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Follicular B Cells in Thyroids of Mice with Spontaneous Autoimmune Thyroiditis Contribute to Disease Pathogenesis and Are Targets of Anti-CD20 Antibody Therapy

So-Hee Hong* and Helen Braley-Mullen*,†

B cells are required for development of spontaneous autoimmune thyroiditis (SAT) in NOD.H-2h4 mice where they function as important APCs for activation of $CD4^+$ T cells. Depletion of B cells using anti-CD20 effectively inhibits SAT development. The goals of this study were to characterize the B cells that migrate to thyroids in SAT, and to determine whether anti-CD20 effectively targets those B cells in mice with established SAT. The results showed that most thyroid-infiltrating B cells in mice with SAT are follicular (FO) B cells. Expression of CD80, CD86, and CD40 was significantly increased on FO, but not marginal zone, splenic B cells after SAT development. Thyroid-infiltrating and peripheral blood B cells had lower expresion of CD20 and CD24 compared with splenic and lymph node FO B cells. Despite reduced CD20 expression, anti-CD20 depleted most B cells in thyroids of mice with established SAT within 3 d. B cell depletion in thyroids of mice given anti-CD20 was more complete and longer lasting than in spleen and lymph nodes and was comparable to that in blood. Circulation of B cells was required for effective and rapid removal of B cells in thyroids because preventing lymphocyte egress by administration of FTY720 abrogated the effects of anti-CD20 on thyroid B cells. Therefore, the FO subset of B cells preferentially contributes to SAT development and persistence, and anti-CD20 targeting of FO B cells effectively eliminates B cells in the target organ even though thyroid B cells have decreased CD20 expression. ***The Journal of Immunology*****, 2014, 192: 897–905.**

Spontaneous autoimmune thyroiditis (SAT) develops when mice are given NaI in their drinking water (1, 2). The immunopathology of thyroiditis of NOD.H-2h4 mice is similar to that of Hashimoto's thyroiditis with infiltration of the thyroid by $CD4^+$ and $CD8^+$ T cells, B cells, and other mononuclear cells. Both $CD4^+$ T cells and B cells are required for SAT development (2, 3), and levels of mouse thyroglobulin (MTg)–specific autoantibodies generally correlate with SAT severity scores (1). B cells are required for developmennt of most spontaneous autoimmune diseases, including SAT (4, 5). B cell–deficient NOD.H-2h4 mice do not develop SAT (5, 6). Moreover, wild-type NOD.H-2h4 mice depleted of B cells by treatment from birth with anti-IgM are SAT resistant, and when B cell–deficient mice are given B cells as adults, they produce anti-MTg Abs but do not develop SAT (5). B cells likely contribute to SAT by acting as APCs that support development and expansion of pathogenic $CD4^+$ T cells. However, it is not known which specific B cell subset regulates onset and progression of SAT.

Recent studies suggest that marginal zone (MZ) B cells are important for development of autoimmune diseases such as diabetes and systemic lupus erythematosus (SLE) (7–9). NOD mice have increased numbers of MZ B cells compared with non–autoimmune-prone mice, and MZ B cells migrate to pancreatic lymph nodes (LNs) and increase in number when diabetes develops (8, 9). Moeover, preferential depletion of MZ B cells by anti-CD21/35 significantly reduced the incidenece of cyclophosphamide-induced type 1 diabetes in NOD mice (10). In some murine lupus models, MZ B cells expand and enter into the follicular region (7, 11). In contrast, other studies suggest that follicular (FO) B cells are important for development of diabetes in NOD mice because depletion of splenic FO B cells by anti-CD20 prevents or delays diabetes onset, although most MZ B cells are spared (12, 13). Therefore, the role of MZ and FO B cells in the pathogenesis of autoimmune diseases remains elusive.

CD20 is a 35-kDa transmembrane protein expressed on immature B cells beginning at the pre–B cell stage and on all mature B cells (14). It is not expressed on plasma cells. Thus, CD20 is considered a pan–B cell antigenic marker (14, 15). Rituximab is a chimeric mAb against CD20 that is U.S. Food and Drug Administration–approved for treatment of non-Hodgkin's B cell lymphomas (16) and some autoimmune diseases, including rheumatoid arthritis and SLE (17, 18). There has been a growing interest in the use of rituximab for treating autoimmune diseases, because it effectively depletes peripheral B cells and is generally well tolerated (19). Rituximab has been used clinically to treat patients with SLE, Sjögren's syndrome, vasculitis, multiple sclerosis, Grave's disease, idiopathic thrombocytopenia, dermatomyostis, and polymyositis (20, 21). Many patients have extended periods of clinical remission without serum autoantibody reduction (22).

Although rituximab is effective for therapy of autoimmune diseases, many aspects of its mechanism of action, and even the true extent of depletion of B cells in lymphoid tissue and effector sites, are unclear because human studies are generally restricted to as-

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Abbreviations used in this article: dLN, draining lymph node; FO, follicular; LN, lymph node; MFI, mean fluorescence intensity; MTg, mouse thyroglobulin; MZ, marginal zone; SAT, spontaneous autoimmune thyroiditis; SLE, systemic lupus erythematosus; S1P, sphingosine 1-phosphate.

sessment of B cell depletion in peripheral blood, which makes up <2% of peripheral B cells (23). Indeed, recent studies showed that B cells downregulate CD20 when they enter the pancreas during development of diabetes in NOD mice (12), suggesting that anti-CD20 may not be effective for depleting B cells in target organs. Thus, how and when anti-CD20 affects B cells in the target organ and secondary lymphoid organs needs to be answered.

The goal of this study was to determine which B cell subsets are most important for SAT progression, and to determine whether B cells that have migrated to the target organ (the thyroid) can be depleted by anti-CD20 in mice with established SAT. The results demonstrate that FO B cells preferentially migrate to thyroids in mice with SAT. Although thyroid B cells had lower expression of CD20 compared with splenic and LN B cells as reported in the diabetes model (12), anti-CD20 quickly and effectively depleted thyroid-infiltrating FO B cells and ameliorated established SAT. Thyroid-infiltrating B cells are similar to B cells in the blood, both in their phenotypic characteristics and sensitivity to depletion by anti-CD20. Thyroid and peripheral B cells were depleted more quickly and effectively than splenic and draining LN B cells even though they express less CD20 than do splenic and LN B cells. Importantly, depletion of thyroid-infiltrating B cells could be prevented using a sphingosine 1-phosphate (S1P) receptor agonist that inhibits egress of B cells from the target organ. These results provide new and important information regarding the mechanisms by which anti-CD20 can inhibit B cells in a target organ and thus effectively inhibit an established autoimmune disease.

Materials and Methods

Mice

NOD.H-2h4 mice express H-2K^k, I-A^k, and D^b on the NOD background. Mice were bred and maintained in the animal facility at the University of Missouri as previously described (5). Both male and female mice were used in these studies, but all mice in a given experiment were the same sex. All animal protocols were approved by the University of Missouri Animal Care and Use Committee.

