B cell–deficient NOD.H-2h4 mice have CD4$^+$CD25$^+$ T regulatory cells that inhibit the development of spontaneous autoimmune thyroiditis

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Wild-type (WT) NOD.H-2h4 mice develop spontaneous autoimmune thyroiditis (SAT) when given 0.05% NaI in their drinking water, whereas B cell–deficient NOD.H-2h4 mice are SAT resistant. To test the hypothesis that resistance of B cell–deficient mice to SAT was due to the activity of regulatory CD4$^+$CD25$^+$ T (T reg) cells activated if autoantigen was initially presented on non–B cells, CD25$^+$ T reg cells were transiently depleted in vivo using anti-CD25. B cell–deficient NOD.H-2h4 mice given three weekly injections of anti-CD25 developed SAT 8 wk after NaI water. Thyroid lesions were similar to those in WT mice except there were no B cells in thyroid infiltrates. WT and B cell–deficient mice had similar numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells. Mice with transgenic nitrophenyl-specific B cells unable to secrete immunoglobulin were also resistant to SAT, and transient depletion of T reg cells resulted in severe SAT with both T and B cells in thyroid infiltrates. T reg cells that inhibit SAT were eliminated by day 3 thymectomy, indicating they belong to the subset of naturally occurring T reg cells. However, T reg cell depletion did not increase SAT severity in WT mice, suggesting that T reg cells may be nonfunctional when effector T cells are activated; i.e., by autoantigen-presenting B cells.

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Abbreviations used: MTg, mouse thyroglobulin; NOD, nonobese diabetic; NP, nitrophenyl; SAT, spontaneous autoimmune thyroiditis; Tg, transgenic; T reg, regulatory T; Tx, thymectomy.

All NOD.H-2h4 mice develop spontaneous autoimmune thyroiditis (SAT) when given NaI in their drinking water (1–3). Thyroid inflammation is chronic, with infiltration of thyroids by lymphocytes, including CD4$^+$ and CD8$^+$ T cells, and B220$^+$ B cells (3–6). All mice that develop SAT produce anti–mouse thyroglobulin (MTg)-specific autoantibodies, and IgG1 and IgG2b autoantibody levels generally correlate with SAT severity scores (1, 5). We previously showed that B cell–deficient NOD.H-2h4 mice did not develop SAT (5). Although adult B cell–deficient mice reconstituted with B cells or given passive anti-MTg autoantibodies did not develop SAT, T cells from B cell–deficient mice could function as effector cells if B cells were provided during the maturation of T cells from bone marrow precursors (5). These results suggested that B cells were required for the early activation of CD4$^+$ T cells, functioning either as important APCs for activation of CD4$^+$ effector T cells or to amplify responses of effector T cells so they could manifest their pathogenic potential. Because the defect in adult B cell–deficient mice could not be corrected by reconstitution of B cells or anti-MTg autoantibodies (5), we hypothesized that CD4$^+$ effector T cells initially activated in the absence of B cells might be rendered unresponsive so they were unable to induce SAT when B cells were provided to adults. Unresponsiveness of effector T cells could be due to induction of anergy or to preferential activation of regulatory T (T reg) cells when autoantigen is initially presented in the absence of B cells.

T cells specific for self-antigens not negatively selected in the thymus can be present in the periphery at birth (7–9). In some strains of mice, nontolerant potentially autoreactive T cells can be activated and lead to spontaneous autoimmune disease. Activation of autoreactive T cells requires or is facilitated by B cells in several systems (10–20). In most cases, activation

