B Cell Depletion Inhibits Spontaneous Autoimmune Thyroiditis in NOD.H-2h4 Mice

Shiguang Yu, Robert Dunn, Marilyn R. Kehry and Helen Braley-Mullen

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B cell depletion inhibits spontaneous autoimmune thyroiditis in NOD.H-2h4 Mice

Shiguang Yu,*† Robert Dunn,§ Marilyn R. Kehry,§ and Helen Braley-Mullen2*†‡

B cells are important for the development of most autoimmune diseases. B cell depletion immunotherapy has emerged as an effective treatment for several human autoimmune diseases, although it is unclear whether B cells are necessary for disease induction, autoantibody production, or disease progression. To address the role of B cells in a murine model of spontaneous autoimmune thyroiditis (SAT), B cells were depleted from adult NOD.H-2h4 mice using anti-mouse CD20 mAb. Anti-CD20 depleted most B cells in peripheral blood and cervical lymph nodes and 50–80% of splenic B cells. Flow cytometry analysis showed that marginal zone B cells in the spleen were relatively resistant to depletion by anti-CD20, whereas most follicular and transitional (T2) B cells were depleted after anti-CD20 treatment. When anti-CD20 was administered before development of SAT, development of SAT and anti-mouse thyroglobulin autoantibody responses were reduced. Anti-CD20 also reduced SAT severity and inhibited further increases in anti-mouse thyroglobulin autoantibodies when administered to mice that already had autoantibodies and thyroid inflammation. The results suggest that B cells are necessary for initiation as well as progression or maintenance of SAT in NOD.H-2h4 mice. The Journal of Immunology, 2008, 180: 7706–7713.

Spontaneous autoimmune thyroiditis (SAT)1 in NOD.H-2h4 mice is a chronic organ-specific autoimmune disease characterized by infiltration of the thyroid by CD4+ and CD8+ T cells, B cells, and plasma cells and destruction of thyroid follicles by infiltrating inflammatory cells. All NOD.H-2h4 mice develop SAT when they are given 0.05% NaI in their drinking water (1–6). All mice produce anti-mouse thyroglobulin (MTg)-specific autoantibodies, and autoantibody charges. This article must therefore be hereby marked

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3 Abbreviations used in this paper: SAT, spontaneous autoimmune thyroiditis; MTg, mouse thyroglobulin; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; MZ, marginal zone; FO, follicular; CLN, cervical lymph node; hCD20, human CD20.

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Mice were given a single injection of 300 μg of anti-CD20 IgG1 or IgG2a or the IgG2a isotype control i.v. CD19+/B cells in total lymphocytes from 3–6 individual mice. Similar results were obtained in another experiment.

Table I. B cell depletion by anti-CD20 IgG1 and IgG2a

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th></th>
<th></th>
<th></th>
<th>Spleen</th>
<th></th>
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<tr>
<td></td>
<td>4 days</td>
<td>2 wk</td>
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<td>2 wk</td>
<td>3 wk</td>
<td>1 wk</td>
<td>2 wk</td>
<td>3 wk</td>
</tr>
<tr>
<td>Isotype control</td>
<td>25.5 ± 3.6</td>
<td>19.6 ± 4.9</td>
<td>44.2 ± 1.5</td>
<td>45.3 ± 1.8</td>
<td>48.1 ± 1.2</td>
<td>20.7 ± 2.9</td>
<td>26.4 ± 6.2</td>
<td>33.2 ± 0.6</td>
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<td>IgG1 anti-CD20</td>
<td>3.6 ± 1.2</td>
<td>2.6 ± 1.5</td>
<td>14.7 ± 1.2</td>
<td>18.6 ± 2.7</td>
<td>28.7 ± 2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2a anti-CD20</td>
<td>0.5 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td></td>
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</tbody>
</table>

* Mice were given a single injection of 300 μg of anti-CD20 IgG1 or IgG2a or the IgG2a isotype control i.v. CD19+ B cells in blood and spleen were determined by flow cytometry at different times after injection as indicated. Results represent mean percentages of CD19+ B cells in total lymphocytes from 3–6 individual mice.
increased to 60–70% of those in mice given either of the isotype control mAbs (Table I and data not shown). In general, B cells in blood began to increase ∼4 wk after a single injection of 250–300 μg of anti-CD20 but did not return to levels seen in mice given isotype control or no mAb for 7–8 wk (data not shown). Although the extent of splenic B cell depletion was variable in the many individual mice examined in these experiments, the overall conclusion is that both IgG1 and IgG2a anti-CD20 mAbs effectively deplete B cells in blood and CLNs of NOD.H-2h4 mice, but depletion of splenic B cells is incomplete.

