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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 HT 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.

Keywords

B lymphocytes; autoimmune thyroid disease; anergy; flow cytometry

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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, 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 $\mu\text{g}/10^6$ 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 minutes at 4°C. Cells were then fixed with 2% formaldehyde at 4°C, followed by incubation with streptavidin-AlexaFlour647 for 20 minutes 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 minutes at 4°C. Samples were then passed over magnetized LS columns (Miltenyi), which were washed 3 times with 2mL 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 X20 (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 hypothyroidism, or if there is still enough functioning thyroid tissue, can result in the production of anti-TSH-R antibodies, leading to hyperthyroidism. Hence, while the clinical manifestation for GD and HT varies, it seems reasonable the initial trigger(s) (e.g. loss of tolerance of thyroid-reactive B cells and T cells) may be shared between the two, with only production of anti-TSH-R antibodies changing the course of disease.

TSH, T4, T3, and antibody titers for Tg, TPO, and TSH-R were assayed for each patient within 4 weeks of their blood draw (Table 1). We also recruited 10 long-standing (L/S) AITD patients (5 GD and 5 HT) that had been diagnosed more than a year earlier, and 19

closely age/sex matched healthy controls. For each AITD subject that was recruited on a given day, we recruited a healthy control so that they could be analyzed simultaneously. Hence, for all analyses a paired Student's *t* test was used. In addition, whenever possible we enriched and analyzed both Tg and TPO-specific B cells from each subject.

3.2 Enrichment for thyroid antigen-specific B cells is specific

Since antigen-specific B cells normally occur at a very low frequency (<0.1%) in the peripheral blood, we utilized our previously published method to enrich thyroid antigen-specific B cells in recent onset AITD, long standing AITD, and healthy controls [23] enabling more refined analysis of phenotype. As depicted in figure 1A, biotinylated Tg or TPO was added to the cells along with antibodies against other cell surface markers to delineate various B cell subpopulations, followed by streptavidin Alexa647. Anti-Tg/TPO B cells were then enriched using anti-Alexa647 magnetic beads followed by analysis by flow cytometry. In a typical PBMC sample, around 0.8% of total B cells bound Tg and 0.4% bound TPO (Figure 1B). Enrichment of these cells yielded a ~20–150-fold enrichment of antigen-binding cells.

In order to verify that enriched cells were reactive with Tg and TPO antigens, and not some other component of the adsorbent, such as streptavidin Alexa647, we tested the effect of omission of the thyroid antigens from the enrichment scheme (Figure 1B). An insignificant number of cells was enriched under these conditions supporting the thyroid antigen specificity of antigen binding. In addition, no antigen binding cells were found among CD19[−] cells, which are primarily comprised of T cells (Figure 1B). Finally, addition of ~50 fold molar excess of unlabeled Tg/TPO before addition of the biotinylated antigen blocked binding by ~90% (data not shown), further confirming specificity of antigen binding.

3.3 Total Tg and TPO-binding B cell frequency is increased in blood of AITD subjects

Comparative analysis of total Tg and TPO-binding B cells in peripheral blood of early-onset AITD, long-standing AITD, and healthy controls, revealed no statistically significant differences in frequency, whether we compared AITD patients as a group or by disease classification (HT or GD) or duration of time since diagnosis (E/O or L/S) (Figure 1C). However, while not statistically significant, the frequency of Tg and TPO-reactive B cells per 10⁷ PBMCs is increased in the AITD patients as a group, independent of their classification or time since diagnosis [Tg: Mean (per 10⁷ PBMCs) (95% CI) 3,565 (349.7, 6779); TPO: Mean (per 10⁷ PBMCs) (95% CI) 21,817 (9,061, 34,573)] compared to healthy controls [Tg: Mean (per 10⁷ PBMCs) (95% CI) 1,814 (228.5, 3,400); TPO: Mean (per 10⁷ PBMCs) (95% CI) 14,221 (6,625, 21,814)].

3.4 Loss of anergic Tg/TPO-reactive B cells from peripheral blood in early onset AITD subjects

In order to analyze the distribution of Tg/TPO-specific B cells in various B cell subpopulations, we enriched antigen binding cells as in figure 1 and applied the gating strategy shown in figure 2A. As depicted, we were able to determine the proportion of antigen-binding cells that reside in the naïve, memory, and plasmablast subpopulations, as well as the previously described anergic (B_{ND}) and double negative (DN) B cell

subpopulations [21, 25, 26]. As mentioned previously, B_{ND} cells are characterized as mature naïve (CD19⁺ CD27⁻) but have greatly reduced surface IgM while retaining normal levels of IgD [21]. Previous studies have shown that these cells are autoreactive, but refractory to stimulation through their BCR based on decreased intracellular calcium flux and phosphorylation of BCR signaling pathway intermediaries, such as Syk, PLC γ 2, and Akt (data not shown) [21, 22, 27]. The double negative (DN) B cells are CD27 and IgD negative, but despite lack of expression of the CD27 memory B cell marker are class-switched memory B cells [26]. It is thought that these cells arise outside the germinal center or are a distinct B cell lineage that never acquires the CD27 marker [26, 28]. These DN cells have been found to be increased in SLE and RA patients [26, 29].