B cell depletion

The anti-CD20 Ab 18B12 and isotype control (IgG2a) were provided by Biogen Idec. Seven- to 10-wk-old mice were given 0.08% NaI in the drinking water to facilitate development of SAT (1). Mice were given a single i.p. injection of 250–300 μg anti-CD20 or isotype control 9–10 wk after mice were given NaI water. At this time all mice had SAT, which reaches maximal severity 8 wk after administration of NaI in the drinking water (3). In some experiments, mice were given an additional injection of anti-CD20 as indicated in the figures. The amount of anti-CD20 was the same as that used in our earlier experiments (24). To block egress of lymphocytes, mice with SAT were given the S1P receptor agonist FTY720 (Cayman Chemicals, Ann Arbor, MI) (1 mg/kg daily for 4 d) beginning 4 h before anti-CD20 treatment.

Scoring of SAT severity

After 8–10 wk on NaI water, thyroids were removed and one thyroid lobe from each mouse was fixed in formalin, sectioned, and stained with H&E as previously described (6). Thyroid histopathology scores were based on the percentage of thyroid follicles replaced by infiltrating lymphocytes as previously described in detail (6). Briefly, a score of 0 indicates a normal thyroid or a few inflammatory cells infiltrating the thyroids. A severity score of 1 is defined as an infiltrate of at least 125 inflammatory cells in one or several foci, and a score of 2 represents 10–20 foci of cellular infiltration, each the size of several follicles, with replacement or destruction of up to a fourth of the gland. A score of 3 indicates that a fourth to a half of the thyroid follicles are replaced by infiltrating inflammatory cells, and a score of 4 indicates that more than half of the thyroid follicles are replaced or destroyed by inflammatory cells.

Flow cytometry

Cells were obtained from peripheral blood, spleen, cervical LNs, and thyroids and suspended in 100 μl FACS buffer (PBS, 0.5% BSA, and 0.05% NaN_3). An appropriate amount of each fluorochrome-conjugated Ab was added together with 1 μg 2.4G2 (anti-FcR Ab). After 30 min incubation at 4°C, cells were washed three times with FACS buffer and data were acquired using CyAn (BD Biosciences, San Jose, CA) followed by data analysis with FlowJo (Tree Star, Ashland, OR) or Summit v5.0. All conjugated Abs were obtained from eBioscience (San Diego, CA) or BioLegend (San Diego, CA) as follows: anti-mouse CD19-FITC (MB19-1), B220-allophycocyanin (RA3-6B2), CD24-FITC (30-F1), CD23-FITC (B3B4), CD21-PE-Cy7 (7E9), CD138-allophycocyanin (281-2), CD20-PE (AISB12), GL7-Alexa Fluor 488, CD45-PE or FITC (30F11), CD1d-PE (1B1), IgM-PE (II/41), CD80-PE (16-10A1), and CD86-PE (GL1). The name of the hybridoma clone is indicated in parentheses. Thyroid-infiltrating cells were isolated from single thyroid lobes of individual mice by treating thyroids with Liberase (0.08 U/ml; Roche Diagnostics, Indianapolis, IN) for 45 min at 37°C. Cells were counted and stained as described above. The other thyroid lobe from each mouse was fixed in formalin and stained with H&E to assess SAT severity.

Confocal laser-scanning double immunofluorescence microscopy

Thyroids were snap-frozen in liquid nitrogen, cut into 5-μm sections, air dried, and fixed with cold acetone for 10 min and stored –70°C until used. Slides were incubated with 5% BSA in PBS for 30 min to block nonspecific binding. To detect apoptosis of B220$^+$ B cells, the following Abs were used: rabbit anti-mouse active caspase-3 (1:500; R&D Systems, Minneapolis, MN) and rat anti-mouse B220 (1:700; Caltag Laboratories, Burlingame, CA). B220 was visualized by Alexa Fluor 488–conjugated anti-rat IgG (1:1000; Molecular Probes, Grand Island, NY). Active caspase-3 cells were visualized by Alexa Fluor–568 conjugated anti- rabbit IgG. Confocal images were acquired using a Zeiss LSM510 upright confocal microscope.

Statistical analysis

A two-tailed Student *t* test was used to determine the significance of differences in SAT experiments with two experimental groups. The significance of differences in experiments with three or more experimental groups was determined by the Kruskal–Wallis nonparametric test using GraphPad Prism software (GraphPad Software, La Jolla, CA). A p value <0.05 was considered significant.

Results

Increased expression of costimulatory molecules on FO B cells in mice with SAT

B cell development proceeds in the spleen to give rise to two mature subsets, FO and MZ B cells (25). FO B cells express high levels of IgD and CD23 and lower levels of CD21 and IgM, whereas MZ B cells express high levels of CD21, IgM, and CD1d but do not express CD23 (26) (Fig. 1A). Recent studies suggest that MZ B cells are important in diabetes pathogenesis in NOD mice (8, 9) and in mouse lupus models (7, 11). However, other studies suggest that FO B cells may be more important for development of diabetes since they are the primary subset depleted by anti-CD20Ab (12, 13). FO B cells are the predominant B cell subset in spleens of most strains of mice, including NOD.H-2h4 mice (24, 27) (Fig. 1A), and the ratios of FO and MZ B cell subsets do not differ for naive mice or mice with SAT (data not shown). Because B cells are effective APCs and can induce autoantigen-specific T cell responses by providing costimulatory signals (27), CD80, CD86, CD40, and MHC class II expression levels were compared on each B cell subset from naive mice and mice with SAT. Expression of CD80 and CD86 was increased ~2-fold in the FO B cell subset of mice with SAT (5.76 ± 1.3% versus 10.6 ± 3%) (Fig. 1B, 1C), compared with naive mice. Although expression of these molecules was higher on MZ B cells, they did not differ between naive mice and mice with SAT (Fig. 1B, 1C). More than 90% of MZ and FO B cells express CD40 and MHC class II, and expression of these molecules on splenic B cells of SAT mice was not significantly different from those of naive mice (data not shown). However, FO B cells in

