved antigens, and we therefore did not hypothesize that this population would be decreased in DiGeorge patients. However, the numbers of MZ-like B-cells were significantly decreased in patients aged 2 years and older ($p < 0.01$) (see Fig. 7).

To follow-up on this finding, we measured anti-alpha-galactosyl IgM antibodies in the serum of DiGeorge patients and controls. Natural antibodies are antibodies against conserved antigens that are produced by the immune system regardless of outside stimulation. Anti-alpha-galactosyl IgM is one of the most prevalent natural antibodies. We hypothesized that the levels of anti-alpha-galactosyl IgM reflect the functional capacity of natural effector B-cells and the T-independent humoral compartment in general.

Indeed, we observed a severe decrease in anti-alpha-galactosyl IgM antibodies in DiGeorge patients compared with controls (patient median 12.3 U/ml, SD 22.0 U/ml, control median 33.3 U/ml, SD 17.6 U/ml, $p < 0.001$) (see Fig. 8).

5. Discussion

Here, in a cohort of patients with DiGeorge syndrome, we show that there is an impairment of B-lymphocyte maturation, skewing of B-cell subpopulations that changes dramatically with age, a trend from hypogammaglobulinemia toward hypergammaglobulinemia later in life and, surprisingly, an impaired T-independent humoral response. We further demonstrate that DiGeorge patients suffer from a decreased ability of B-lymphocytes to undergo proper maturation, which manifests as a decreased number of switched memory B-cells and an increased population of naïve B-cells, which are findings that have been published previously [5–7]. In this study, we show that this difference is unremarkable in infancy but becomes significant at approximately

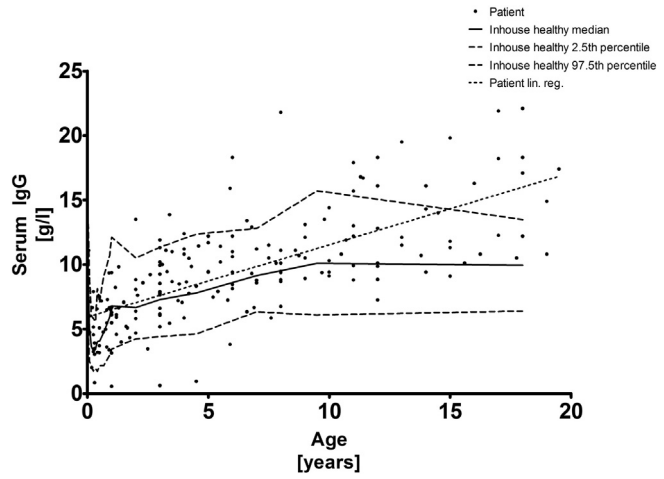

Fig. 5. Serum IgG. Serum IgG levels in DiGeorge patients were compared with in-house healthy reference values (dashed and full lines). The calculated linear trendline for the patient values is included (dotted line). There is a clear trend toward hypergammaglobulinemia later in life, with 17/47 (36%) samples from patients of 10 years and older showing values above the healthy 97.5th percentile. The presented data are for 195 samples from 69 patients.

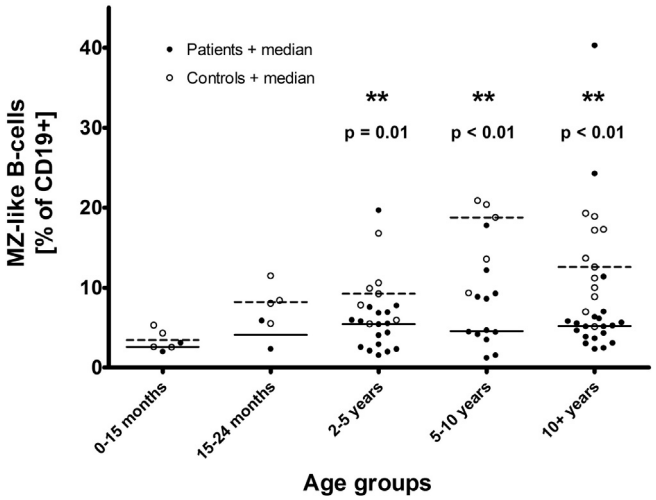

Fig. 7. MZ-like B-cells. The percentage of MZ-like B-cells ($CD19^+CD27^+IgD^+$) among all B-lymphocytes is decreased in patients of all ages, and significantly so in patients of 2 years and older ($p < 0.01$). For this analysis, 53 samples from 32 patients were compared with 31 healthy controls.

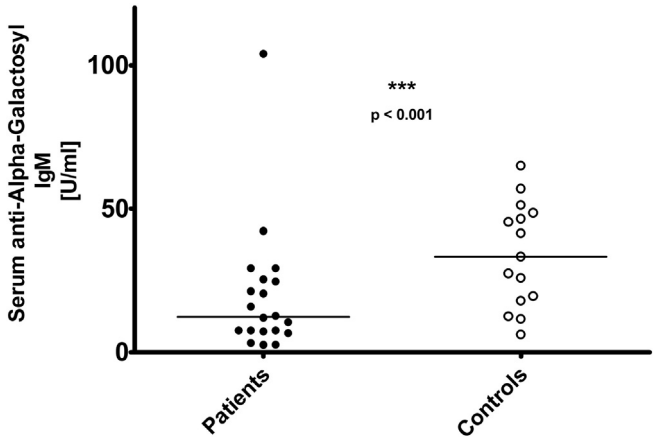

Fig. 8. Serum anti-alpha-galactosyl IgM. Serum anti-alpha-galactosyl IgM levels are severely and significantly decreased in patients compared with controls (patient median: 12.3 U/ml, SD 22.0 U/ml, control median 33.3 U/ml, SD 17.6 U/ml). For this analysis, 20 samples from 17 patients were compared with 15 healthy controls.