MZ B cells in NOD.H-2h4 mice are resistant to depletion by anti-CD20

Because some splenic B cells were resistant to depletion by anti-CD20, it was of interest to determine whether a particular subpopulation of splenic B cells might be resistant to depletion by anti-CD20. To address this issue, three-color flow cytometry was used to determine the phenotypes of residual splenic B cells in mice given anti-CD20 7 days previously (Fig. 2). B220<sup>+</sup>CD21<sup>high</sup>CD23<sup>low</sup> cells represent MZ B cells, B220<sup>+</sup>CD21<sup>intermediate</sup>CD23<sup>high</sup> cells represent follicular (FO) B cells and B220<sup>+</sup>CD21<sup>intermediate</sup>CD23<sup>high</sup> represent transitional 2 (T2) MZ precursor B cells (34–36). Most FO and T2 B cells were depleted in spleens of anti-CD20 treated NOD.H-2h4 mice. Most residual splenic B cells in mice given anti-CD20 were MZ B cells, although some FO and T2 B cells were also resistant to depletion by both isotypes of anti-CD20 (Fig. 2). As mentioned above, both anti-CD20 mAbs effectively depleted 80–90% of B cells in CLNs and blood, and this is consistent with the fact that MZ B cells are noncirculating; i.e., B cells in blood and LN are primarily FO B cells (Ref. 34 and data not shown).

**Effect of anti-CD20 on development of SAT**

To determine whether anti-CD20 could inhibit development of SAT, 7-wk-old NOD.H-2h4 mice were given 300 μg of IgG1

![Diagram](image-url)
anti-CD20 or the IgG1 isotype control i.v. One week later, they were given NaI in their water to initiate development of SAT (Table II). Anti-CD20 and isotype control injections were repeated 3 and 6 wk later, and thyroids were removed 2 wk after the last injection. During the experiment, analysis of peripheral blood by flow cytometry showed that >80% of B cells in blood were depleted at all times tested (data not shown). SAT was only marginally inhibited in mice given IgG1 anti-CD20, and anti-Mtg autoantibody responses were slightly, but not significantly, lower than in isotype controls (Table II). Similar results were observed in two other experiments (not shown). At the time thyroids were removed, ~50% of splenic B cells were depleted in the anti-CD20-treated mice compared with isotype controls (Table II).

Previous results of others suggest that depletion of B cells by anti-CD20 involves FcγR-dependent pathways, and IgG2a mAbs are the most effective isotype for depletion of B cells in vivo (37, 38). The results in Fig. 1 and Table I indicate that the 18B12 IgG2a mAb was more effective than the IgG1 mAb for depletion of splenic B cells. To determine whether the IgG2a 18B12 mAb would be more effective for inhibiting development of SAT, 7-wk-old mice were given 300 μg anti-CD20 IgG2a i.v. After 1 wk, they were given NaI in their drinking water, and injections of anti-CD20 IgG2a or isotype control were repeated 3 and 6 wk later. Thyroids were removed 2 wk after the last injection (Table III). Peripheral blood B cells were monitored several times during the experiment, and CD19+B cells in blood were almost completely depleted (80–90%) at all times (data not shown). In multiple experiments, three of which are shown in Table III, mice given IgG2a anti-CD20 had significantly reduced SAT severity scores compared with isotype controls. Anti-Mtg autoantibody responses were also significantly lower in anti-CD20-treated mice in the three experiments in Table III. At the time thyroids were removed, depletion of splenic B cells in individual mice was variable, ranging from a low of 25–30% to as high as 80–90% in some mice. Interestingly, there was no correlation between the SAT severity scores and percentages of splenic B cells in individual mice at the end of the experiments (data not shown), suggesting that complete depletion of splenic B cells was not necessary for effective disease suppression. Consistent with the results in Fig. 2, most of the residual splenic B cells in anti-CD20-treated mice in the experiments in Table III were MZ B cells (data not shown). Thus, depletion of most circulating (predominantly FO) B cells by IgG2a anti-CD20 reduces development of SAT, suggesting that mature CD20+B cells are important for development of SAT. The results also suggest that effective suppression of SAT and anti-Mtg autoantibody responses is possible even though some B cell subsets, especially MZ B cells, are relatively resistant to depletion.