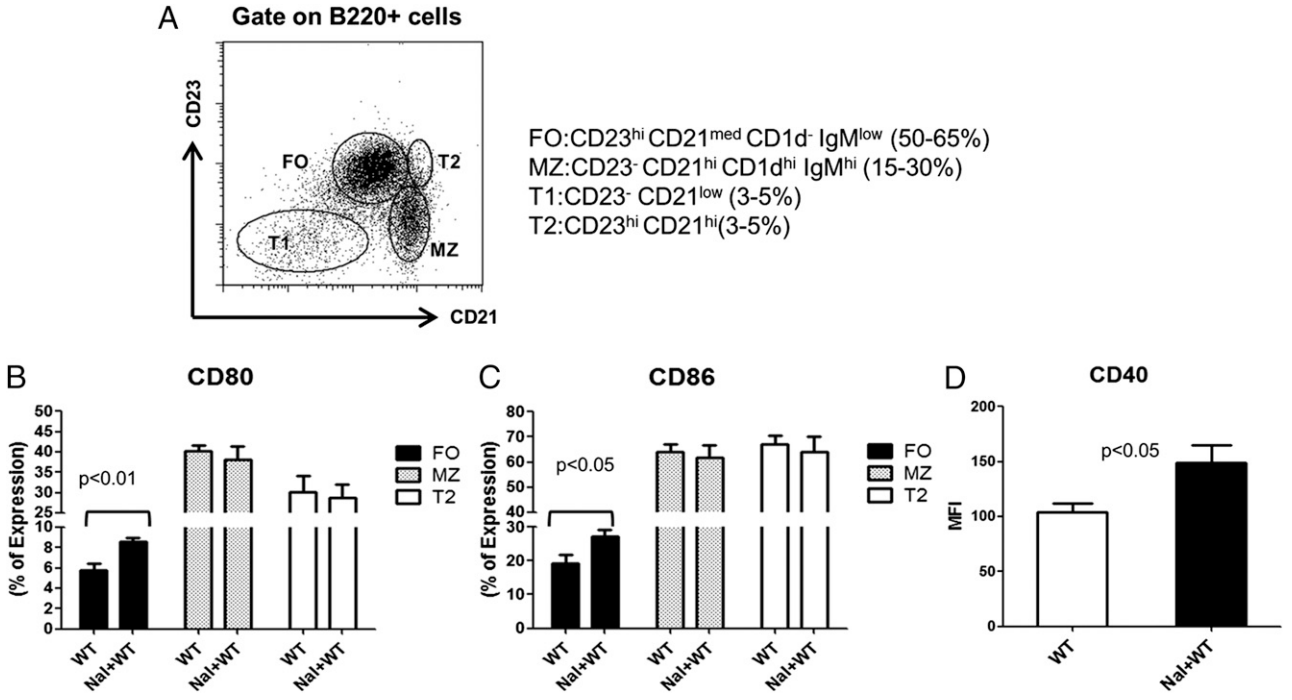

FIGURE 1. Expression of CD40, CD80, and CD86 by B cells of NOD.H-2h4 mice with or without SAT. (**A**) NOD.H-2h4 splenocytes were stained for CD21, CD23, and B220 surface markers. The percentages of cells within T1 ($CD23^{-}CD21^{low}$), T2 ($CD23^{+}CD21^{hi}$), FO ($CD23^{+}CD21^{lo}$), and MZ ($CD23^{-}CD21^{hi}$) gates for a typical spleen are shown. (**B** and **C**) Expression levels of CD80 and CD86 on FO and MZ B cells were determined by flow cytometry. Splenic $B220^{+}$ B cells from naive and mice with SAT (severity scores of 2+) were gated and MZ and FO B cells were identified by CD23 and CD21 expression. Results are means ± SEM of $n = 3$–6/group and are pooled from two experiments. (**D**) MFI of CD40 expression in $B220^{+}$ B cells in cervical LNs of naive mice and mice with SAT score of 2+ was determined by FACS analysis. Results are shown as means ± SEM of $n = 3$–6/group and are pooled from two experiments.

draining LNs (dLNs) of mice with SAT had 1.5-fold increased CD40 mean fluorescence intensity (MFI) levels compared with naive B cells (104 ± 7.7 versus 149 ± 15, Fig. 1D). These results suggest that FO B cells may contribute to SAT onset because their expression of costimulatory molecules increases when SAT develops.

FO B cells preferentially migrate into thyroids of mice with SAT

To determine which subsets of B cells infiltrate thyroids of mice with SAT, thyroid-infiltrating cells were isolated using Liberase as described in *Materials and Methods*. Spleen and LN cells were also incubated with Liberase to ensure that any differences were not simply related to the treatment. The integrity of CD23, CD21, B220, CD1d, and IgM surface molecules was not affected by Liberase (data not shown). Flow cytometric analysis indicated that nearly 80% of thyroid-infiltrating B cells were $CD23^{hi}CD21^{lo}$ FO B cells, and most thyroid B cells were $CD1d^{-}$ and IgM^{lo} (Fig. 2A–C). Confocal microscopy also confirmed that most thyroid-infiltrating B cells are IgM^{lo} and IgD^{hi} (data not shown), which is consistent with the FO phenotype (28). FO B cells were also the predominant B cell subset when thyroids were examined at both earlier (6 wk) and later times (16–18 wk) after mice were given NaI water (Fig. 2D). Therefore, FO B cells are the major thyroid-infiltrating B cell subset in SAT.

It is likely that the thyroid-infiltrating FO B cells are activated and may play a role in promoting activation/differentiation of the $CD4^{+}$ T effector cells because thyroid B cells express relatively high levels of the costimulatory molecule CD80. They also produce TNF-α and IL-6, cytokines known to be important proinflammatory cytokines produced by activated APCs (Supplemental Fig. 1).

Anti-CD20 quickly and effectively depletes thyroid-infiltrating B cells and reduces SAT severity

Our previous results showed that transient B cell depletion by anti-CD20 in NOD.H-2h4 mice for the first 4–6 wk after birth prevents development of SAT in adult mice, and prolonged treatment with anti-CD20 in adult mice inhibits development of SAT and could inhibit SAT and further increases in anti-MTg autoantibodies when administered to mice with mild SAT (24, 29). However, it was not determined whether anti-CD20 could inhibit established SAT or whether it effectively depleted B cells in the target organ.

To address this question, groups of age- and sex-matched NOD.H-2h4 mice were given NaI in their drinking water for 9–10 wk to induce SAT. In each experiment, thyroids were removed from four to six mice at this time and they had SAT severity scores ranging from 1 to 4 (average, 2.2 + 0.27; $n = 19$) (data not shown). The remaining mice were given 250–300 μg anti-CD20 or isotype control, and B cell subsets in peripheral blood, secondary lymphoid organs, and thyroids were analyzed at various times to determine the kinetics and efficacy of B cell depletion (Fig. 3A, 3B). As shown in Fig. 3A and 3C, >80% of B cells in thyroids and peripheral blood were depleted 3 d after injection of anti-CD20, whereas maximum B cell depletion in spleen and dLNs was observed 1–2 wk after injection of anti-CD20 (Fig. 3A). Maximal B cell depletion was similar (> 80%) in dLNs, blood, and thyroids, but it was less complete in spleen where 30–40% of $B220^{+}$ splenic B cells were resistant to depletion (Fig. 3A), as shown previously by ourselves and others (12, 24). Although absolute numbers of both FO and MZ B cells were decreased by anti-CD20, most MZ B cells were resistant to depletion by anti-CD20 (24, 29, and data not shown).