2–5 years of age. We hypothesize that this might be due to an impaired ability of young patients to generate a long-lasting memory of antigens to which they are exposed at high levels during pre-school age. This contrasts with some previous studies showing a decreased population of naïve B-cells in pediatric patients [7].

We further demonstrate dysregulation of the humoral compartment in the form of hypergammaglobulinemia later in life, again contrasting with some previous studies that have detected hypogammaglobulinemia [10,11,26]. In this skewed population, B-cells are supported in terms of survival and antibody production by BAFF, a cytokine whose levels are commonly observed to be increased in autoimmune disorders such as SLE, RA, CGD and others [18–20]. We also show that BAFF levels are increased in DiGeorge patients. This is a novel finding in DiGeorge syndrome, though it is in good accord with the well-established increased prevalence of autoimmune disorders in these patients [11, 26–28], as well as findings of increased BAFF in other primary immunodeficiencies such as CVID [29].

Finally, we report the surprising finding of low numbers of MZ-like B-cells, sometimes referred to as natural effector B-cells [30–32]. These cells share some characteristics with memory B-cells, such as a long half-life and surface expression of CD27, but they carry B-cell receptors with limited diversity and limited somatic mutations, produce only class M immunoglobulins and are part of an innate-like T-cell-independent response to conserved non-protein antigens [33]. Given the T-lymphopenia observed in our patients, we expected these cells to be either compensatorily increased or unaffected. However, the MZ-like B-cell population was not found to increase with age in DiGeorge patients, in contrast to the healthy controls. We followed-up on this finding with the measurement of serum anti-alpha-galactosyl IgM antibodies, which are considered natural antibodies against conserved bacterial antigens (presumably produced by the MZ-like B-cells in this case). The levels of these antibodies were also found to be severely decreased in DiGeorge patients, thus demonstrating the functional side of the T-independent humoral response deficiency. We consider it reasonable to assume, that the decreased numbers of MZ-like B-cells are the cause of this low level of natural IgM antibodies.

The development of natural effector MZ-like B-cells occurs in the spleen and involves many inter- and intra-cellular pathways [12–14,34]. The importance of the spleen, with its complex architecture and cellular populations, for the development of MZ-like B-cells cannot be overstated, as shown by overwhelming post-splenectomy infection (OPSI) by encapsulated bacteria in post-splenectomic patients. There are scarce reports of white pulp atrophy in the spleens of DiGeorge patients [3], but the precise nature of this condition and whether it is the reason for the decreased MZ-like B-cell population are unknown at present.

6. Conclusion

The B-lymphocyte compartment is severely compromised in DiGeorge syndrome, with increased numbers of naïve and decreased switched memory B-cells. This dysregulation manifests as a trend from hypogammaglobulinemia toward hypergammaglobulinemia later in life as well as an increased prevalence of autoimmunity. In DiGeorge patients, the survival and proliferation of B-cells are supported by increased BAFF levels. Surprisingly, the numbers of T-independent MZ-like B-cells are decreased in these patients, as are the levels of natural anti-alpha-galactosyl IgM antibodies. Further studies on the function of the spleen and MZ-like B-cell maturation in DiGeorge patients are needed to elucidate the reasons for the decreased T-independent humoral response.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2015.08.013.

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Follicular Helper T Cells in DiGeorge Syndrome

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DiGeorge syndrome is an immunodeficiency characterized by thymic dysplasia resulting in T cell lymphopenia. Most patients suffer from increased susceptibility to infections and heightened prevalence of autoimmune disorders, such as autoimmune thrombocytopenia. B cells in DiGeorge syndrome show impaired maturation, with low switched-memory B cells and a wide spectrum of antibody deficiencies or dysgammaglobulinemia, presumably due to impaired germinal center responses. We set out to evaluate circulating follicular helper T cells (cTFHs) in DiGeorge syndrome, as markers of T–B interaction in the germinal centers in a cohort of 17 patients with partial DiGeorge and 21 healthy controls of similar age. cTFHs were characterized as $CXCR5^{+}CD45RA^{-}$ $CD4^{+}$ T cells using flow cytometry. We verify previous findings that the population of memory $CD4^{+}$ T cells is relatively increased in diGeorge patients, corresponding to low naïve T cells and impaired T cell production in the thymus. The population of $CXCR5^{+}$ memory $CD4^{+}$ T cells (cTFHs) was significantly expanded in patients with DiGeorge syndrome, but only healthy controls and not DiGeorge syndrome patients showed gradual increase of CXCR5 expression on cTFHs with age. We did not observe correlation between cTFHs and serum IgG levels or population of switched memory B cells. There was no difference in cTFH numbers between DiGeorge patients with/without thrombocytopenia and with/without allergy. Interestingly, we show strong decline of PD1 expression on cTFHs in the first 5 years of life in DiGeorge patients and healthy controls, and gradual increase of PD1 and ICOS expression on $CD4^{-}$ T cells in healthy controls later in life. Thus, here, we show that patients with DiGeorge syndrome have elevated numbers of cTFHs, which, however, do not correlate with autoimmunity, allergy, or production of immunoglobulins. This relative expansion of cTFH cells may be a result of impaired T cell development in patients with thymic dysplasia.

Keywords: immunodeficiency, DiGeorge, T cells, follicular helper T cells, PD1, ICOS, memory, thymus

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INTRODUCTION

DiGeorge syndrome is a primary immunodeficiency characterized by thymic dysplasia and T-cell lymphopenia (1). Its most common cause is a 3 Mb deletion on the 22nd chromosome (del22q11.2), which among others encompasses also the *TBX* gene, responsible for the formation of thymic anlage, a basic structural foundation of the thymus, and its further fetal development (2). This

failure to develop a proper niche for the generation of mature thymocytes results in T-cell lymphopenia and increased susceptibility to infection in patients with DiGeorge syndrome. Other clinical symptoms of this syndrome include congenital heart disease, hypoparathyroidism, developmental retardation, and an increased prevalence of autoimmune disease (3–7).