Interestingly, analysis of the frequency of Tg and TPO-reactive B cells in the naïve, DN, memory, and plasmablast populations in blood of AITD subjects and healthy controls revealed no significant differences between groups (data not shown). This is similar to observations in studies of insulin-binding cells in type 1 diabetics. In that study we found based on analysis of affinity of recombinant BCR cloned from single insulin binding B cells that most antigen-binding cells enriched by this method have such a low affinity that they are ignorant of ambient antigen. We suspect the same is true in the case of enriched thyroid antigen-binding B cells, especially since Tg and insulin are present in the blood at similar concentrations, ~50 pmol/L [30, 31] and TPO is not thought to circulate in the blood at detectable levels. Hence, cells with low affinity for Tg or TPO should be ignorant and thus occur in all major B cell subpopulations depending on their antigen experience with higher affinity crossreactive antigens.

The frequency of Tg and TPO-reactive B cells that reside in the B_{ND} compartment was significantly decreased in early-onset AITD subjects compared to long-standing AITD and healthy controls (Figure 2B). These results suggest a breach in tolerance occurs early in AITD and is transient, since in patients diagnosed for more than one year prior to analysis the anergic B cell population returns to near normal levels. The transient nature of this loss suggests that an environmental trigger may initiate a breach in tolerance of thyroid antigen-reactive B cells.

When we analyzed the frequency of total (Tg/TPO⁻) anergic B_{ND} cells, we found a significant decrease in the early onset AITD subjects compared to the long-standing and healthy controls (Figure 2C). Given that the transient loss of anergic thyroid antigen-reactive B cells is likely due to an environmental factor, one would expect this event to affect the anergy of autoreactive B cells regardless of their autoantigen specificity. Hence, this finding suggests loss of anergic B cells is a general phenomenon that occurs early in disease and may even precede it, as is the case in T1D. It has been reported that a general feature of B_{ND} cells is polyreactivity [21]. Thus it is possible that loss of all B_{ND} cells reflects the fact that all of these cells are significantly reactive with Tg/TPO and become activated due to interaction with thyroid antigens during development of AITD.

3.5 Frequency of anergic Tg-reactive B cells is inversely correlated with autoantibody titers

Early diagnosis of AITD depends in part on the presence of autoantibodies targeting thyroid antigens. Thus it presupposes that a breach in B cell tolerance must occur prior to production

of autoantibodies. Hence, we suspected loss of B cell anergy in early onset AITD subjects might correlate with appearance of autoantibodies in blood. In figure 3, the frequency of anergic Tg/TPO binding B cells is plotted against the subject's respective Tg/TPO antibody titer levels. We found a decreased frequency of anergic B cells correlated with appearance of Tg antibody (Tg correlation coefficient -0.756) and to a much lesser extent with TPO (correlation coefficient -0.018). The inverse correlation was significant for Tg ($p = 0.015$).

3.6 Tg-reactive B cells show evidence of activation in early onset AITD subjects

The inverse correlation between loss of anergic Tg, and to a lesser extent TPO, reactive B cells and appearance of autoantibodies suggests activation and differentiation of these cells to become plasmablasts and plasma cells. Hence, we next wanted to investigate whether the Tg/TPO-reactive B cells showed evidence of activation in AITD patients by analyzing the expression level of CD86, a marker for B cell activation. Interestingly, the expression of CD86 on Tg-reactive B cells in early onset AITD subjects was significantly increased compared to healthy controls (Figure 3B). This finding was somewhat surprising, given that based on our T1D study we hypothesized the majority of the Tg-reactive B cells we enrich have such a low affinity for their antigen they are virtually ignorant. When we analyzed the expression of CD86 in the various Tg-binding B cell subpopulations, we found that in early onset AITD subjects CD86 was significantly increased on all major subpopulations, including the few remaining B_{ND}, naïve, and memory B cells, compared to healthy controls (Figure 3D). However, the fold increase in CD86 expression was highest in the B_{ND} population, which we know have high antigen affinity. Studies in mice and humans have shown that under certain circumstances anergic B cells can upregulate CD86 *in vitro* in response to anti-BCR stimulation [27, 32] or IL-4/CD40L [19, 21, 33, 34], likely reflecting subversion of anergy. These findings also suggest Tg-reactive B cells in AITD subjects may have an increased capacity to become activated and present antigen to Tg-reactive T cells, since CD86 is also a co-stimulatory molecule required for T cell activation.