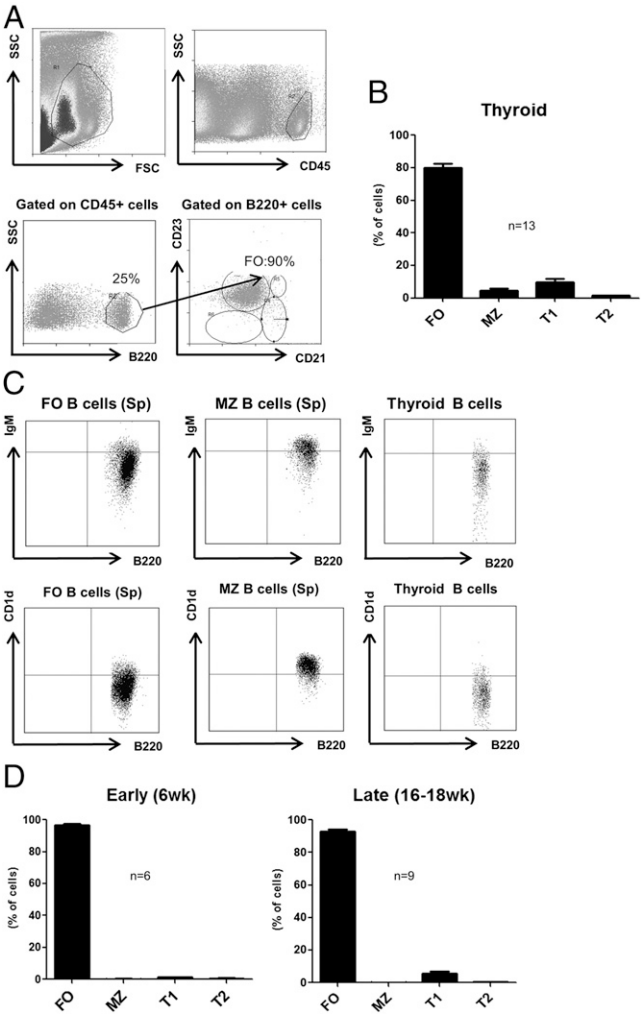

FIGURE 2. Most thyroid-infiltrating B cells are FO B cells. (**A**) Thyroid-infiltrating cells were isolated from single thyroid lobes of mice with SAT by incubating thyroids with Liberase (0.08 U/ml) for 45 min. Cells were stained with CD45, B220, CD21, CD23, and Abs and analyzed by flow cytometry. The gating strategy for a typical sample is shown. (**B**) Bar graph represents mean frequency ± SEM of B cell subsets in the thyroid. Results are from three independent experiments, with a total combined $n = 13$/group. (**C**) Cells were isolated from Liberase-treated spleens and thyroids of mice with SAT. Cells were stained with anti-CD1d, anti-IgM, and B220 Abs and assayed by flow cytometry. Results are representative of five independent samples. (**D**) Thyroid B cells were isolated from NOD.H-2h4 mice given NaI water for 6 wk ($n = 6$) or 16–18 wk ($n = 9$) and stained with CD45, B220, CD21, CD23, and Abs as in (A).

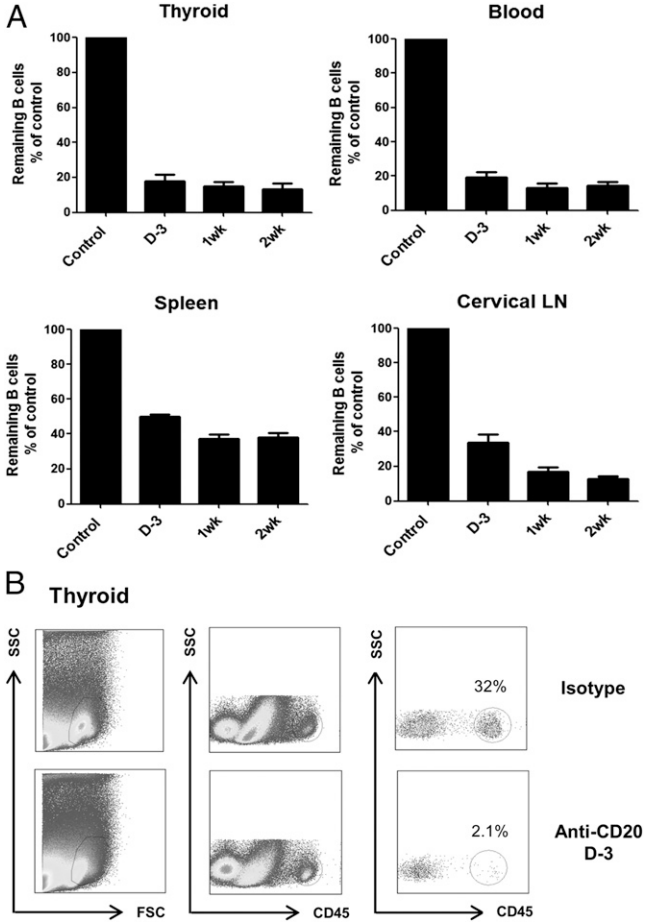

FIGURE 3. Kinetics of B cell reduction in peripheral lymphoid organs, blood, and thyroids in response to anti-CD20. (**A**) NOD.H-2h4 mice were given NaI water at 8 wk of age. Nine weeks later, mice were given IgG2a isotype Ab or 250–300 μg anti-CD20 IgG2a Ab i.p. Thyroids were removed at the indicated times and the percentages of $B220^+$ cells in peripheral blood, LN, spleen, and thyroid were determined by flow cytometry. Data are shown as means ± SEM of $n = 3$–6/group and are pooled from two experiments. (**B**) Representative plot for a mouse given isotype control or anti-CD20 three days earlier.

Histologic comparison of thyroids from isotype control and anti-CD20–treated mice indicated that SAT severity scores were significantly reduced (2.28 ± 0.18 versus 1.28 ± 0.28, $n = 7$, Fig. 4A) from 3 d for at least 3 wk after a single injection of anti-CD20 (Fig. 4). SAT severity scores of isotype control mice did not differ from those of the controls whose thyroids were removed at the time anti-CD20 or isotype control injections were initiated (9 wk control; Fig. 4A, *right panel*, 4C). Although B cells began to repopulate the spleen around 3 wk after anti-CD20 treatment (data not shown), B cells in blood and thyroids remained depleted longer. Seven weeks after anti-CD20 treatment, B cells in spleen and dLNs had repopulated up to nearly 70–80% of the preinjection control, whereas B cells in blood and thyroids were still reduced by 50–60% (Supplemental Fig. 2A). These results indicate that circulating FO B cells in thyroids and peripheral blood are the most effective targets of anti-CD20. Although SAT severity scores were generally lower than those of controls 7 wk after anti-CD20 (Supplemental Fig. 2B), they were increased compared with those seen 1–3 wk after anti-CD20. A longer period of B cell depletion could be maintained by a second injection of anti-CD20 after 3 wk (Supplemental Fig. 2C).

As reported previously (24), B cell depletion reduces both B cells and T cells in the thyroid infiltrates. Although the percentage of $CD4^+$ T cells in thyroids was not decreased by anti-CD20, absolute $CD4^+$ T cell numbers were significantly reduced in thyroids of mice given anti-CD20 (Supplemental Fig. 3A, 3B). Importantly, T cell numbers were not reduced in spleens of anti-CD20–treated mice (Supplemental Fig. 3C).