The immune system has been studied thoroughly in DiGeorge syndrome, with a specific focus on T cells and their development. While only 1.5% of patients present with complete DiGeorge syndrome and suffer from life-threatening severe T-cell lymphopenia (8), even patients with partial DiGeorge syndrome show T-cell lymphopenia and decrease of thymic output with low naïve T cells, recent thymic emigrant T cells reflected by low number of T-cell receptor excission circles (4, 9, 10). The impaired T-cell development is further shown to cause oligoclonality within the T-cell compartment (11). Taken together with information on the humoral immune compartment in DiGeorge syndrome patients, including impaired response to vaccination, hypogammaglobulinemia (12, 13), and dysfunctional maturation of B-cells (4, 14, 15), these findings reflect the dysregulation of T–B-cell interactions in DiGeorge syndrome.

The principal subset of T cells crucial for the proper development of germinal center response, B-cell class-switching, and establishment of humoral memory are the follicular helper T cells. These cells are characterized by expression of chemokine receptor CXCR5, which allows their homing along the CXCL13 chemokine gradient produced mainly by follicular dendritic cells in germinal centers (16), thus ensuring their temporospatial colocalization with naïve B cells during the germinal center response to antigen. TFH cells produce IL-21 and express B-cell costimulatory molecules such as ICOSL, CD40L, and others, which promote B-cell proliferation, affinity maturation, and class-switching (17). While TFHs are mostly present in the secondary lymphoid organs, the peripheral blood contains a small population of cells that are generally accepted to be the circulating counterparts of TFH cells [thus circulating follicular helper T cells (cTFHs)] (18, 19). Numerous phenotypic characteristics have been proposed and used, generally including memory marker CD45RO or the absence of CD45RA, the chemokine receptors CXCR5, CCR6, CXCR3, activation/costimulation molecules PD1 and ICOS or the transcription factor Bcl-6. Similarly to changing proportions of naïve vs memory and other T-cell subsets during an individual's life (20), the amount and quality of cTFHs is likely to change over time and has already been shown to decrease in the elderly (21).

There have been several reports describing cTFHs in various primary immunodeficiencies (22–24), but limited information is available on cTFHs in patients with DiGeorge syndrome, even though the combination of dysregulated T-cell development, impsaired humoral immunity, and immune dysregulation makes this syndrome a prime candidate for evaluation of cTFH cells. An earlier report by Derfalvi et al. found increased percentage of $CXCR5^+ICOS^+$ CD4 T cells in DiGeorge syndrome patients both below 17 years of age and adults (25). However, no further age-specific resolution was provided or clinical correlation discussed. We, therefore, investigated the cTFH population in pediatric patients with partial DiGeorge syndrome.

MATERIALS AND METHODS

Patients

We present the results of 17 patients with partial DiGeorge syndrome (age 0.5–21 years, mean 7.6 years, 12 females, 5 males), compared to 21 healthy controls (age 0.1–22 years, mean 11.6 years, 9 females, 12 males). Basic patient data are summarized in **Table 1**. All of the patients harbor a del22q11.2 deletion verified through multicolor fluorescent *in situ* hybridization using the DiGeorge/VCFS TUPLE 1/22q Deletion Syndrome LPU004 probe (Cytocell, Cambridge, UK), and at the time of diagnosis, they fulfilled the ESID diagnostic criteria for DiGeorge syndrome. This study was carried out in accordance with the recommendations of the Ethical Committee of the second Faculty of Medicine, Charles University in Prague and University Hospital in Motol, Czech Republic. The protocol was approved by the Ethical Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Flow Cytometry

Peripheral blood was taken as part of other routine investigations into EDTA-coated tubes, peripheral blood mononuclear cells were isolated using Ficoll-Paque gradient and stained with anti-CD3 Alexa Fluor 700 (clone MEM-57), anti-CD4 Pacific Blue (clone MEM-241, both from Exbio, Czech Republic), anti-CD45RA PE-Cy7 (clone HI100), anti-CXCR5 Alexa Fluor 488 (clone J252D4), anti-PD1 APC (clone EH12.2H7, all from BioLegend, San Diego, CA, USA), and anti-ICOS PE (clone ISA-3, ThermoFisher, MA, USA). Data were acquired on BD FACSAria II cytometer (BD Biosciences, USA) and analyzed using FlowJo VX (FlowJo, LLC, USA) and GraphPad Prism 6 (GraphPad Software, USA). Gating strategy is shown in **Figure 1A**.

RESULTS

Patients with DiGeorge Syndrome Have High Memory $CD4^+$ T-Cells due to Comparative Decrease of Naive $CD4^+$ T-Cells

Our cohort of DiGeorge syndrome patients has typically low absolute T cell lymphopenia, which becomes less pronounced with age (**Figure 1A**). Reflecting this finding and we also observe low absolute memory $CD4^+$ T cells (**Figure 1B**); however, the low thymic output of naïve T celly compartment (**Figures 1C,D**). This increase starts already at birth, remains constant throughout childhood s as shown by other groups (4, 9) results in relative increase of the memorand adolescence, and is highly significant (linear regression intercept $p = 0.0002$, slope $p = 0.54$) (**Figure 1C**).

cTFHs Are Expanded in DiGeorge Syndrome

To correct for the relative increase of memory $CD4^+$ T cells in DiGeorge, we compared the percentages of $CXCR5^+$ memory $CD4^+$ T cells (cTFHs) of all memory $CD4^+$ T cells (gating strategy shown in **Figure 2A**). We found that patients with DiGeorge

TABLE 1 | Cohort characteristics.