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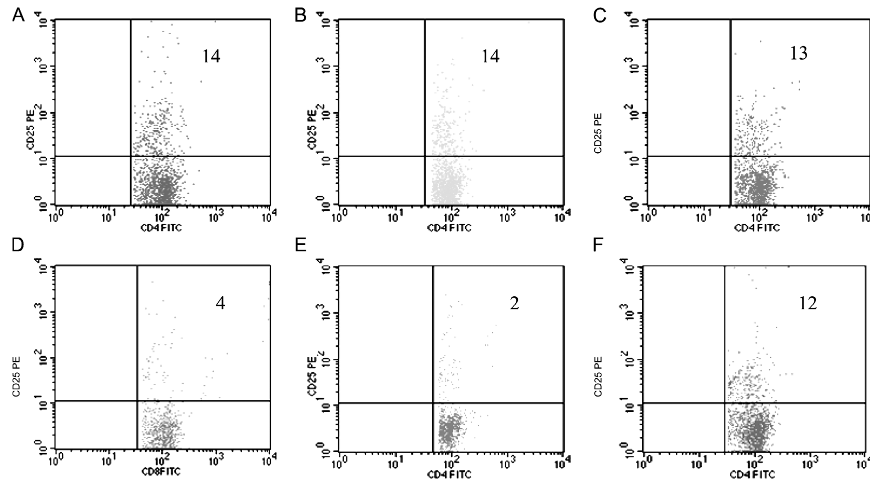

Figure 1. Flow cytometric analysis of CD4$^+$CD25$^+$ splenic T cells from 6-wk-old WT (A), B cell–deficient (B), and NP Tg (C) NOD.H-2h4 mice, CD8$^+$CD25$^+$ splenic T cells from NP Tg (D) mice, and CD4$^+$CD25$^+$ splenic T cells from NP Tg mice given anti-CD25 2 (E) or 7 (F) d previously as analyzed by flow cytometry. Results are representative of 5–10 mice analyzed in each group. The percentages of CD25$^+$ cells in the gated CD4$^+$ or CD8$^+$ cells are indicated in the top right of each panel.

of self-reactive lymphocytes in the periphery is prevented by naturally occurring T reg cells, a subset of thymus-derived CD4$^+$ T cells that constitutively express CD25 (7–9). Day 3 thymectomy (Tx) in mice that do not normally develop spontaneous autoimmune disease results in development of organ-specific autoimmune diseases, including thyroiditis due to elimination of CD4$^+$CD25$^+$ T reg cells (21, 22). B cell–deficient NOD.H-2h4 mice might not develop SAT if B cells are required for optimal activation of autoreactive T cells and if naturally occurring T reg cells are preferentially activated if B cells are not available to present autoantigen. This study was undertaken to test this hypothesis by asking if B cell–deficient mice would develop SAT if CD25$^+$ T reg cells were transiently eliminated.

RESULTS

CD25$^+$CD4$^+$ T cells are not elevated in B cell–deficient mice

To begin to determine if increases in peripheral CD4$^+$CD25$^+$ T reg cells might explain the resistance of B cell–deficient mice to SAT, percentages of CD4$^+$CD25$^+$ T cells were compared in the spleens and peripheral blood of 4- and 8-wk-old B cell–deficient and WT mice. Although there was some variation, the percentages of CD4$^+$CD25$^+$ T cells were similar (averaging 10–15% of CD4$^+$ T cells) for 4–8-wk-old WT and B cell–deficient mice (Fig. 1, A and B) not given NaI water as well as for older mice given NaI water for 8 wk (not depicted). Although most CD4$^+$CD25$^+$ cells in B cell–deficient mice could have been T reg cells, while some CD4$^+$CD25$^+$ cells in WT mice could have been activated CD25$^+$ effector T cells, the two populations could not be distinguished by CD25 expression. These results indicate that differences in absolute numbers of peripheral CD4$^+$CD25$^+$ T cells in naive B cell–deficient versus WT mice do not explain their different susceptibility to SAT.

SAT develops in B cell–deficient mice given anti-CD25 mAb

B cell–deficient NOD.H-2h4 mice given NaI water do not develop SAT, whereas all WT mice develop SAT and produce anti-MTg autoantibodies under the same conditions (Table I). To determine if the inability of B cell–deficient

Table I. Development of SAT in B cell–deficient NOD.H-2h4 mice given anti-CD25 mAb

Mice[a]	SAT severity[b]				Anti-MTg[c]	
	0	1+	2+	3+	IgG_1	IgG_{2B}
NOD.H-2h4μ^{null}	5	0	0	0	0.060 ± 0.005	0.003 ± 0.001
μ^{null} (anti-CD25)	0	2	3	0	0.062 ± 0.009	0.001 ± 0.001
NOD.H-2h4 WT	1	2	6	2	0.307 ± 0.071	0.393 ± 0.087
WT (anti-CD25)	5	3	2	0	0.234 ± 0.133	0.355 ± 0.061