Thyroid and peripheral blood B cells differ phenotypically from B cells in dLNs and spleen

To begin to identify the mechanisms underlying the rapid and effective B cell depletion in thyroids and peripheral blood, B cells in peripheral blood, lymphoid organs, and thyroids were analyzed to determine whether they differ in expression of particular surface markers. Similar to splenic and LN B cells, thyroid and peripheral blood B cells express high levels of both CD19 and B220 (Fig. 5A

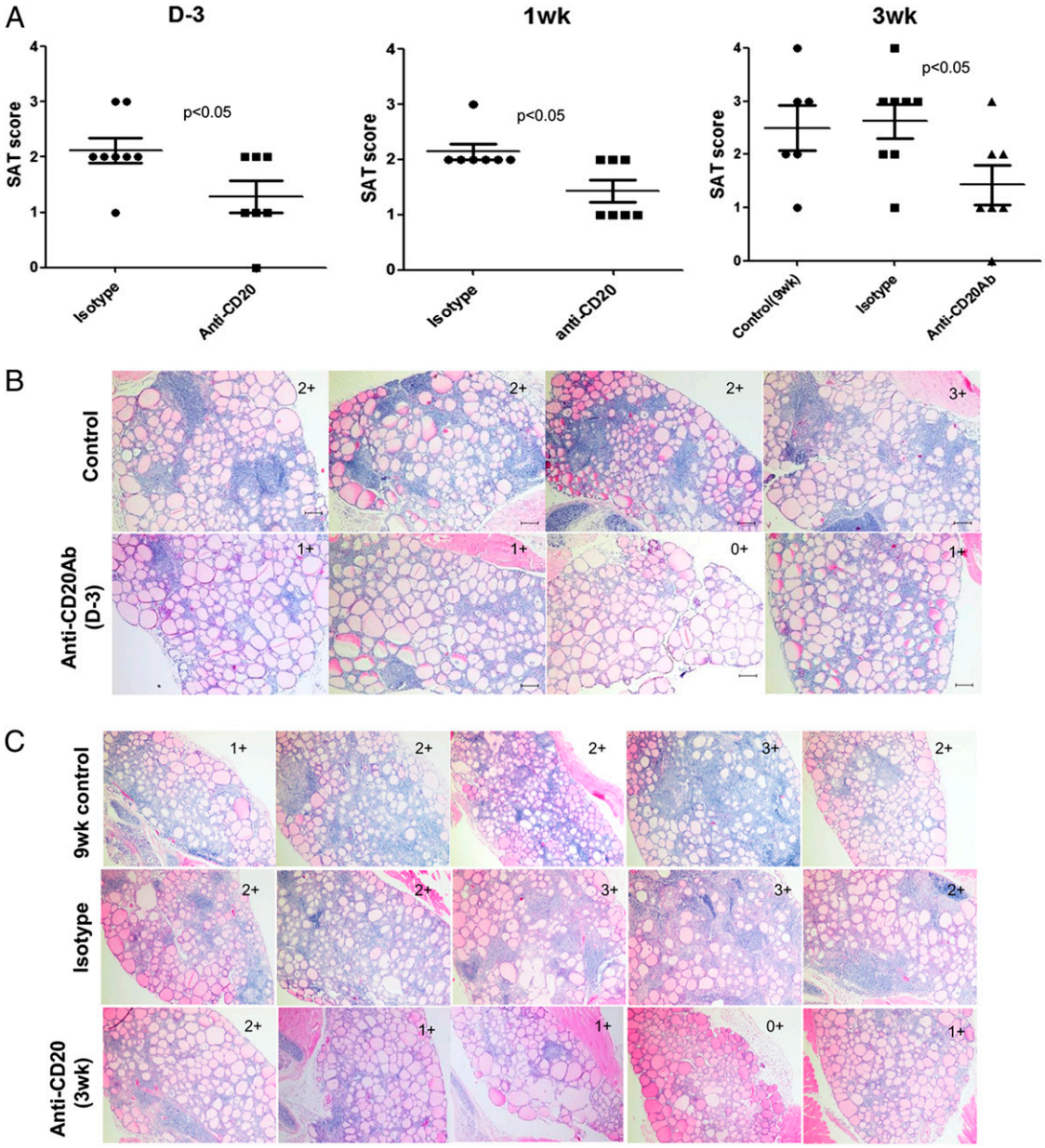

FIGURE 4. Reduction of SAT severity scores after B cell depletion. NOD.H-2h4 mice were given NaI water for 9 wk. Thyroids were removed from six mice to assess SAT severity at 9 wk (9 wk control, *right panel*). The remaining mice were given isotype control or anti-CD20 Ab (250–300 μg) i.p. (**A**) Thyroids were removed from groups of five to seven mice 3, 7, and 21 d after injection of anti-CD20 or isotype control, and SAT severity was graded on a scale of 0–4 as described in *Materials and Methods*. Each symbol represents one mouse. (**B** and **C**) Representative H&E-stained thyroid sections from each group of mice. Original magnification ×100.

and data not shown), but thyroid and blood B cells express lower levels of CD24 compared with splenic or LN B cells (45.8 ± 3.1, 88.4 ± 2.1, 78.6 ± 3, and 36.3 ± 4.27%, respectively; Fig. 5A, 5B). CD24 expression usually declines when B cells are activated and induced to further maturation (30), as well as when they enter the germinal centers in the secondary lymphoid follicles (31). These results suggest that circulating and thyroid-infiltrating B cells could be more mature than most splenic and LN B cells.

Effective depletion of B cells by anti-CD20 generally requires that the cells highly express CD20 (32–34). Unexpectedly, however, a lower percentage of peripheral blood and thyroid-infiltrating B cells expressed CD20 (72.7 ± 3.7% in blood and 56 ± 4.9% in thyroids) compared with splenic and LN B cells (90 ± 2.1 and 90 ± 1.77%, respectively; Fig. 5C), and the intensity of CD20 expression (MFI) was also much lower for thyroid and peripheral blood B cells (Fig. 5D). These results suggest that the effective depletion of thyroid B cells by anti-CD20 does not require that the B cells highly express CD20. Jilani and et al. (35) demostrated that lymphocytes from chronic lymphocytic leukemia patients regain CD20 expression after 24 h in culture. To determine whether thyroid B cells could regain CD20 expression after culture, cells were isolated from thyroids and cultured for 24 h, and CD20 expression on $B220^+$ cells was examined. As shown in Fig. 5E, the population of $CD20^{hi}$ B cells greatly increased after 24 h culture. Although this study did not allow us to determine whether $CD20^-$ thyroid B cells can reacquire CD20 expression in vivo, the results do suggest that CD20 is not lost but is simply downregulated when B cells migrate to the thyroid. When B cells are removed from the thyroid environment, they can re-express CD20 on their surface.