Patient ID	Age (years)	Lymphocytes ($\times 10^9$/l)	CD3 (% of lympho)	CD3 ($\times 10^9$/l)	CD4 (% of lympho)	CD4 ($\times 10^9$/l)	IgG (g/l)	IgM (g/l)	Thrombocytopenia	Allergy	SwM B cells (% of B cells)
Patient 1	0.2	8.32	34	2.83	12	1.00	2.47	0.30	No	No	NA
Patient 2	0.6	1.84	19	0.35	15	0.28	6.64	0.38	No	No	NA
Patient 3	1.6	5.32	67	3.56	28	1.49	9.56	0.59	No	No	NA
Patient 4	1.6	1.91	62	1.18	45	0.86	3.98	0.51	Yes	No	NA
Patient 5	3.5	2.10	53	1.11	34	0.71	12.6	0.28	No	No	8.4
Patient 6	4.5	NA	45	NA	23	NA	11.1	0.78	No	No	NA
Patient 7	5.2	3.49	54	1.88	34	1.19	7.55	0.22	No	No	11.7
Patient 8	5.3	2.67	51	1.36	27	0.72	11.2	0.59	No	No	6.6
Patient 9	5.5	2.55	72	1.84	43	1.10	9.77	0.83	No	Yes	NA
Patient 10	5.6	5.63	73	4.11	33	1.86	11.5	0.62	No	No	10.7
Patient 11	7.0	1.90	40	0.76	22	0.42	9.55	0.65	No	No	5.6
Patient 12	10.5	1.78	62	1.10	34	0.61	12.4	0.88	No	No	14.2
Patient 13	11.0	2.77	58	1.61	26	0.72	11.7	1.11	Yes	No	6.7
Patient 14	13.0	1.89	60	1.13	44	0.83	9.93	0.58	Yes	Yes	9.9
Patient 15	14.5	1.68	67	1.13	40	0.67	17.7	1.62	Yes	Yes	NA
Patient 16	19.5	1.57	71	1.11	43	0.68	10.1	0.41	No	Yes	1.6
Patient 17	20.5	2.01	61	1.23	45	0.90	20.2	3.03	Yes	No	13.3

Table describing patients with DiGeorge syndrome included in this study, including basic laboratory and clinical data.

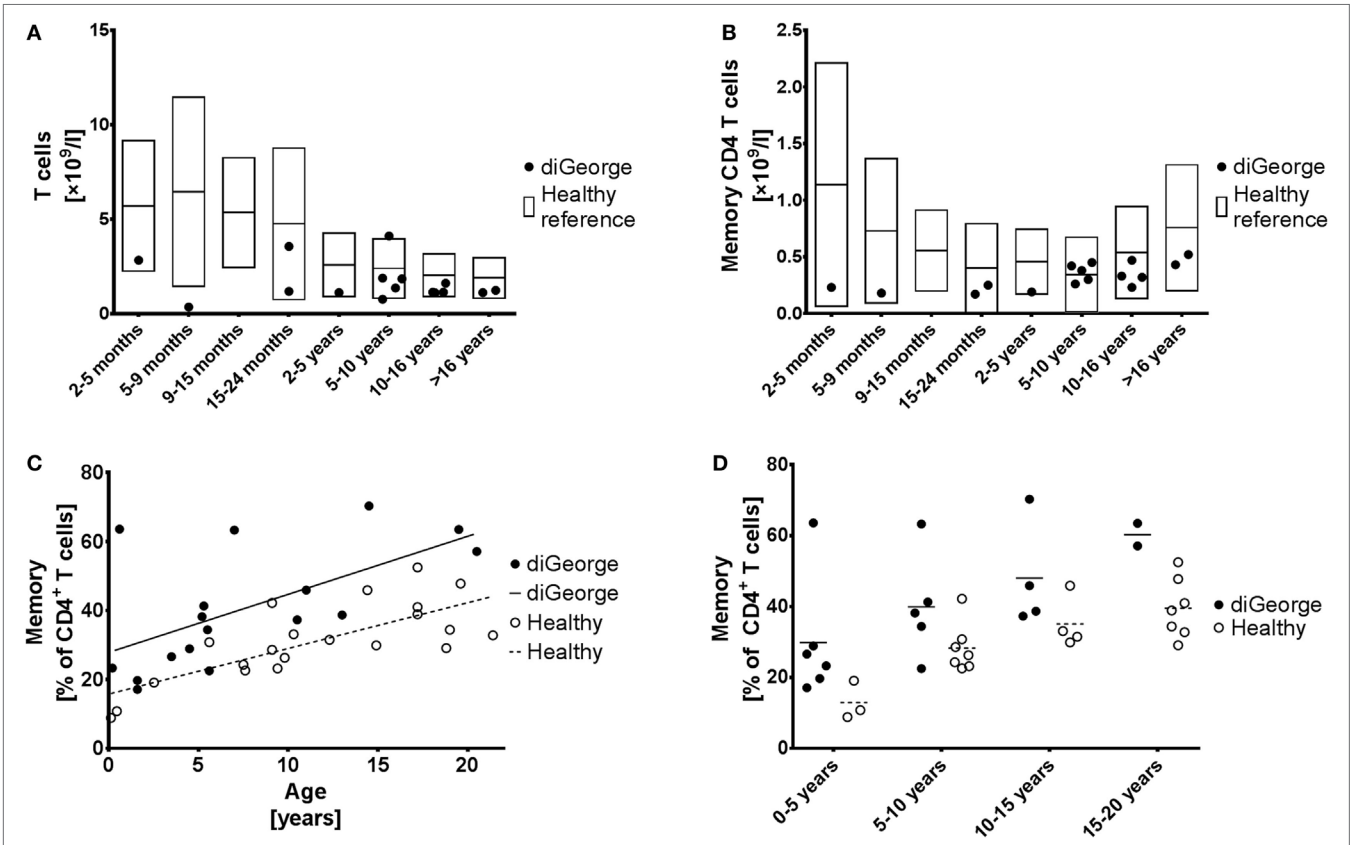

FIGURE 1 | Memory CD4⁺ T cells. **(A)** Absolute T cell numbers and **(B)** absolute memory CD4⁺ T cell numbers in patients with DiGeorge syndrome, compared to published healthy reference values (20), shown as mean and 90% range. **(C)** Proportion of memory CD4⁺ T cells of all CD4⁺ T cells compared to age, shown with linear regression trendlines and **(D)** divided into several age groups.