Mice in lines 2 and 4 received 0.5 mg anti-CD25 mAb 10, 17, and 24 d after birth. Mice in lines 1 and 3 received normal rat Ig on days 10, 17, and 24.
[a]Female NOD.H-2h4WT or B cell–deficient (μ^{null})mice were given 0.05% NaI in their water beginning at 8 wk of age.
[b]Numbers of mice with various degrees of severity of SAT 8 wk after NaI water. P-values: line 1 versus line 2, $P < 0.0002$; line 2 versus line 3, $P > 0.6$; line 3 versus line 4, $P < 0.01$.
[c]Anti-MTg IgG_1 and IgG_{2B} expressed as OD_{410} ± SEM.

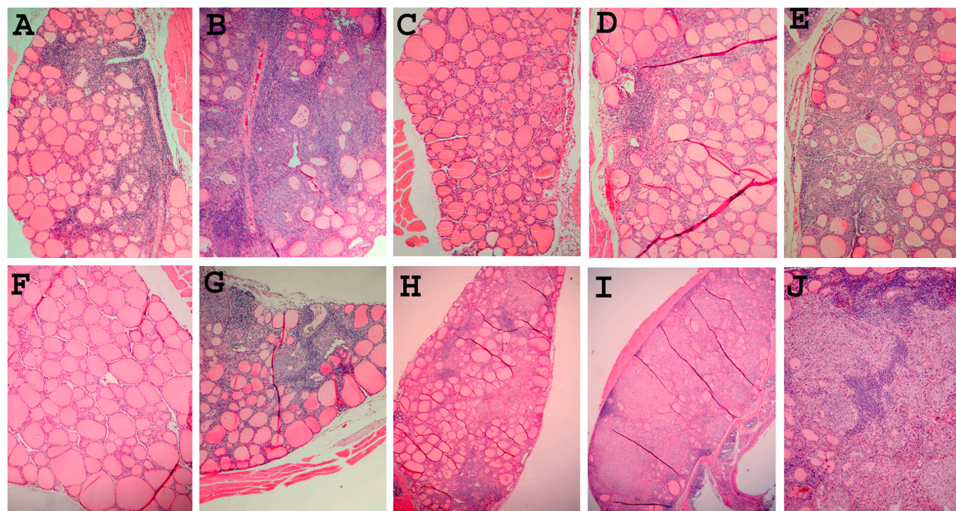

Figure 2. Hematoxylin and eosin–stained thyroids from WT, B cell–deficient, and NP Tg NOD.H-2h4 mice 2 mo after NaI water. (A) WT mouse with 2+ SAT, (B) WT mouse with 4+ SAT, and (C) B cell–deficient mouse given rat Ig; severity score, 0. (D and E) B cell–deficient mice given anti-CD25; D, 1+; E, 2+. (F) Rat Ig–treated NP Tg mouse; severity score, 0. (G–J) NP Tg mice given anti-CD25; G, 2+; H, 3+; I and J, 4–5+. Magnification: A–G and J, 100; H and I, 40.

mice to develop SAT might be due to $CD4^+CD25^+$ T reg cells that suppressed the activation or function of effector T cells, B cell–deficient mice were given three weekly injections of anti-CD25 mAb beginning at 10 d of age (23). This resulted in depletion of most $CD4^+CD25^+$ T cells 2–3 d after the last injection of mAb (Fig. 1 E). $CD4^+CD25^+$ T cells gradually returned, and nearly normal numbers of $CD4^+CD25^+$ splenic T cells were detected 7–10 d after the last antibody injection (Fig. 1 F). B cell–deficient mice given three weekly injections of anti-CD25 beginning at 10 d of age developed SAT, whereas B cell–deficient mice given rat Ig did not develop SAT (Table I). The SAT severity scores of anti-CD25–treated B cell–deficient mice were comparable to those of rat Ig–treated WT NOD.H-2h4 mice ($P > 0.6$ for the experiment shown in Table I). Thyroid lesions in both WT and B cell–deficient mice predominantly had lymphocyte infiltration (Fig. 2, A and B vs. D and E), but all lymphocytes in the thyroids of B cell–deficient mice were T cells (Fig. 3, D–F), whereas many B cells were also present in WT thyroids (Fig. 3 B). WT mice given anti-CD25 beginning at 10 d of age consistently developed less severe SAT than controls (Table I, line 3 vs. line 4; $P < 0.01$). This may be due to elimination of $CD25^+$ effector T cells that could have developed before anti-CD25 was administered. These results suggest that the inability of B cell–deficient mice to develop SAT can be explained, at least in part, by the activity of $CD25^+$ T reg cells that limit the ability of effector $CD4^+$ T cells to expand or become activated. B cell–deficient mice clearly have $CD4^+$ T cells that can function as effector cells for SAT if $CD25^+$ T cells are transiently depleted so their T cells are not anergic. Because CD25 depletion is transient, the results also suggest that T reg cells do not efficiently inhibit effector T cells after they have been activated.