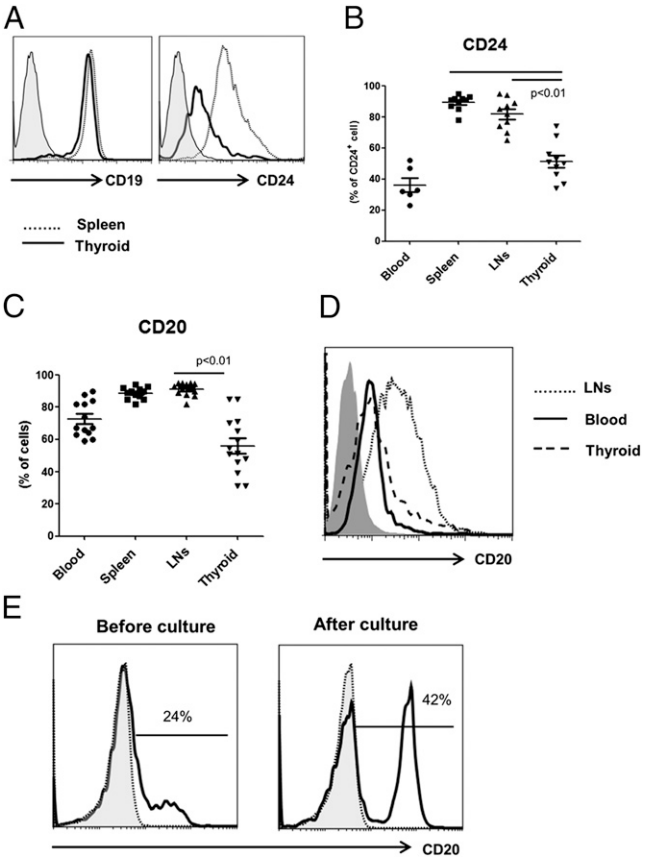

FIGURE 5. Thyroid and peripheral blood B cells have reduced CD20 and CD24 expression compared with splenic or LN B cells. NOD.H-2h4 mice were given NaI water at 8 wk of age. Ten to 12 wk later, CD20 expression on B cells in blood, secondary lymphoid organs, and thyroids was examined by flow cytometry. (**A**) Line graph shows overlay of CD24 and CD19 expression on $B220^+$ B cells. Gray indicates isotype control; black, thyroid B cells; black dot, splenic B cells. Results are representative of six samples. (**B**) Graph represents the mean percentage ± SEM of CD24-expressing cells in $B220^+$ gated cells. Data are from two independent experiments, with a total combined of $n = 6$ or 10 per group. (**C**) Mean percentage ± SEM of CD20-expressing cells in $B220^+$ gated cells in blood, spleen, LNs, and thyroids. Results are from three independent experiments, with a total combined $n = 14$/group. Each symbol represents an individual mouse. (B and C) $p < 0.01$. (**D**) Graph shows overlay of CD20 expression on $B220^+$ B cells. Gray indicates isotype control; long dash, thyroid B cells; black, peripheral blood B cells; dot, cervical LN B cells. Results are representative of 14 individual samples. (**E**) Cells were obtained from thyroids using Liberase and separated into two fractions. One was immediately assayed for expression of CD20 by $B220^+$ cells and the other was cultured for 24 h in RPMI 1640 media containing 10% FBS at 5% CO_2 before being assayed for CD20 expression. Results are representative of five individual samples. Gray indicates isotype control; black, $B220^+CD45^+$ cells.

Because thyroid B cells had reduced expression of CD20 and CD24, it was important to determine whether they were plasma cells. Terminally differentiated plasma cells do not express CD20, whereas most mature B cells and some preplasmablasts and plasmablasts express CD20 (36, 37). Plasma cells express CD138 (syndecan-1) (38), and a recent study in the NOD model of diabetes showed that B cells became $CD20^-$ and expressed CD138 when they infiltrated the pancreas (12, 38) However, very few of the $CD20^-B220^+$ cells in thyroids of mice with SAT express CD138, and most $CD138^+$ cells were $B220^-$ (Fig. 6A). This result was also confirmed by immunohistochemical staining (data not shown).

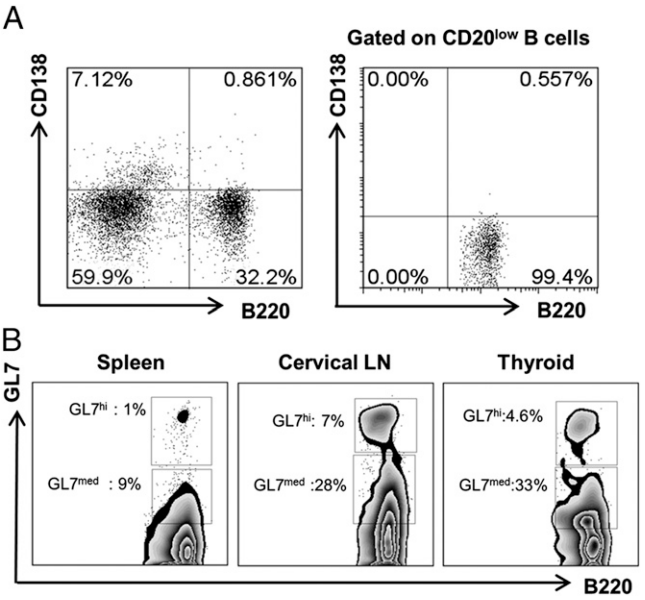

FIGURE 6. Analysis of thyroid B cell phenotype. (**A**) *Left panel*, $CD45^+$ thyroid-infiltrating cells were gated and stained with CD138 (syndecan-1) and B220 Abs. *Right panel*, Thyroid $CD20^-$ B cells were gated and CD138 expression was analyzed by flow cytometry. (**B**) The percentages of GL7-expressing cells among $B220^+$ cells in spleen, cervical LNs, and thyroids of mice with SAT (severity scores of 2–3+). The results are representative of five to six individual thyroids.

Several studies have reported that lymphoid structures resembling secondary lymphoid organs develop in the target organ of autoimmune/chronic inflammatory diseases (24, 39–41), including the thyroid in SAT (23). Consistent with our earlier results (3), thyroid B cells express the germinal center B cell marker GL7. Percentages of GL7 intermediate and high populations in thyroid B cells were higher than those of splenic B cells and comparable to those in dLNs (Fig. 6B).

Lymphocyte circulation is important for reduction of $CD20^{lo}$ B cells in thyroids

The ability of anti-CD20 to effectively reduce B cells in thyroids of mice with SAT, even though thyroid B cells express much less CD20 than splenic or LN B cells, raises a question as to the mechanism of B cell depletion in the target organ. Others have shown that anti-CD20 depletes B cells primarily through Ab-dependent cellular cytotoxicity, complement-dependent lysis, and apoptosis (32–34). All three mechanisms would require expression of CD20 on the B cell surface, suggesting that a different mechanism might be functioning in this model. To determine whether anti-CD20 leads to apoptosis of $CD20^-$ B cells in thyroids, active caspase-3 levels of thyroid B cells were examined by immuoflouresence staining. Very few active caspase-3^+ B cells were detected in thyroids of isotype control or anti-CD20–treated mice (Supplemental Fig. 4), suggesting that it is unlikely that most B cells are undergoing apoptosis and dying in the thyroids.

