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Activation of thyroid antigen-reactive B cells in recent onset autoimmune thyroid disease patients

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ABSTRACT

Autoimmune thyroid disease (AITD), including Hashimoto's thyroiditis (HT) and Graves' disease (GD), is the most common autoimmune disorder in the United States, affecting over 20 million people. At the time of diagnosis, both HD and GD are characterized by the accumulation of B and T lymphocytes in the thyroid gland and production of autoantibodies targeting the thyroid, indicating that a breach in tolerance of autoreactive lymphocytes has occurred. However, few studies have sought to understand the underlying pathogenesis of AITD that ultimately leads to production of autoantibodies and loss of thyroid function. In this study, we analyzed the phenotype of thyroid antigen-reactive B cells in the peripheral blood of recent onset and long standing AITD patients. We found that in recent onset patients thyroid antigen-reactive B cells in blood no longer appear anergic, rather they express CD86, a marker of activation. This likely reflects activation of these cells leading to their production of autoantibodies. Hence, this study reports the early loss of anergy in thyroid antigen-reactive B cells, an event that contributes to development of AITD.

1. Introduction

While autoimmune thyroid disease (AITD) is rarely life threatening, it is one of the most common autoimmune disorders and is associated with many serious comorbidities. The AITD constellation includes Hashimoto's thyroiditis (HT) and Graves' disease (GD), and common comorbidities include weight gain/loss, heart abnormalities, nervousness/agitation, and increased risk for thyroid cancer [1]. In addition, AITD is commonly seen in patients with other autoimmune disorders, such as type 1 diabetes (T1D), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [2–4]. Secondary AITD is an immune related Adverse Event (irAE) commonly seen following treatment with immune-checkpoint antibodies, such as anti-PD-1/PD-L1 and anti-CTLA4 [5–7]. In addition, up to 40% of multiple sclerosis patients treated with alemtuzumab (anti-CD52), which depletes both B and T cells, develop secondary AITD. The majority of these patients develop GD [8,9]. Hence, studies aimed at understanding the underlying pathogenesis of AITD are needed.

Both HT and GD are characterized by the production of autoantibodies directed against the thyroid that either lead to hypothyroidism in HT or hyperthyroidism in GD. Autoantibodies against thyroglobulin (Tg) and thyroid peroxidase (TPO) are common in the serum of both HT and GD patients, while autoantibodies against the thyroid-stimulating hormone receptor (TSH-R) are more characteristic in GD patients. Mouse models of HT and GD have demonstrated an important role for B cells in the pathogenesis of disease [10–13]. In humans and mice, B cells are thought to not only produce autoantibodies, but also act as antigen presenting cells to T cells. Analysis of the thyroid gland in AITD patients has revealed the presence of B cells in and around the thyroid, forming lymphoid follicles, which are not present in healthy controls [14,15]. While we know B cells are essential in the pathogenesis of AITD, nothing is known regarding changes in the status of thyroid antigen reactive B cells during disease development.

Development of a B cell mediated autoimmunity, such as AITD, requires a breach in tolerance that allows self-reactive B cells to become activated and participate in disease. In healthy individuals,

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up to 70% of B cells produced in the bone marrow bind to self-antigens. However, most autoreactive B cells appear to be eliminated before reaching the periphery as indicated by the fact that autoreactive B cell frequency in the peripheral lymphoid compartments is ~20% [16]. In the bone marrow, B cells that bind to self with a high avidity undergo a process of receptor editing, in which they rearrange their B cell antigen receptor (BCR) immunoglobulin light chains until self-reactivity is eliminated. If editing fails to eliminate self-reactivity, these cells can undergo apoptosis [17]. B cells that have moderate avidity for self-antigen can escape these tolerance checkpoints in the bone marrow and enter the periphery where chronic antigen binding induces anergy. Anergic B cells are by definition unresponsive to antigenic stimulation and are characterized by down regulation of surface IgM and an inability to become activated and produce antibodies [18–20]. Though most knowledge of B cell anergy has come from studies in mice, an anergic B cell population has been identified in the peripheral blood of healthy individuals [21]. These cells bear markers consistent with mature naïve ($CD19^{+}$ $CD27^{-}$) phenotype while expressing low surface IgM expression but normal levels of IgD. They are termed B_{ND} ("B cells that are naïve IgD+").

