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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 ($p < 0.01$).