An alternative possibility is that following administration of anti-CD20, B cells might exit the thyroid and re-enter the circulation. If so, the reduction in thyroid B cells in mice given anti-CD20 could occur in response to the depletion of circulating B cells in the blood. To address this possibility, mice with SAT were injected with the S1P receptor agonist, FTY720, 4 h before and daily following anti-CD20 treatment as described in *Materials and Methods*. B cell

percentages in secondary lymphoid organs, blood, and thyroids were analyzed 5 d after anti-CD20 treatment. Downregulation of S1P receptors on T and B lymphocytes by FTY720 results in defective egress of these cells from lymphoid organs (42–44), and lymphocytes are unable to recirculate to peripheral inflammatory tissues but remain in the lymphoid compartment (45). FTY720 treatment completely blocked egress of B cells in both the thyroids and dLNs. Although B cells in dLNs were effectively depleted by ant-CD20, they were not reduced in dLNs of mice given FTY720 (Fig. 7A), as the percentage of LN B cells increased from 5.7 ± 1.0 to 23.4 ± 2.5%. Most importantly, inhibition of B cell recirculation also completely abolished the reduction of B cells in thyroids of anti-CD20–treated mice. Mice given FTY720 and anti-CD20 had B cell percentages (Fig. 7A) and SAT severity scores (Fig. 7B) comparable to those of isotype controls. In the same experiment, mice given anti-CD20 without FTY720 had greatly reduced B cells in their thyroids (Fig. 7A) and reduced SAT severity scores (Fig. 7B). Treatment with FTY720 and isotype control had no effect on SAT severity scores or B cell percentages in the thyroid (Fig. 7C, 7D). These results are consistent with the idea that effective depletion of thyroid B cells by anti-CD20 is dependent on their ability to enter the circulation. When they re-enter the circulation, their expression of CD20 may increase so that they can be effectively depleted.

Discussion

B lymphocytes have a fundamental role in the pathogenesis of autoimmune diseases as precursors of autoantibody-producing cells and as APCs that can produce cytokines and chemokines that activate pathogenic T cells (4, 46, 47). Therefore, B cell depletion may have beneficial effects on autoimmune diseases by interfering with these pathogenic functions in addition to inhibiting autoantibody production. There is currently insufficient data regarding the precise mechanisms by which B cell depletion inhibits autoimmune diseases in humans because it is difficult to study events taking place in secondary lymphoid organs and inflamed effector sites in patients during treatment. Thus, studies with animal models are important to understand the mechanisms by which B cell depletion therapy can inhibit autoimmune diseases.

In the present study, using the murine autoimmune disease model of SAT, we provide new information regarding the mechanisms of B cell depletion by anti-CD20, with particular emphasis on depletion of B cells in the target organ. The results indicate that circulating FO B cells are the main B cell subset in the thyroid and are also the major target of anti-CD20. FO B cells had increased costimulatory molecule expression both in spleen and LNs with development of SAT. This suggests that FO B cells may contribute to activation of autoreactive T cells by providing positive second signals. Moreover, FO B cells are preferentially recruited into inflamed thyroids, where they express costimulatory molecules and

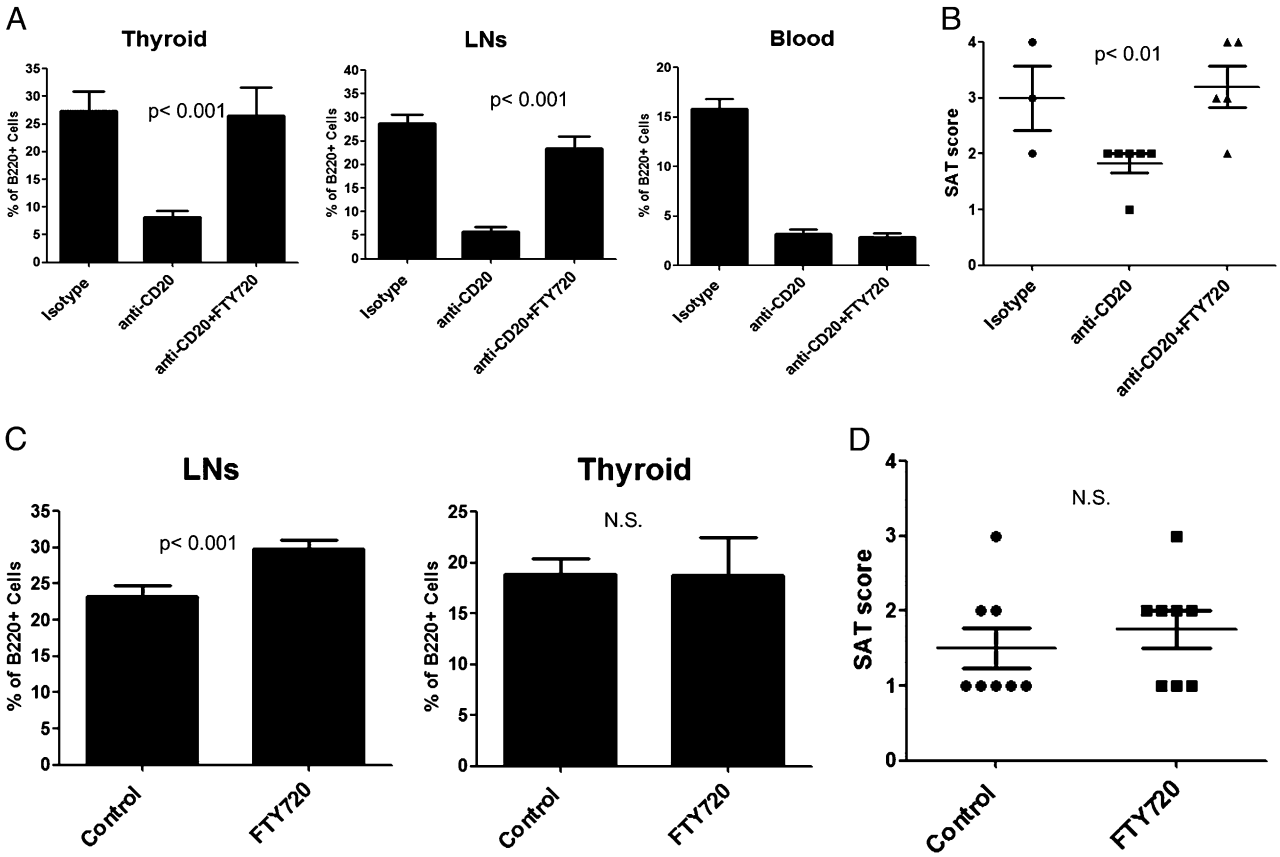

FIGURE 7. Anti-CD20 does not deplete thyroid B cells when they cannot exit the thyroid. (**A**) NOD.H-2h4 mice were given NaI water. Ten weeks later, one group of five mice was given FTY720 i.p. (1 mg/kg every 4 d). After 4 h, these mice, as well as a group of mice not given FTY720, were given 300 μg anti-CD20, and another group of mice was given isotype control. B cell percentages in thyroids, LNs, and peripheral blood were analyzed 5 d later. Results are expressed as the mean percentages of $B220^+$ cells ± SEM of five mice per group. Data are representative of two independent experiments. (**B**) Preventing B cell egress from thyroids maintains SAT severity scores comparable to those of mice given isotype control. Each dot represents an individual mouse. (**C**) NOD.H-2h4 mice were given NaI water. Nine weeks later, one group of five mice was given FTY720 i.p. (1 mg/kg every 4 d). After 4 h, these mice as well as a group of mice not given FTY720 were given 300 μg isotype control. B cell percentages in thyroids and LNs were analyzed 5 d later. (**D**) SAT severity scores of mice given isotype control with or without FTY720. Each dot represents an SAT score of individual mouse.

produce proinflammatory cytokines (Supplemental Fig. 1). Similarly, others have shown that 90% of B cells infiltrating the pancreatic islets of NOD mice are FO B cells that are IgD^{hi} and IgM^{lo} (48). These results are consistent with the fact that most circulating B cells are FO B cells, although circulating peripheral memory B cells in humans were shown to have an MZ phenotype (49), and MZ B cells were shown to migrate to the pancreatic LNs of NOD mice during diabetes development (8).