Mice with B cells unable to secrete Ig do not develop SAT unless they are given anti-CD25

To further examine the mechanisms by which B cells regulate development of SAT, we asked if transgenic (Tg) mice with nitrophenyl (NP)-specific B cells that cannot secrete Ig (12) would develop SAT. The percentage of splenic $B220^+$ B cells was similar for WT and NP Tg mice (not depicted), and $CD4^+CD25^+$ cells in the spleens of NP Tg mice (Fig. 1 C) were comparable to those of WT and B cell–deficient mice (Fig. 1, A and B). Very few $CD8^+$ T cells in the spleens of NP Tg (Fig. 1 D), WT, or B cell–deficient mice (not depicted)

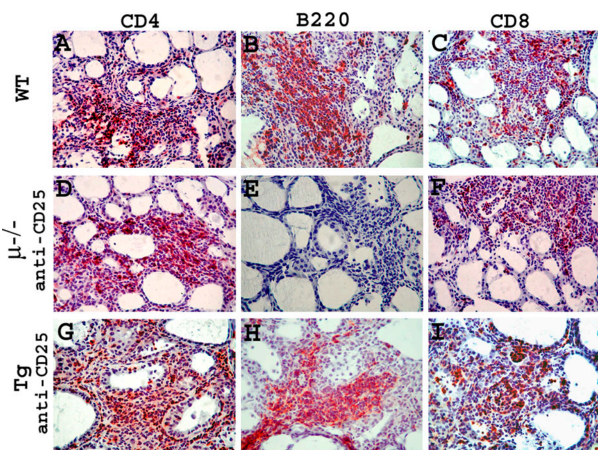

Figure 3. Immunohistochemical staining for CD4, CD8, and B220 in WT mice and in B cell–deficient and NP Tg mice given anti-CD25 beginning at 10 d of age as described in Materials and methods. All mice were given 0.05% NaI water at 8 wk of age. All infiltrating lymphocytes in B cell–deficient thyroids are CD4 and $CD8^+$ T cells. WT and NP Tg thyroids also have many $B220^+$ B cells. Magnification: 400.

Table II. Development of SAT in NP Tg mice given anti-CD25 mAb

Mice[a]	SAT severity[b]					Anti-MTg[c]	
	0	1+	2+	3+	4+	IgG_1	IgG_{2B}
NP Tg	9	2	0	0	0	0.060 ± 0.005	0.003 ± 0.001
NP Tg (anti-CD25)	0	0	3	4	4	0.062 ± 0.009	0.001 ± 0.001
NP Tg (anti–IL-10)	4	1	0	0	0	0.010 ± 0.004	0.052 ± 0.053

[a]Female NOD.H-2h4 NP Tg μnull mice were given 0.05% NaI water beginning at 8 wk of age. Mice received 0.5 mg anti-CD25 (PC61), anti–IL-10 (JESS 2A5), or normal rat Ig 10, 17, and 24 d after birth as indicated.
[b]Numbers of mice with various degrees of severity of SAT 8 wk after NaI water. P-values: line 1 versus line 2, $P < 10^{-9}$; line 1 versus line 3, $P > 0.9$.
[c]Anti-MTg IgG1 and IgG2B expressed as OD410 ± SEM.