It is not clear why the IgG1 anti-CD20 was less effective than the IgG2a mAb for inhibiting SAT development, because neither mAb effectively depleted MZ B cells, and differences in the extent of depletion of FO and T2 B cells by the two mAbs were fairly minimal (Fig. 2). IgG1 anti-CD20 was also shown by others to be less effective than IgG2a for depletion of B cells in NOD mice (38).

### Table II. Effect of IgG1 anti-CD20 on development of SAT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SAT Severity</th>
<th>Anti-Mtg IgG1(OD)</th>
<th>Splenic B Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotype control</td>
<td>0 1+ 2+ 3+ 4+</td>
<td>0.567 ± 0.042</td>
<td>45 ± 3.5</td>
</tr>
<tr>
<td>IgG1 anti-CD20</td>
<td>0 1+ 2+ 3+ 4+</td>
<td>0.342 ± 0.046</td>
<td>16 ± 3.9</td>
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### Table III. IgG2a anti-CD20 inhibits development of SAT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SAT Severity</th>
<th>Anti-Mtg IgG2a(OD)</th>
<th>Splenic B Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotype control</td>
<td>0 1+ 2+ 3+ 4+</td>
<td>0.626 ± 0.200</td>
<td>41 ± 2.5</td>
</tr>
<tr>
<td>IgG2a anti-CD20</td>
<td>0 1+ 2+ 3+ 4+</td>
<td>0.124 ± 0.049</td>
<td>21 ± 5.1</td>
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</table>

### Table IV. Effect of anti-CD20 given to mice with SAT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SAT Severity</th>
<th>Anti-Mtg IgG1(OD)</th>
<th>Splenic B Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotype control</td>
<td>0 1+ 2+ 3+ 4+</td>
<td>0.938 ± 0.05</td>
<td>39.3 ± 0.9</td>
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<tr>
<td>IgG2a anti-CD20</td>
<td>0 1+ 2+ 3+ 4+</td>
<td>0.030 ± 0.01</td>
<td>5.7 ± 0.3</td>
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</tbody>
</table>