syndrome have significantly elevated proportion of cTFH within the memory compartment compared to healthy controls (Welch's *t*-test, $p = 0.02$) (**Figure 2B**).

However, whereas this proportion increased with age in healthy controls (linear regression, $p = 0.01$, $R^2 = 0.29$) (**Figure 2C**), there was no significant increase of cTFH/memory CD4⁺ T cell

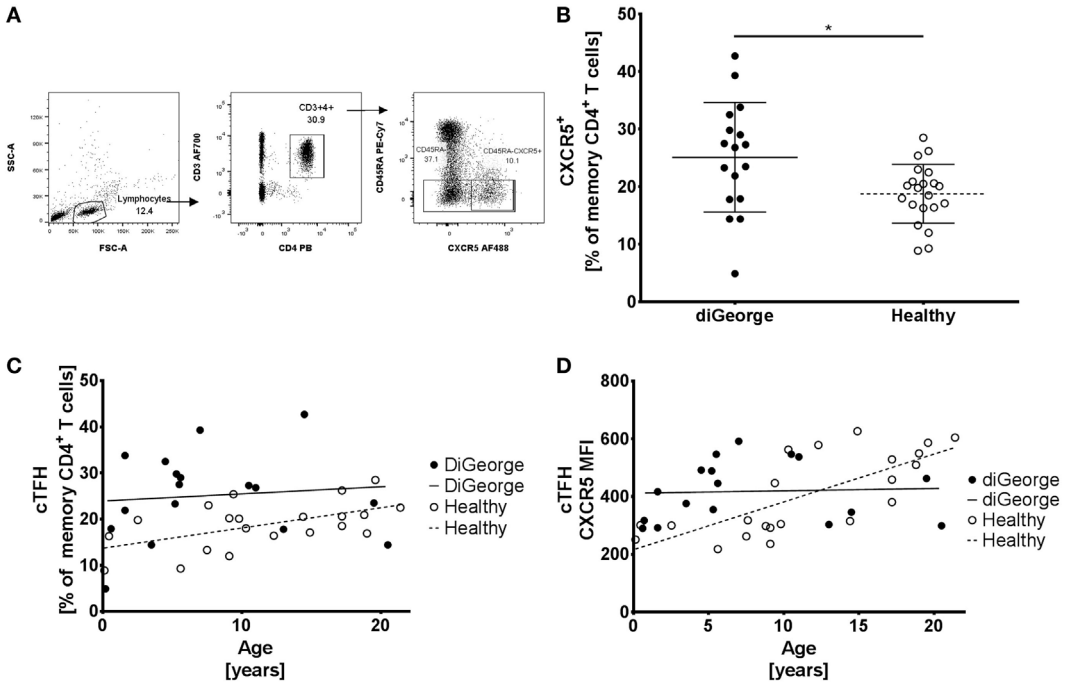

FIGURE 2 | Gating strategy, circulating follicular helper T cells (cTFHs), and CXCR5 expression **(A)** Gating strategy of CD45RA$^-$ (memory) CD4$^+$ T cells and CD45RA$^-$CXCR5$^+$ (cTFH) CD4$^+$ T cells. **(B)** Proportion of cTFH in memory CD4$^+$ T cells in DiGeorge syndrome patients and healthy controls shown in summary (lines denote mean ± SD) and **(C)** as development over time with linear regression trendlines. **(D)** Expression of CXCR5 on cTFHs over time (lines denote linear regression trendlines).

proportion over time in DiGeorge patients (linear regression, $p = 0.69$, $R^2 = 0.01$) (**Figure 2C**). This trend is further corroborated by gradual increase of CXCR5 expression on cTFHs in healthy patients (linear regression, $p = 0.0001$, $R^2 = 0.54$) but not DiGeorge patients (linear regression, $p = 0.68$, $R^2 = 0.01$) (**Figure 2D**).

cTFH Are Not Markers of Humoral Immune Dysregulation in DiGeorge Syndrome

In order to evaluate whether cTFH population reflects the humoral immune dysregulation seen in patients with DiGeorge syndrome, we compared it to serum IgG levels, switched memory B cells, thrombocytopenia, and allergy.

2/17 patients (12%) in our cohort suffered from hypogammaglobulinaemia (Patients 1 and 4), but hypergammaglobulinemia is seen in 7/17 patients (41%) (Patients 5, 6, 8, 10, 12, 15, and 17). However, we observed no correlation between the elevated cTFHs seen in **Figure 2B** and serum IgG levels (linear regression, $p = 0.19$, $R^2 = 0.11$) (**Figure 3A**).

Similarly, despite the observed elevated cTFHs, there is a block in B cell maturation with low class-switched memory B cells in DiGeorge syndrome (11, 14). We compared cTFH numbers with switched memory B cells measured as part of previous investigations (0–2 years prior to evaluation of cTFH counts) (14), but saw no correlation (**Figure 3B**) (linear regression, $p = 0.44$, $R^2 = 0.10$).