expressed CD25. Tg mice, like B cell–deficient mice, developed no or very mild SAT after receiving NaI water (Table II), produced no detectable serum anti-MTg autoantibodies (Table II), and were serum Ig negative (not depicted). However, NP Tg mice developed severe SAT ($P < 10^{-9}$ compared with controls), but no detectable anti-MTg autoantibodies, after being given anti-CD25 to transiently deplete T reg cells. Treatment of NP Tg mice with another rat anti–mouse mAb, i.e., anti–IL-10 (Table II), using the injection schedule used for anti-CD25 had no effect on SAT. Thyroid lesions in anti-CD25–treated NP Tg mice were histologically like those in WT mice, although severity scores were often higher. Some NP Tg mice had very severe thyroid inflammation, with proliferation of thyroid epithelial cells (Fig. 2, I and J) and fibrosis (not depicted). Thyroid infiltrates in anti-CD25–treated NP Tg mice included many $B220^+$ B cells (Fig. 3 H) as well as $CD4^+$ and $CD8^+$ T cells (Fig. 3, G and I). The presence of B cells in the thyroids of anti-CD25–treated NP Tg mice is of interest because their B cells are NP specific (12, 20), do not secrete Ig, and presumably do not include specificities for thyroid proteins. This suggests that trafficking of B cells to the thyroid can be independent of specificity for thyroid proteins and/or secretion of autoantibodies, and production of autoantibodies in the thyroid or in the circulation is not necessary for optimal thyroid damage.

Depletion of $CD25^+$ T cells does not result in SAT in other SAT-resistant NOD.H-2h4 mice

Because B cell–deficient and NP Tg mice, both normally resistant to SAT, developed SAT after anti-CD25 treatment, it was of interest to determine if other SAT-resistant mice also developed SAT after depletion of $CD25^+$ T cells. To address this question, IFN-γ–deficient mice (24) and WT NOD.H-2h4 mice that have mutated and do not develop SAT (25) were given three weekly injections of anti-CD25 beginning at 10 d of age and 0.05% NaI water at 8 wk. Thyroids were examined 8 wk later (Table III). Anti-CD25 did not result in SAT in these mice, suggesting that their resistance to SAT is probably not due to T reg cell activity. More importantly, the results indicate that the transient lymphopenia induced by CD25 depletion (26–28) is not sufficient for development of SAT. It is not known why SAT-resistant WT NOD.H-2h4 mice given anti-CD25 had higher anti-MTg autoantibody responses than controls (Table III).

Timing of CD25 depletion is not critical for SAT development in B cell–deficient or NP Tg mice

Our previous study suggested that B cells were required during the first 4–6 wk after birth for development of SAT in WT mice (5). To determine if early CD25 depletion was necessary for B cell–deficient mice to develop SAT, B cell–deficient and NP Tg mice were given three weekly injections of anti-CD25 beginning 11 d, 4 wk, or 8 wk after birth (Table IV). Mice given anti-CD25 11 d or 4 wk after birth all developed SAT with similar severity scores. Because CD25 depletion is transient (Fig. 1 F), normal numbers of $CD25^+$ T cells were present when mice began NaI water. In preliminary experiments, B cell–deficient mice given anti-CD25 beginning at 8 wk developed minimal SAT (not depicted), and NP Tg mice given anti-CD25 beginning at 8 wk developed

Table III. All SAT-resistant NOD.H-2h4 mice do not develop SAT after treatment with anti-CD25 mAb

Mice[a]	SAT severity[b]					Anti-MTg[c]	
	0	1+	2+	3+	4+	IgG_1	IgG_{2B}
NOD.H-2h4 WT	0	1	2	2	0	0.238 ± 0.096	0.331 ± 0.046
SAT-resistant WT	5	3	2	0	0	0.182 ± 0.074	0.085 ± 0.049
SAT-resistant WT (anti-CD25)	4	2	0	0	0	0.413 ± 0.064	0.386 ± 0.036
IFN-$\gamma^{-/-}$ NOD.H2h4	4	0	1	0	0	0.218 ± 0.040	0.213 ± 0.031
IFN-$\gamma^{-/-}$ (anti-CD25)	3	1	0	0	0	0.306 ± 0.079	0.388 ± 0.076

[a]Female NOD.H-2h4WT, SAT-resistant WT (reference 25), or IFN$\gamma^{-/-}$ (reference 24) NOD.H-2h4 mice were given 0.05% NaI water beginning at 8 wk of age. Mice received 0.5 mg anti-CD25 (PC61) or normal rat Ig 10, 17, and 24 d after birth as indicated.
[b]Number of mice with various degrees of severity of SAT 8 wk after NaI water.
[c]Anti-MTg IgG1 and IgG2B expressed as OD410 ± SEM.