### Notes:
- a Seven-week-old NOD:H-2k4 mice were given 300 μg of IgG1 anti-CD20 or the IgG1 isotype control i.v. and the injections were repeated i.p. 3 and 6 wk later. Each isotype vs anti-CD20 pair represents a separate experiment. All mice were given 0.05% NaI in their water at 8 wk of age, and thyroids were removed 2 wk after the last injection.
- b Numbers of mice with various degrees of severity of SAT 8 wk after NaI water (p < 0.05, line 1 vs line 2).
- c Anti-Mtg IgG1 (1/100 serum dilutions) expressed as OD410 ± SEM (p = 0.06; line 1 vs line 2).
- d Percentage of splenic CD19+B cells at termination of the experiments (mean ± SEM of all mice in the group) (p < 0.01 for each experiment).
- e In Expt. 1 (lines 1–3), mice were given NaI water at 8 wk of age. Five weeks later, thyroids were removed from one group of mice (line 1), and the remaining mice were given the IgG2a isotype control (line 2) or 300 μg anti-CD20 IgG2a i.v. (line 3). Injections were repeated i.p. 3 and 6 wk later, and thyroids were removed 2 wk after the last injection (11 wk after beginning NaI water). In Expt. 2 (lines 4–6), mice were given NaI water at 8 wk of age. Thyroids were removed from one group of mice 8 wk later (line 4) and the remaining mice were given anti-CD20 IgG2a or the IgG2a isotype control as in Expt. 1. Thyroids were removed 2 wk after the last injection (14 wk after beginning NaI water).
- f Numbers of mice with various degrees of severity of SAT before and after administration of anti-CD20 or IgG2a isotype control. p < 0.05 (line 2 vs line 3 and line 5 vs line 6).
- g Anti-Mtg IgG1 (1/100 serum dilutions) expressed as OD410 ± SEM (p < 0.09, line 2 vs line 3 and p < 0.01, line 5 vs line 6) and p < 0.05, line 1 vs line 3, line 4 vs line 6.
- h Percentage of splenic CD19+B cells (mean ± SEM of all mice in the group). (p < 0.01).
lines 4–6) weeks after NaI water. Thyroids and serum were obtained from groups of five mice in each experiment to confirm the extent of inflammation and levels of autoantibodies present when anti-CD20 injections began (Table IV). Thyroid lesions and anti-Mtg autoantibodies were present in most mice 5 (Table IV, line 1) or 8 (Table IV, line 4) weeks after NaI water when anti-CD20 injections began. Other groups of mice in the same experiments were given three injections of IgG2a anti-CD20 or the IgG2a isotype control at 3-wk intervals, and experiments were terminated 2 wk after the last injection. SAT severity was significantly reduced in both experiments in mice given IgG2a after inflammation had begun to develop (Table IV, line 2 vs line 3 and line 5 vs line 6). When B cell depletion was initiated in mice that had begun to develop SAT and produce autoantibody, circulating anti-Mtg autoantibodies in anti-CD20-treated mice were only slightly higher than those present before anti-CD20 treatment (Table IV, line 1 vs line 3 and line 4 vs line 6), whereas anti-Mtg autoantibodies in mice given isotype control mAb continued to increase (Table IV). When thyroids were removed for evaluation of thyroid histopathology, 40–60% of splenic B cells were depleted in anti-CD20-treated mice (Table IV), and as mentioned above, there was no correlation between the percentage of splenic CD19+ B cells present at the time of thyroid removal with the SAT severity scores. This suggests that depletion of circulating B cells, which was always essentially complete throughout the experiment, was the most important predictor of disease suppression in anti-CD20-treated mice. Depletion of B cells in blood and spleens of mice given anti-CD20 IgG2a after disease had begun to develop was comparable with that shown for naive mice in Table I (data not shown).

Reduction of both T and B cells in thyroids after anti-CD20 treatment
IgG2a anti-CD20 markedly inhibited SAT development, and inflammatory cell infiltration of the thyroid was therefore reduced (Fig. 3). When anti-CD20 injections were initiated after SAT had begun to develop, T and B cells had already migrated to the thyroid. Infiltration of both B and T cells was reduced in mice given anti-CD20, and clustering of T and B lymphocytes which was evident in isotype controls was minimal (Fig. 3). These results suggest that B cells may be required for maintenance of inflammation in SAT, and when circulating B cells (including those that migrate to the thyroid) are depleted, effector CD4+ T cells are not retained in the inflammatory site.

Discussion
The results of this study indicate that development of SAT and anti-Mtg autoantibody responses were reduced in NOD.H-2b4
mice given anti-CD20 IgG2a mAb. SAT severity scores were also reduced in mice given anti-CD20 IgG2a if treatment was initiated after disease had begun to develop (Table IV). Because plasma cells reportedly do not express CD20 (21, 39), anti-MTG autoantibody responses in anti-CD20-treated mice were comparable with those present when treatment began, whereas Ab responses continued to increase in isotype controls. In these experiments (Table IV), SAT severity scores were relatively mild (2+ in most mice) when treatment was initiated. It is not known whether anti-CD20 would be effective in mice with more severe SAT.