To investigate the influence of cTFHs on clinical phenotype of patients, we compared cTFH numbers in patients with/without thrombocytopenia (**Figure 3C**)—the most commonly seen autoimmune complication in DiGeorge syndrome—and with/without allergy (**Figure 3D**). There was no difference in cTFHs between these cohorts, suggesting that cTFHs are not good markers of autoimmunity, allergy, or dysgammaglobulinemia in patients with DiGeorge syndrome.

Expression of PD1 and ICOS Is Preserved on cTFHs of DiGeorge Syndrome Patients

The phenotypic and functional identity of cTFHs has been previously characterized using the extended surface expression of important surface molecules, such as the inhibitory checkpoint-molecule PD1 (26) and the costimulatory receptor ICOS (27, 28). We, therefore, evaluated the surface expression of PD1 and ICOS on cTFHs in DiGeorge patients compared to healthy controls, but saw no significant difference (multiple *t*-tests with Benjamini FDR approach, PD1 $p = 0.57$, ICOS $p = 0.22$) (**Figure 4A**).

PD1 and ICOS Expression

Two healthy and three DiGeorge outliers with very high PD1 and ICOS expression on cTFHs can be seen in the summary data (**Figure 4A**). These are very young (<2 years old) patients and controls. Observing this trend, we evaluated the development of PD1 and ICOS expression in DiGeorge patients and healthy controls with age.

We observed a significant decrease of PD1 expression on cTFHs (linear regression, $p = 0.01$, $R^2 = 0.78$) (**Figure 4B**), but not CXCR5$^-$ memory CD4$^+$ T cells (**Figure 4C**) or CD4$^-$ T cells (**Figure 4D**) in the first 5 years of life in patients with DiGeorge syndrome. This trend seems to be similar in healthy controls, but is not significant, possibly due to low number of samples ($n = 3$) and, therefore, low statistical power.

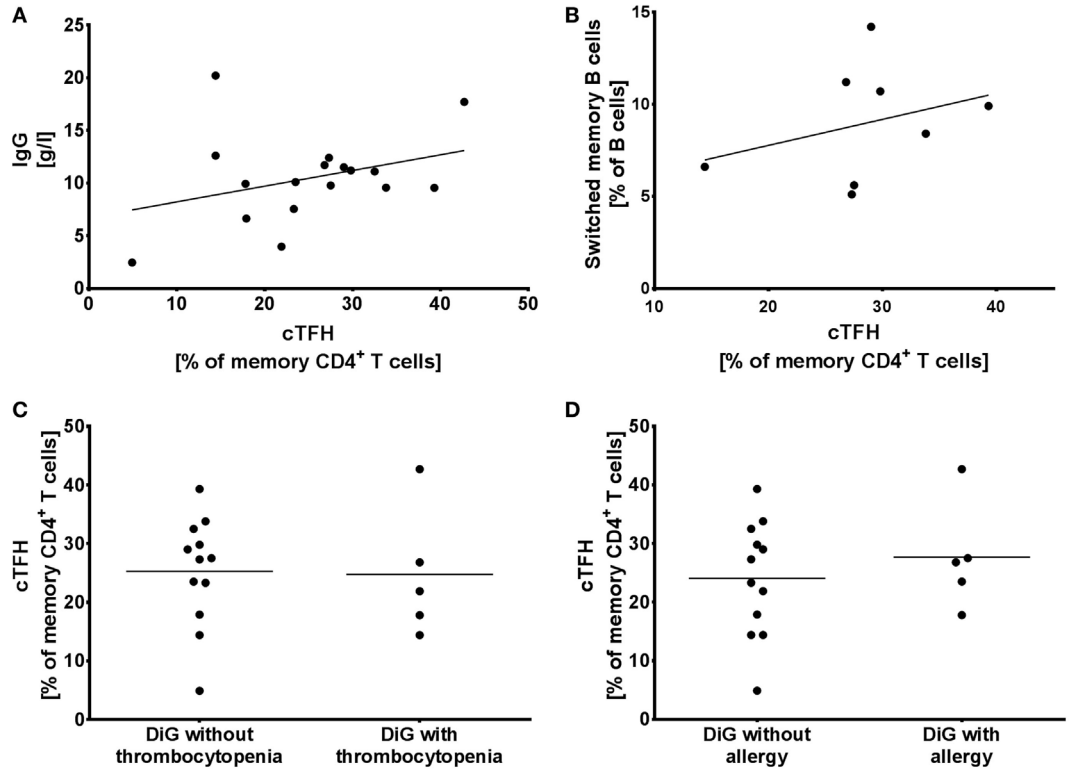

FIGURE 3 | Circulating follicular helper T cells (cTFH) in humoral immune dysregulation, autoimmunity, and allergy. **(A)** Proportion of cTFHs in memory CD4+ T cells compared to serum IgG levels and **(B)** switched memory B cells in DiGeorge syndrome patients, shown with linear regression trendlines. **(C)** cTFHs in DiGeorge syndrome patients without/with thrombocytopenia and **(D)** without/with allergy (lines denote mean).

PD1 expression further decreases later in life on cTFHs (linear regression, $p = 0.04$, $R^2 = 0.37$) (**Figure 4B**), but not CXCR5$^-$ memory CD4$^+$ T cells (**Figure 4C**) and CD4$^-$ T cells (**Figure 4D**) in DiGeorge syndrome. In healthy controls, there is no subsequent decrease in cTFHs, but on the contrary, there is significant increase of PD1 expression later in life on both CXCR5$^-$ memory CD4$^+$ T cells (linear regression, $p = 0.01$, $R^2 = 0.29$) and CD4$^-$ T cells (linear regression, $p = 0.0003$, $R^2 = 0.56$), which is not present in DiGeorge patients.