Table IV. SAT in B cell–deficient and NP Tg mice given anti-CD25 mAb at various times

Mice[a]	SAT severity[b]				
	0	1+	2+	3+	4+
Controls (μ-)	5	0	0	0	0
anti-CD25 day 11 (μ-)	0	3	1	1	1
anti-CD25 4 wk (μ-)	0	0	5	0	1
anti-CD25 8 wk	1	3	2	0	0
NP-Tg Controls	3	2	1	0	0
anti-CD25 day 11 (NP Tg)	0	0	4	3	3
anti-CD25 8 wk (NP Tg)	1	1	2	1	0

[a]Female μ^{null} (lines 1–3) or NP Tg (lines 5–7) mice were given 0.05% NaI water at 8 wk. Mice in line 4 received NaI water for 8 wk beginning at 10 wk of age to allow for recovery of $CD25^+$ cells after the last injection of anti-CD25. Mice in lines 2, 4, 6, and 7 received three weekly injections of 0.5 mg anti-CD25 beginning at the indicated time after birth. Mice in lines 1 and 5 received rat Ig.
[b]Number of mice with various degrees of severity of SAT 8 wk after NaI water.

less severe SAT than those given anti-CD25 at day 11 (Table IV, lines 6 and 7). Because mice all began NaI water at 8 wk of age, CD25 depletion was maintained during the first 3 wk when effector T cell activation begins (1). Because activated effector $CD4^+$ T cells also express CD25, it was important to determine if mice given anti-CD25 beginning at 8 wk of age would develop SAT if more time was allowed for optimal development of SAT. Therefore, mice given anti-CD25 at weeks 8, 9, and 10 were given NaI water at 11 wk of age, and thyroids were removed 8 wk later. Most mice developed SAT, although the severity scores were slightly lower than in mice given anti-CD25 earlier (Table IV, line 4 vs. lines 2 and 3). These results suggest that if sufficient time is allowed for SAT development, effector T cell function can be manifest even when T reg cells are eliminated in adults.

T reg cells that inhibit SAT in B cell–deficient and NP Tg mice are eliminated by day 3 Tx

Because naturally occurring T reg cells are eliminated by Tx at 3 d of age (21, 22, 29), we asked if day 3 Tx B cell–deficient and NP Tg mice would develop SAT (7–9, 21, 22, 30). Groups of WT and Tg mice were thymectomized at 3 d, given NaI water at 8 wk, and thyroids were removed 8 wk later (Table V). Day 3 Tx had no effect on SAT development in WT mice. These results are consistent with those obtained using anti-CD25 (Table I) and suggest that T reg cells do not inhibit SAT in WT mice. However, most Tx NP Tg (Table V) and B cell–deficient (not depicted) mice developed SAT, indicating that effector T cells can be activated and/or expanded when T reg cells are eliminated by day 3 Tx. The two NP Tg mice in Table V that did not develop SAT had thymic remnants at autopsy and were not lymphopenic. In contrast, all Tx WT mice and the six NP Tg mice that developed SAT had minimal or no residual thymus and were T cell lymphopenic, with a 30–50% reduction in $CD4^+$ and $CD8^+$ T cells in peripheral blood and spleen relative to non-Tx littermates and a correspondingly increased percentage of $B220^+$ B cells (not depicted).