The above findings were only found in the Tg-reactive B cell population in the early onsets. When we analyzed CD86 expression in the TPO-binding B cells (Figure 3C) or the non-Tg/TPO binding B cells (data not shown), or analyzed long standing AITD subjects (Figure 3B/C), no statistical differences were found. These results are consistent with our finding that loss of anergic B cells was found to be correlated with autoantibody production in the Tg but not TPO-binding cells. Moreover, given that Tg circulates in blood, while TPO does not, activation of Tg-reactive B cells may be one of the earliest events in the pathogenesis of AITD. Stratifying the AITD subjects based on their subclass (HT or GD) did not make a difference, and therefore, they were grouped together for the above analyses.

4. CONCLUSIONS

Recently diagnosed AITD subjects were found to have a significant decrease in the frequency of anergic Tg and TPO-reactive B cells in peripheral blood, as well as total anergic B cells when compared to long standing AITD subjects and healthy controls. This finding suggests that anergy of B cells reactive with these autoantigens is lost early and during disease development and may not be limited to B cells reactive with the autoantigen

targets of the disease. This finding is most consistent with an activation by an environmental trigger, such as exposure to excessive amounts of iodine, especially since both the environment and genetics are important contributors to development of AITD. In addition, recent GWAS studies have identified several risk alleles associated with development of AITD, some of which could be helping drive loss of B cell tolerance. A polymorphism in the *PTPN22* gene, which encodes the lymphoid tyrosine phosphatase (Lyp) protein, is highly associated with AITD [35, 36], and Lyp has been shown to be a negative regulator of B cell signaling [37]. In addition, individuals who carry this polymorphism have increased numbers of autoreactive B cells in their periphery [38]. Hence, we suspect a combination of susceptibility genes and the environment are involved in driving loss of B cell anergy. Studies underway in our lab seek to better understand the contribution of genetic risk alleles to loss of anergy.

Frequency of anergic B cells is inversely correlated with autoantibody levels, particularly anti-Tg antibodies, demonstrating a break in B cell anergy likely precedes differentiation into autoantibody producing cells, something known to occur in mice but has not been shown in humans [39]. In addition, Tg-reactive B cells in AITD subjects expressed higher levels of the activation marker CD86 than healthy controls, suggesting early activation of these cells and competence to act as antigen-presenting cells to Tg-reactive T cells. Although all Tg-binding B cell populations showed increased CD86 expression compared to healthy controls, the increase in CD86 expression was highest in the few remaining B_{ND} cells in the early onset AITD subjects. Historically anergic B cells in both mice and human have been shown to be negative for CD86 expression, but express this activation marker *in vitro* when anergy is broken by stimulation with potent anti-BCR antibodies or IL-4/CD40L [19, 21, 27, 32]. Further, Chang et al. found that an equivalent population to B_{ND} cells, presumable in the process of losing anergy, has increased expression of CD86 *ex vivo* in SLE patients but not healthy controls [40]. Hence, we hypothesize that the few Tg-reactive B_{ND} cells left in the peripheral blood of early onset AITD subjects may have recently received strong interaction with a cross-reactive exogenous stimulus or multimerized Tg, possibly in the form of immune complexes, leading to CD86 upregulation. Given that loss of anergic Tg-reactive B cells inversely correlated with Tg antibody titers, this seems plausible. Alternatively, these cells may be recently activated mature naïve Tg-reactive B cells that upon seeing their antigen have internalized their BCR, with reduced mIgM emulating the phenotype of B_{ND} cells.

Dogma suggests that autoreactive B cells become anergic in the periphery due to chronic antigen binding in the absence of second signals provided by “helper” T cells (signal 1 without signal 2). Hence, properly timed provision of T cell help may prevent onset of anergy. The gene conferring the highest susceptibility for AITD is in the *HLA* locus. It has been shown that the HLA class II genes DR3 and DR4 confer the highest risk [36] and in our study 18/22 AITD subjects carried one or both of these genes (supplemental figure 1). It is hypothesized that these class II molecules are structurally permissive for binding of Tg and TSH-R peptides that lead to cognate interaction with T cells, giving rise to loss of anergy and development of AITD [41]. Hence, it is possible that loss of B cell anergy in early onset AITD patients enables B cells to present antigen and receive cognate T cell help. We suspect other factors are in play, such as the environment and other genetic risk alleles

that allow escape from tolerance of thyroid-specific B and T cells, setting the stage for cognate interactions.