There are no significant changes in ICOS expression on any measured T cell population of DiGeorge syndrome patients (**Figures 4F–H**). In healthy controls, however, there is a highly significant gradual increase of ICOS expression on CD4$^-$ T cells in children older than 5 years. There seems to be a trend similar to the sharp PD1 decrease in first 5 years of life on cTFHs and CXCR5$^-$ memory CD4$^+$ T cells in healthy controls, but it is not significant.

We verified the observed strong decline of PD1 expression on cTFHs cells during the first 5 years of life on paired samples obtained from two DiGeorge syndrome patients on two consequent visits (**Figure 4E**).

DISCUSSION

We explore in this manuscript the presence and phenotype of TFH cells in the context of thymic pathology in patients with DiGeorge syndrome. As our understanding of the immune system grows more detailed, valuable opportunities present themselves at times to elucidate facets of long-known diseases that were not fully understood when those diseases were first described. One such opportunity was the description of the circulating population of follicular helper-like T-cells, which express the chemokine receptor CXCR5 and show functionally and transcriptionally distinct properties.

The inability of immune system in DiGeorge syndrome to produce naïve T-cells in normal quantities has been recognized for a long time and is believed to result from thymic dysplasia. We corroborate these findings on our cohort of pediatric DiGeorge syndrome patients, showing that there is a relative, but not absolute expansion of mature T-cells.

Circulating follicular helper T cells have already been studied in several primary immunodeficiencies, especially with emphasis on T cell B cell cooperation. For example, patients with hyper-IgM syndrome due to CD40L deficiency had low cTFHs (23, 29). The importance of B cells for cTFH generation was also shown in patients with BTK deficiency, who also had low cTFHs (30). Finally, the influence of cTFH on immune system dysregulation was also shown in CVID patients, who suffer from impaired antibody production and low-switched memory B cells, and in whom, elevated Th1 subset of cTFHs was associated with complicated course of the disease (24).

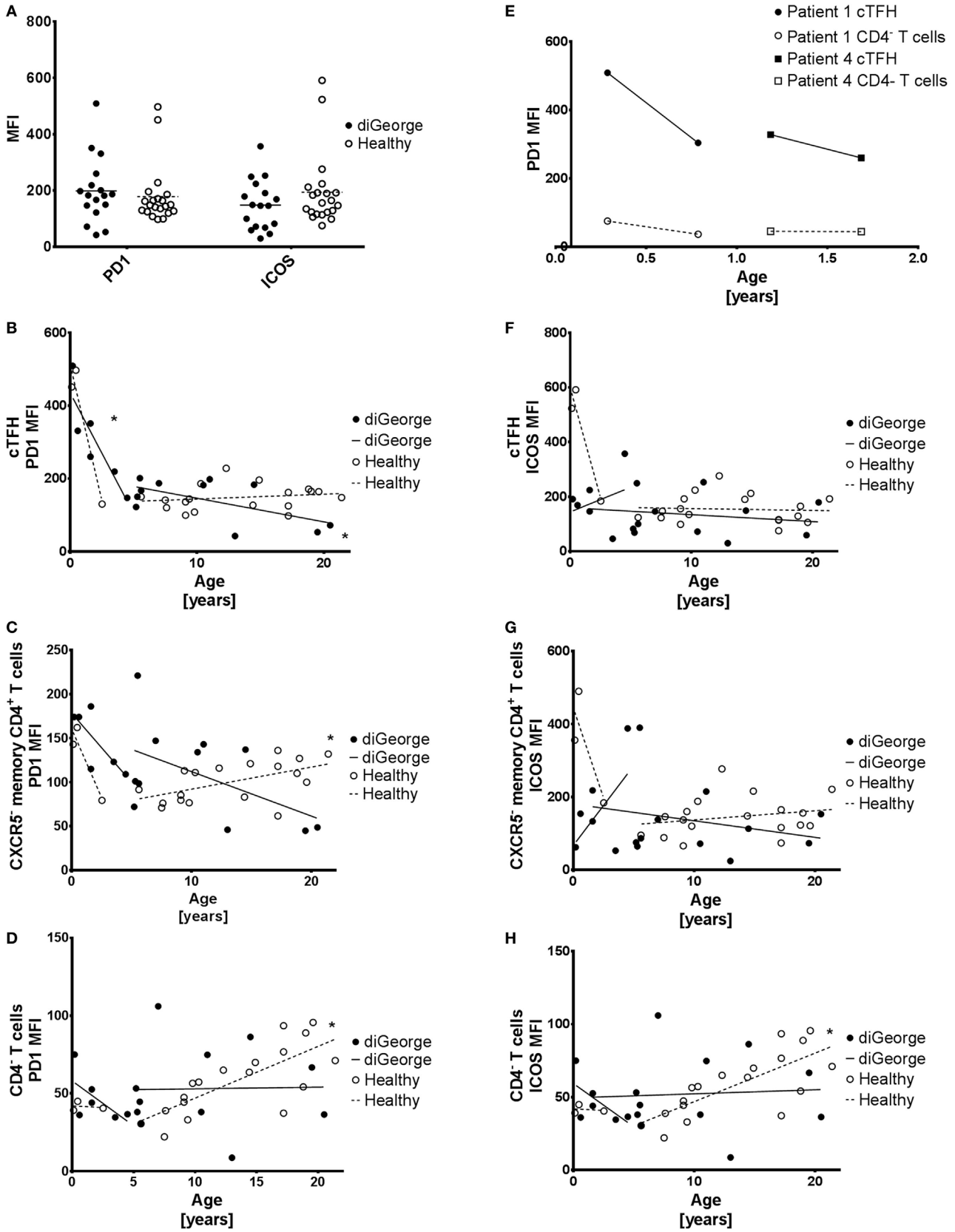

FIGURE 4 | Continued

FIGURE 4 | PD1 and ICOS expression on circulating follicular helper T cells (cTFH), CXCR5$^-$ memory CD4$^+$ T cells, and CD4$^-$ T cells. **(A)** Summary graph showing PD1 and ICOS expression on cTFHs of DiGeorge patients and healthy controls. **(B)** Expression of PD1 on cTFHs, **(C)** CXCR5$^-$ memory CD4$^+$ T cells, and **(D)** CD4$^-$ T cells with linear regression trendlines. * denotes significant correlation. **(E)** Change in PD1 expression over time on paired samples from two patients with DiGeorge syndrome on two consequent visits. **(F)** Expression of ICOS on cTFHs, **(G)** CXCR5$^-$ memory CD4$^+$ T cells, and **(H)** CD4$^-$ T cells with linear regression trendlines. * denotes significant correlation.