WT and B cell–deficient mice have similar numbers of $CD4^+CD25^+Foxp3^+$ T cells

The results presented so far suggest that $CD25^+$ T reg cells may be preferentially activated in SAT-resistant B cell–deficient and NP Tg NOD.H-2h4 mice, whereas SAT-susceptible WT mice may have fewer $CD25^+$ T reg cells and/or effector $CD4^+$ T cells are preferentially activated in WT mice. Foxp3 is expressed by $CD4^+CD25^+$ T cells with T reg cell function, but not by effector $CD4^+CD25^+$ T

Table V. Effect of day 3 Tx on development of SAT in WT and NP Tg mice

Mice[a]	SAT severity[b]					Anti-MTg[c]	
	0	1+	2+	3+	4+	IgG1	IgG2B
WT controls	0	0	6	2	0	0.007 ± 0.007	0.013 ± 0.005
WT day 3 Tx[d]	1	0	3	3	1	0.013 ± 0.008	0.031 ± 0.009
NP Tg controls	5	1	0	0	0	0.021 ± 0.010	0.017 ± 0.007
NP Tg day 3 Tx[d]	2[e]	2	4	0	0	0.010 ± 0.004	0.052 ± 0.053

[a]Female and male WT and NP Tg mice were thymectomized at 3 d of age. Thymectomized and littermate control nonthymectomized mice were given 0.05% NaI water beginning at 8 wk of age.
[b]Numbers of mice with various degrees of severity of SAT 8 wk after NaI water. P-values: line 1 versus line 2, $P > 0.5$; line 3 versus line 4, $P < 0.001$, excluding the two animals in line 4 with residual thymus.
[c]Anti-MTg IgG1 and IgG2B expressed as OD410 ± SEM.
[d]Most thymectomized mice had fewer CD4+ and CD8+ T cells and a correspondingly increased percentage of B220+ B cells in their spleens as determined by flow cytometry.
[e]Mice were not lymphopenic and had residual thymus at autopsy.

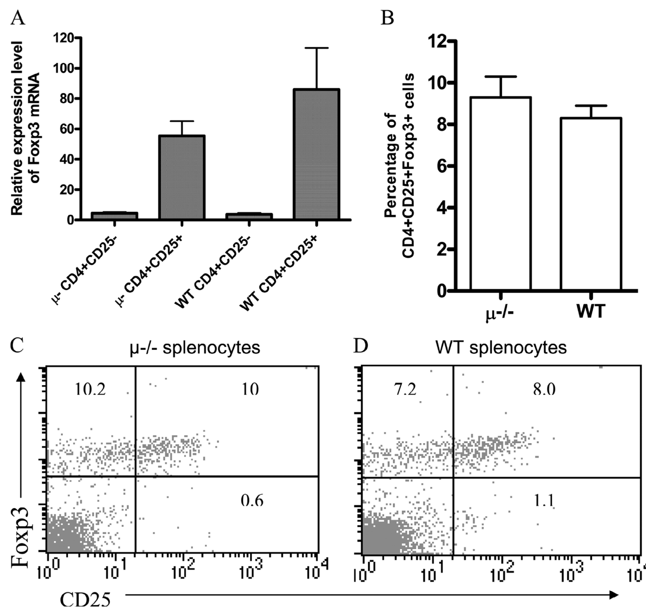

Figure 4. $CD4^+CD25^+$ T cells from WT and B cell–deficient mice express similar amounts of Foxp3. (A) Expression of Foxp3 mRNA by $CD4^+$CD25-depleted and $CD4^+$CD25-enriched cells from 6–8-wk-old naive WT and B cell–deficient NOD.H-2h4 mice. There was no significant difference in expression of Foxp3 by $CD25^+$ cells from WT and B cell–deficient mice ($P > 0.3$). (B) Spleen cells from WT and B cell–deficient mice express similar numbers of $CD4^+CD25^+Foxp3^+$ cells when analyzed by flow cytometry using a Foxp3 staining kit as described in Materials and methods. Bars represent mean percentages of $CD4^+CD25^+Foxp3^+$ cells from five individual 8-wk-old B cell–deficient (μ−/−) or WT mice. (C and D) Representative Foxp3 staining results for a B cell–deficient (C) or WT (D) mouse.