An unexpected and important result of these experiments was that anti-CD20 depleted B cells in thyroids of mice with SAT very effectively and quickly even though B cells in thyroids express less CD20 than B cells in secondary lymphoid organs. Similar to B cells in blood, 80% of thyroid B cells were depleted in 3 d. Concomitant with effective reduction of thyroid B cells, SAT severity scores were also significantly reduced in 3 d. After 3wk, as B cells repopulate, SAT severity scores also began to increase, but a second injection of anti-CD20 resulted in more prolonged reduction in SAT severity. (Supplemental Fig. 2C).

Because thyroid B cells were effectively depleted by anti-CD20, it was surprising that they expressed much less surface CD20 compared with dLN and splenic B cells (Fig. 5C, 5D). It is generally accepted that stimulation of B cells by Ag results either in cell death or proliferation and differentiation into plasma cells (50). It is thus likely that FO B cells receive T cell help and become activated by Ag, and when they migrate to the thyroid they form germinal centers and differentiate into plasmablasts and lose CD20 expression. Loss of CD20 expression by B cells after they migrate to the target organ was also shown for B cells in pancreatic islets in NOD mice (12). Although islet B cells and B cells infiltrating the thyroid in SAT both lose CD20 expression, islet B cells differ from those in the thyroid in that they are not susceptible to depletion by anti-CD20, and most express CD138 indicating a plasma cell phenotype (12). The difference in sensitivity to depletion of islet versus thyroid B cells by anti-CD20 may be related to the extent of loss of CD20, which is much greater for islet B cells than for B cells in thyroids. Although 40–50% of thyroid B cells still express CD20, 98% of islet-associated $B220^+$ cells are $CD20^-CD138^+$ cells (12). Unlike B cells resident in secondary lymphoid organs, thyroid B cells express lower levels of CD24 (Fig. 5A, 5B) and express more CD44 (data not shown). These patterns are similar to the phenotype of plasma cells. Because very few of the $CD20^{lo}$ thyroid B cells express CD138, they may be less mature than islet-infiltrating B cells, and this may influence their sensitivity to depletion by anti-CD20. Although differentiated plasma cells downregulate surface expression of CD20 and express CD138 (51, 52), early steps of differentiation of B cells into plasma cells are poorly known. Because $CD20^-$ thyroid B cells have reduced expression of mature B cell (CD24) and plasma cell markers (CD138), they could be an intermediate state between mature B cells and plasma cells (e.g., plasmablasts). Several studies in humans have demostrated that during B cell differentiation into plasma cells, CD20 expression is lost at the plasmablast stage, and CD138 upregulation occurs at the late plasma cell stage (51–53).

The loss of CD20 expression by thyroid B cells is in apparent contrast to the effective depletion of thyroid B cells by anti-CD20. Anti-CD20 is thought to deplete B cells by Ab-dependent cellular cytotoxicity, complement-dependent lysis, or apoptosis (38, 40). Our results suggest that thyroid B cells are not undergoing apoptosis following anti-CD20 (Supplemental Fig. 4), and the other mechanisms would require expression of CD20 on the B cell surface. The results of this study suggest that the effective depletion of B cells in thyroids of mice with SAT occurs in response to the depletion of peripheral B cells, and thyroid B cells re-enter the circulation in response to the B cell lymphopenia in the periphery. It is known that effector T cells migrate effectively from the bloodstream into sites of inflammation and can exit extralymphoid tissue through afferent lymph (54), and FO B cells in a highly vascularized organ such as the thyroid may typically circulate in this way. The percentage of remaining thyroid B cells correlated with remaining B cell percentages in blood in individual mice, and the recovery rate of thyroid B cells was similar to that of peripheral blood B cells. In rheumatoid arthritis patients, there is evidence that post–germinal center B cells recirculate between blood and the pathologic site (55, 56). Therefore, the reduction in the supply of B cells in peripheral blood in mice given anti-CD20 may prevent reseeding of pathogenic B cells in thyroids. The fact that blocking B cell egress into the circulation by an S1P1 receptor agonist abrogates the effects of anti-CD20 on thyroid B cells (Fig. 7) supports the notion that thyroid B cells have to exit the thyroid to be effectively depleted by anti-CD20. These results are consistent with those of Gong et al. (44) who demonstrated that access to the circulation is needed for the effective depletion of FO B cells in LNs and spleen by anti-CD20 because B cells are cleared by the reticuloendothelial system. Although our studies did not allow us to determine whether thyroid B cells reacquire CD20 expresion in vivo after they leave the thyroid so they can be depleted by anti-CD20, the fact that expression of CD20 increases on thyroid B cells after in vitro culture (Fig. 5E) is consistent with this notion.

SAT is characterized by clusters of $CD4^+$ T cells and B cells and development of germinal centers in the thyroid (3). Consistent with those results, some thyroid B cells express the germinal center B cell marker GL7 (Fig. 6). Thyroid-infiltrating B cells have increased expression of CD80 as well as proinflammatory cytokines (TNF-α, IL-6) compared with FO B cells in lymphoid organs (Supplemental Fig. 1). Therefore, crosstalk between T cells and B cells in the thyroid may increase costimulatory molecules and cytokine production by B cells, thus facilitating GC formation in thyroids (57–59).

In summary, our studies have identified the phenotype of B cells in inflamed thyroids and, most importantly, showed that anti-CD20 effectively depletes B cells in thyroids of mice with established SAT even though many thyroid B cells do not express CD20. We show that depletion of thyroid B cells by anti-CD20 is likely due to egress of B cells from the thyroid into the circulation, resulting in reduced severity of an established autoimmune inflammatory response. Because anti-CD20 does not effectively deplete $CD20^-$ B cells that have migrated to the pancreas in NOD mice with diabetes (12), the results suggest that the mechanisms of B cell depletion in target organs may differ depending on the extent of vascularization of the organ. These results provide new information that is relevant for understanding the mechanisms by which B cell depletion effectively treats autoimmune diseases in humans.

Disclosures

The authors have no financial conflicts of interest.

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