In other studies, anti-CD20 given before disease onset prevented development of collagen induced arthritis, fibrosis in tight-skin mice, Sjögren’s syndrome, and diabetes in mice but had little effect in mice with ongoing disease (26–28, 38). However, the same anti-CD20 IgG2a Ab used here effectively inhibited proteoglycan-induced arthritis in mice that had already begun to develop arthritis and autoantibodies (30). The IgG1 18B12 mAb inhibited ongoing SLE in MRL/lpr mice (29), and anti-hCD20 effectively suppressed some clinical signs of SLE and diabetes in transgenic MRL/lpr (29) or NOD (31) mice expressing hCD20 on B cells. The reason(s) for the differences in effectiveness of anti-CD20 in suppressing ongoing disease in different animal models are not known but may be due to differences in requirements for B cells in disease initiation vs their role in maintaining disease in different models. Previous studies have established an important role for B cells in initiation of many autoimmune diseases, where they presumably function as important APCs (7–9, 14–18) to promote activation of pathogenic CD4+T cells (29–31, 40).

Experiments using B cell-deficient mice and mice depleted of B cells beginning at birth indicate that B cells are required for development of many autoimmune diseases including SAT (7–12, 14–18). In those studies, B cell depletion was essentially complete, and in B cell-deficient mice, B cells are completely absent throughout development; this may result in abnormalities that extend beyond simple B cell depletion (40). The results presented here indicate that anti-CD20 given to adult NOD.H-2h4 mice reduced development of SAT and autoantibody responses even though most splenic MZ B cells were resistant to depletion. These results are consistent with those of others (29, 31, 38), indicating that B cells in autoimmunity-prone mouse strains are relatively resistant to depletion by anti-CD20 compared with those in non-autoimmunity-prone strains. B cells in autoimmunity-prone mice could be more resistant to depletion because autoreactive B cells are presumably activated before development of autoimmune diseases, and activated B cells could be more resistant to depletion by anti-CD20 (29). Alternatively, the relatively greater resistance of B cells from autoimmunity-prone mice, particularly those of NOD mice, to depletion by anti-CD20 could be due to deficient FcγRI binding of IgG2a anti-CD20 and/or decreased numbers of splenic monocytes in NOD mice compared with C57BL/6 mice (38). Because NOD.H-2h4 mice differ from NOD mice only at the MHC locus (2, 32), the defects in FcγRI effector functions in NOD mice (38, 41, 42) are presumably also present in NOD.H-2h4 mice. The relative resistance of some splenic B cells to depletion by anti-CD20 in our studies might also be explained by the fact that some NOD.H-2h4 mice given anti-CD20 IgG2a generated an immune response against the variable domain of the IgG2a anti-CD20 Ab, because circulating anti-idiotype Abs were detected in some mice (data not shown). This explanation, however, is unlikely to account for the relative resistance to depletion of splenic B cells in most mice because only a very small percentage of sera had detectable anti-idiotypic Abs, these Abs were never detected in mice given IgG1 anti-CD20, and MZ B cells were resistant to depletion in all mice.

Previous studies by others indicated that circulating mature B cells were almost completely depleted by anti-hCD20 in transgenic mice expressing hCD20 on B cells after injection of anti-hCD20, but most MZ B cells were resistant to depletion (25). The results presented here indicate that the CD21highCD23low splenic MZ B cell compartment in NOD.H-2h4 mice was almost completely resistant to depletion by anti-CD20, whereas most splenic FO and transitional T2 B cells were depleted (Fig. 2). The resistance of MZ B cells to depletion by anti-CD20 is not due to lack of CD20 expression by MZ B cells or to lack of binding of the mAb to MZ B cells (25, 38). In some models, MZ, T2, and FO B cells were depleted to a similar extent by anti-CD20 (26–30, 38). To our knowledge, our studies are the first to show that splenic MZ B cells are relatively resistant to depletion by anti-CD20 compared with other splenic B cell subsets. This suggests that B cell subsets in different mouse strains might differ in their susceptibility to depletion by anti-CD20. However, other factors are apparently also important because a different anti-CD20 mAb effectively depleted MZ B cells in NOD mice (38), which are closely related to the NOD.H-2h4 mice used here. Importantly, the splenic B cells (primarily MZ B cells) that were not depleted in NOD.H-2h4 mice given IgG2a anti-CD20 were apparently insufficient and/or were not required to initiate development of SAT in NOD.H-2h4 mice.