This is the first study comparing the status of Tg and TPO-reactive B cells in the peripheral blood of early onset AITD, long standing AITD, and healthy controls. Loss of B cell anergy was found in the early onset subjects, and may precede onset of disease, as suggested by loss of anergic B cells in first-degree relatives of T1D patients [22]. Hence, loss of B cell anergy could be an early indicator of increased risk of developing primary or secondary AITD. Studies in our lab are focused at addressing this possibility in high risk first-degree relatives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Anergic thyroid-reactive B cells are lost in early onset AITD patients
- Loss of anergic B cells is inversely correlated with autoantibody production
- Thyroid-reactive B cells in AITD patients have increased expression of CD86

Figure 2. Early onset AITD subjects have a loss of anergic Tg and TPO-binding B cells in their peripheral blood

A. Representative gating strategy for identification and analysis of Tg/TPO-reactive B cells using Tg binding cells as an example. IgM vs IgD gates are drawn based on staining of T (CD19⁻) cells, which are IgM-IgD⁻. **B.** Percentage of anergic Tg and TPO-reactive B_{ND} cells in early onset (E/O) AITD, long standing (L/S) AITD, and healthy controls (H/C). **C.** Percentage of total (Tg/TPO⁻) anergic B_{ND} cells in each subject group.

Figure 3. Loss of anergic Tg-reactive B cells is correlated with autoantibody production and activation

A. Anti-Tg and anti-TPO titers for each early onset AITD subject is plotted with their respective percentage of anergic Tg/TPO-reactive B_{ND} cells to demonstrate correlation between the two. Anti-Tg titers had a significant inverse correlation with % Tg-binding B_{ND} cells; $p=0.015$, Spearman's correlation test). B. Fold increase of geometric mean fluorescence intensity (gMFI) of CD86 on Tg-binding B cells from AITD E/O and AITD L/S patients over healthy controls. Representative histogram of CD86 expression from Tg-binding B cells from AITD E/O (black line) and H/C (gray line). C. Fold increase of CD86 gMFI on TPO-binding B cells from AITD E/O and AITD L/S patients over healthy controls. D. Fold increase in CD86 gMFI on Tg-reactive B_{ND}, mature naïve (MN) (CD27-), and memory (CD27+) B cell subpopulations from early onset AITD subjects compared to healthy controls. CD86 gMFI on AITD subjects was normalized to healthy controls in order to account for changes in voltage settings that occurred during the course of the sample collections. Statistical significance determined by paired Student's *t* tests.

Table 1

Early onset subject test results

Patient ID	Diagnosis	TPO ab (0–34 IU/mL)	TG ab (0.0–4 IU/mL)	TSHR ab (0.00–1.75 IU/L)	TSH (0.450–4.5 uIU/mL)	Thyroxine/T4 Free (0.82–1.77 ng/dL)	Total T3 (71–180 ng/dL)	% B _{ND} (of TG+)	% B _{ND} (of TPO+)
2838	Graves'	1230 (H)	94 (H)	3.7 (H)	0.02 (L)	1.17	116	2.14	0
2899	Graves'	1202 (H)	2	26.75 (H)	81.84 (H)	1.99 (H)	132	3.37	0
2946	Graves'	3.2	<0.9	13.87 (H)	<0.015 (L)	1.96 (N)	179 (H)	0.409	NA
2983	Graves'	202 (H)	5 (H)	33.35 (H)	0.02 (L)	3.52 (H)	359 (H)	0.291	0.778
3000	Hashimoto	2874 (H)	1150 (H)	<0.90	9.11 (H)	0.92	92	0.425	0.659
3066	Graves'	589 (H)	<1	27.2 (H)	0.03 (L)	5.75 (H)	778 (H)	0.32	1.14
3116	Graves'	143 (H)	0	65.9 (H)	<0.006 (L)	6.56 (H)	627 (H)	4.68	0.97
3190	Graves'	109 (H)	247 (H)	9.23 (H)	0.18 (L)	3.53 (H)	219 (H)	0	2.73
3236	Hashimoto	>13000 (H)	2369 (H)	<0.9	8.28 (H)	1.02	154	0	NA
3305	Graves'	142 (H)	2	24.23 (H)	0.04 (L)	3.46 (H)	330 (H)	0.224	0.513
3327	Graves'	1882 (H)	>2200 (H)	5.88 (H)	<0.015 (L)	1.3	124	NA	2.33
3533	Graves'	88 (H)	1	2.92 (H)	0.03 (L)	1.87 (H)	256 (H)	NA	0