However, we show here elevated cTFHs in patients with thymic pathology as part of the DiGeorge syndrome, a finding previously heralded by Derfalvi et al. who also observed elevated bulk and activated cTFHs in DiGeorge syndrome patients (25). Similar trend of elevated cTFH counts was also shown in CVID patients with low-switched memory B-cells (31), and in the context of autoimmune disease such as systemic lupus erythematosus (32), Sjögren's syndrome (33), or rheumatoid arthritis (34). Patients with DiGeorge syndrome also exhibit low class-switched memory B cells as we have shown previously (14); however, in our cohort, we saw no correlation between switched memory B cells and cTFHs, which was also not observed by Derfalvi. While we have not observed any difference in cTFHs between patients with and without history of autoimmune thrombocytopenia, the size of our cohort and lack of patients with other autoimmune complications precludes far-reaching conclusions at this point.

While there have been reports of hypogammaglobulinemia in patients with DiGeorge syndrome (12, 13, 35), which would be expected due to the lack of switched memory B cells, we have shown previously and again in this manuscript that there is a trend toward humoral immune dysregulation and hypergammaglobulinemia in DiGeorge syndrome, especially in adolescents. Although the increased numbers of cTFHs present one possible explanation, we found no correlation between serum IgG levels and cTFH numbers. Such correlation has been shown in the literature for some specific diseases such as IgG4-related disease (36) or rheumatoid arthritis (37), but is otherwise not widely observed, which might reflect the more complex nature of antibody production regulation.

We thus propose that cTFHs are present in patients with DiGeorge syndrome but are dysfunctional in their control and regulation of germinal center response, a hypothesis supported by the observed hypergammaglobulinemia and increased prevalence of autoimmune complications in DiGeorge syndrome and also in our cohort. Thymic dysplasia with loss of central tolerance may lead to production of autoreactive cTFHs resulting in autoimmune complications. Homeostatic proliferation of naïve T cells early in life that has been shown in DiGeorge syndrome (38) may also contribute to the relative expansion of cTFHs, a hypothesis supported by our finding of gradual increase of CXCR5 expression in healthy, but not DiGeorge cTFHs over time, which would indicate early expansion, but impaired long-term maturation of the cTFH compartment.

Finally, we provide data on the changes of PD1 and ICOS expression on various T cell subsets, including cTFH, over time. PD1 is a molecule that has enjoyed a dramatic increase in popularity in recent years, with the advent of checkpoint-blockade treatments in various cancers and with central role in CD8 T-cell exhaustion investigated primarily in chronic viral infections. While much has been documented about the expression of PD1 on cells in adults, there is little to no information available on its expression pattern during childhood. We observed a strongly increased expression of PD1 on cTFHs, and to lesser extent also CXCR5$^-$ memory CD4$^+$ T cells, in infants in both DiGeorge syndrome patients and healthy controls. We then show a much slower and gradual increase of PD1 expression in CXCR5$^-$ memory CD4$^+$ T cells and CD4$^-$ T cells in healthy controls, but not DiGeorge syndrome patients, in older childhood and adolescence.

The trend of gradual PD1 expression increase has also been observed in murine CD4$^+$ T cells (39) and CD8$^+$ T cells (40). High levels of PD1 have also been reported in human neonatal Vdelta2 T cells (41), as well as cord blood Tregs (42). The exact impact of PD1 expression on the function of cTFHs is unclear, however, with both increased (21) and decreased (21) humoral immune response recorded in models with attenuated PD1/PD1L interaction. Interestingly, in their study Derfalvi et al. show increased percentage of CXCR5$^+$CCR7^{lo}PD1^{hi} activated cTFH CD4 T cells in both pediatric and adult DiGeorge syndrome patients. Considering the fact that we did not observe increased PD1 expression on DiGeorge cTFHs compared to controls, this finding by Derfalvi et al. may be attributed to higher proportion of bulk cTFHs in CD4 T cells.

In summary, we present here novel information on cTFHs in patients with thymic pathology and primary immunodeficiency underlying DiGeorge syndrome and provide first data on the change in PD1 and ICOS expression on cTFHs and other T cell subsets during childhood. Our work proposes new challenges for investigation in patients with primary immunodeficiency, which could lead to better understanding of the function of cTFHs, as well as temporal development of PD1 and ICOS expression.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethical Committee of the second Faculty of Medicine, Charles University in Prague and University Hospital in Motol, Czech republic. The protocol was approved by the Ethical Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AK designed the study, performed experiments, analyzed results, and wrote the manuscript. ZP designed the study and performed the experiments. MB and JP assisted with sample acquisition and preparation and provided clinical information. MR performed experiments. SU analyzed the data, interpreted results, and contributed to the discussion. KW contributed to the discussion of data and writing of the manuscript. AŠ interpreted results, assisted with sample acquisition and preparation, and provided clinical information and co-wrote the manuscript. All

authors contributed to manuscript revision, read and approved the submitted version.

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