The finding that anti-CD20 was relatively effective when anti-CD20 was given to mice that had already begun to develop SAT (Table IV) suggests that B cells play a role both in initiation of SAT and in maintaining chronic inflammation in the thyroid. Several studies support an active role for B cells in the diversification of autoreactive T cell responses, and T cell-B cell interactions constitute a positive feedback loop that enables diversification and continuous amplification of autoimmune responses (43–47). B cells also contribute to inflammation by functioning as APCs for activation of autoreactive T cells (8, 14–18) and by producing autoantibodies and proinflammatory cytokines (28, 47–50). In the current study, infiltration of both T and B cells in thyroids was reduced when anti-CD20 was given to mice that had already developed SAT (Fig. 3). This is consistent with a recent report indicating that rituximab depleted intrathyroidal B cells in a patient with Graves’ disease (51). B cells that migrate to the thyroid may promote infiltration and proliferation of autoreactive CD4+ T cells to form tertiary lymphoid organs found in many autoimmune disease target tissues, including thyroids of NOD.H-2h4 mice with SAT (6, 48–50, 52, 53). B cells can be important for the formation and maintenance of these structures (49, 50), and B cell depletion could lead to dissolution of these organized lymphoid infiltrates as suggested by our finding that thyroid-infiltrating cells in anti-CD20-treated mice tended to be scattered, whereas those in mice given isotype control form clusters (Fig. 3), shown previously to be comprised of CD4+ T cells and B220+B cells (6). Because infiltration of both CD4+ T cells and B cells decline after treatment with anti-CD20, T cells apparently die or are not retained in the thyroid after B cells are depleted.

In addition to their role in promoting autoimmune diseases, B cells can function as regulatory cells to suppress progression and/or promote recovery in several murine models of chronic inflammation (54), including inflammatory bowel diseases (54–56), rheumatoid arthritis (36, 57–59), and experimental autoimmune encephalomyelitis (60). In some reports, B cells were shown to negatively regulate inflammatory responses by producing IL-10 (36, 54, 57–60). In some of these studies, IL-10-producing regulatory B cells were MZ-like B cells, characterized by expression of high levels of CD1d and responsiveness to LPS (57), whereas in others, regulatory B cells were reported to be mesenteric (56) or transitional type 2 cells (36, 57, 59) that include precursors of MZ
B cells (34–36). FO, MZ, and transitional B cells could have different roles in development of some autoimmune diseases, with FO B cells functioning to promote disease and MZ or transitional B cells functioning to inhibit disease. Consistent with this notion, constitutive expression of B7-1 on B cells of NOD mice resulted in marked reduction of splenic FO B cells, whereas MZ B cells were largely unaffected. The transgenic mice had reduced insulin and did not develop diabetes (61), suggesting that MZ B cells were not sufficient to initiate development of diabetes and could be functioning as regulatory cells. In the current study, anti-CD20 effectively suppressed SAT development even though most MZ B cells were resistant to depletion. It is possible that the splenic MZ B cells in anti-CD20-treated NOD.H-2h4 mice could contribute to suppression of SAT, e.g., through production of IL-10. We are currently generating IL-10−/− NOD.H-2h4 mice to address a possible role of IL-10 and MZ B cells in suppression of SAT in anti-CD20-treated mice.

Although multiple factors in addition to B cells contribute to autoimmune disease pathogenesis, understanding the contributions of different subsets of B cells to autoimmune diseases is important. More detailed analyses of the mechanisms involved in suppression of autoimmune diseases after B cell depletion in animal models will lead to a better understanding of the potential applications of B cell-depleting therapies for autoimmune and inflammatory diseases in humans. In addition to its effectiveness in suppressing SAT, e.g., through production of IL-10, we are interested in the role of MZ B cells in anti-CD20-treated NOD.H-2h4 mice as a possible source of IL-10 and MZ B cells in suppression of SAT in anti-CD20-treated mice.

References

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