Previously, we analyzed the frequency of anergic insulin-binding B cells in the peripheral blood of subjects along a continuum of type 1 diabetes development, using a method we devised to identify and enrich insulin binding B cells (IBC) using magnetic particles [22,23]. We found that in autoantibody positive and recent onset T1D subjects anergic IBC and total anergic B cells occur at reduced frequency in blood. Interestingly, we found in a portion of first-degree relatives the frequency of anergic B cells is also very low, suggesting that loss of B cell anergy precedes development of autoantibodies and could be a biomarker of increased risk for development of T1D [22]. Another group reported that SLE patients have decreased numbers of anergic anti-nuclear B cells in peripheral blood compared to healthy controls [24]. Based on these studies, we hypothesized loss of anergic B cells, reflecting their activation, could be an early event in other autoimmune disorders, such as AITD, and therefore, could be utilized as a biomarker for individuals at risk of developing primary or secondary AITD. Using our method to enrich antigen-specific B cells, we analyzed the phenotype of Tg and TPO-specific B cells in the peripheral blood of recent onset and long-standing AITD subjects compared to healthy controls. We found that recent onset AITD subjects, but not long standing AITD subjects, have reduced numbers of anergic Tg/TPO-specific B cells in blood, and this is correlated with increased autoantibody titers. In addition, Tg-specific B cells show increased expression of CD86, suggesting activation and increased capacity to function in antigen-presentation to thyroid antigen-reactive T cells. Hence, this is the first study to identify changes in the phenotype of thyroid antigen-reactive B cells in the peripheral blood during AITD development.

2. Material and methods

2.1. Subjects and peripheral blood processing

Samples were obtained with informed consent at the University of Colorado Anschutz Medical Center and the Barbara Davis Center for Childhood Diabetes using protocols approved by the University of Colorado Institutional Review Board. Eligible subjects were male or female, who had been diagnosed with either Graves' disease (GD) or Hashimoto's thyroiditis (HT) within 6 months for early-onset and more than one year ago for long-standing. Presence of antibodies against Tg, TPO, and TSH-R, as well as TSH, Free T4, and Total T3 tests were used to confirm a diagnosis of GD or HT. PBMCs from 12 early-onsets, 10 long-standing, and 19 healthy age/sex matched controls were isolated from heparinized blood by Ficoll-Hypaque fractionation.

2.2. Enrichment of antigen-specific B cells and flow cytometry analysis

In order to maintain consistency of gating, enrichment of antigen-reactive B cells, and changes in day to day flow cytometer settings, each day patient samples were analyzed in parallel with age/sex matched healthy controls. A minimum of 30 million PBMCs were used to enrich for Tg and/or TPO-specific B cells. Blood from all AITD subjects was divided in half and enriched for Tg or TPO-specific B cells, unless an insufficient amount of blood was collected and then only one antigen was used. Native human Tg (AbD Serotec) and recombinant human TPO (Kronus) were biotinylated according to manufacturer's instructions (Thermo Scientific EZ-Link Sulfo-NHS-Biotin).

PBMCs were stained in PBS/1%BSA/0.02%NaAzide with human FcR Blocking Reagent (Miltenyi Biotec), 0.1 μg/10^6 cells Tg-biotin or TPO-biotin or TT-biotin, and mouse monoclonal anti-human antibodies against CD19-BV511 (BioLegend), CD27-BUV395 (BioLegend), IgM-PE (Southern Biotech), IgD-FITC (BD Biosciences), CD38-BV421 (BioLegend), and CD86-BV711 (BioLegend) for 20 min at 4 °C. Cells were then fixed with 2% formaldehyde at 4 °C, followed by incubation with streptavidin-AlexaFlour647 for 20 min at 4 °C. Cells were washed, suspended in MACS buffer (PBS/0.5%BSA/2mMEDTA), and incubated with 1 μL anti-Cy5/Anti-Alexa647 magnetic microbeads (Miltenyi)/10^6 cells for 15 min at 4 °C. Samples were then passed over magnetized LS columns (Miltenyi), which were washed 3 times with 2 mL of MACS buffer, and bound cells were eluted with 6 mL of MACS buffer after removal from the magnet. Flow cytometry was performed on LSR Fortessa ×20 (BD) and data analyzed with FlowJo software ver. 9.9.4. Gates for B_{ND} cells were drawn based on $CD19^{-}$ T cells, which are negative for IgM and IgD.

2.3. Statistics

Data were analyzed using Prism software (GraphPad Software, Inc.). Paired Student's *t* tests were used to determine significance of differences among patient groups. Spearman's Correlation tests were used to determine correlation between two data sets.

3. Results and discussion

3.1. AITD subjects

For this study we recruited AITD patients (10 GD and 2 HT) that were early onset (E/O) based on diagnosis within the previous 6 months. Only subjects that had not begun treatment or had only had minimal treatment with thyroid replacement or antithyroid drugs were enrolled (Table 1), since we suspected treatment could alter the phenotype of their peripheral blood lymphocytes. Initially we sought to recruit an equal number of HT and GD patients. However, this was made extremely difficult by the fact the treatment of HT patients is typically begun prior to entering specialist care. Therefore the majority of HT patients coming to our center did not meet eligibility requirements. Hence, while we present results for both GD and HT patients, our conclusions are most applicable to the GD patients, given the larger subject number, but may also be consistent with the early pathogenesis of HT. From our experience, both disorders likely follow a similar early pathogenesis. For instance, both disorders can display an initial lymphocytic infiltrate into the thyroid gland and/or production of anti-TPO and Tg antibodies. We believe if the inflammation progresses, it can result in

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