cells (31–34). To determine if differences in the numbers of $CD4^+25^+Foxp3^+$ cells might explain why WT mice are susceptible and B cell–deficient mice are resistant to SAT, spleen cells from 6–8-wk-old WT and B cell–deficient mice were separated into $CD4^+$CD25-depleted and $CD4^+$CD25-enriched populations as described in Materials and methods. RNA was isolated and reverse transcribed, and real-time PCR for Foxp3 was performed. $CD4^+CD25^+$ cells from both WT and B cell–deficient mice had a much higher expression of Foxp3 mRNA than CD25-depleted cells from the same mice (Fig. 4 A), and $CD25^+$ T cells in WT and B cell–deficient mice expressed comparable levels of Foxp3. *$CD4^+$* splenic T cells from 8-wk-old WT and B cell–deficient mice were also examined for Foxp3 expression by flow cytometry (Fig. 4, B–D) and immunohistochemical staining (not depicted). These results also indicated that WT and B cell–deficient mice have similar numbers of $CD4^+CD25^+Foxp3^+$ T cells in spleens (Fig. 4, B–D) and cervical lymph nodes (not depicted) at the time they begin NaI water. The percentages of $CD4^+CD25^-Foxp3^+$ cells that may be able to differentiate into $CD25^+$ T reg cells were also similar in the spleens of WT and B cell–deficient mice (Fig. 4, C and D). Thus, differences in the numbers of splenic $CD4^+CD25^+Foxp3^+$ cells do not explain the different susceptibility of WT and B cell–deficient mice to SAT.

DISCUSSION

This study was undertaken to test the hypothesis that resistance of B cell–deficient NOD.H-2h4 mice to SAT was due to naturally occurring T reg cells that inhibited activation and/or expansion of SAT effector T cells. The results indicate that transient depletion of $CD25^+$ T cells by anti-CD25 or depletion of naturally occurring T reg cells by day 3 Tx resulted in SAT in B cell–deficient mice. These results suggest that SAT effector T cells are not anergic in B cell–deficient mice, and if T reg cells are depleted, non–B cells can function as APCs for activation of these effector T cells.

$CD4^+CD25^+$ splenic T cells from B cell–deficient, WT (Fig. 4 A), and NP Tg mice (not depicted) highly expressed mRNA for Foxp3, a marker expressed by $CD4^+CD25^+$ T cells with regulatory function but not by $CD4^+CD25^+$ effector T cells (31–34). $CD4^+CD25^+$ T cells from WT, NP Tg, and B cell–deficient mice all had similar levels of Foxp3 mRNA (Fig. 4 A and not depicted). Flow cytometry results also indicated that the spleens of WT and B cell–deficient mice had similar numbers of $CD4^+CD25^+Foxp3^+$ as well as $CD4^+CD25^-Foxp3^+$ cells (Fig. 4, B–D), suggesting susceptible and resistant mice have comparable numbers of T reg cells. At 8 wk of age, when mice are given NaI water, effector T cells have apparently already expanded in WT mice and they develop SAT 8 wk later. In contrast, effector T cells expand in B cell–deficient and NP Tg mice only if T reg cells are transiently depleted. These results suggest that antigen-specific B cells may be needed to optimally present antigen to effector T cells in the presence of T reg cells, and even if some effector T cells are activated by other APCs, activation is suboptimal in comparison to WT mice. Therefore, even though all three strains have comparable numbers of T reg cells, T reg cell function predominates in mice lacking antigen-specific B cells, resulting in resistance to SAT.

The results presented here suggest that T reg cells have no apparent function in WT mice because depletion of $CD25^+$ T cells and day 3 Tx did not increase SAT severity in WT mice. This suggests that T reg cells in WT mice may have lost their suppressive function as suggested by others (35), or that after effector T cells become activated, they cannot be suppressed by T reg cells. The fact that B cell–deficient mice develop SAT even though $CD25^+$ T cells are depleted only transiently is consistent with the idea that effector T cells may be resistant to suppression by T reg cells. Studies are in progress to directly compare the ability of $CD4^+CD25^+$ T cells from WT and B cell–deficient mice to inhibit T cell responses in vitro and to inhibit development of SAT in vivo. It will also be important to determine if $CD4^+CD25^+$ T cells from B cell–deficient mice can inhibit SAT development in WT mice.

Previous studies established a role for B cells in the activation of autoreactive T cells in several autoimmune diseases. These studies suggested that B cells might function as APCs

for the activation of autoreactive T cells (10, 11, 17–20), or that autoreactive T cells were activated in B cell–deficient mice but did not induce tissue damage (11, 36). Alard et al. (30) and Tung et al. (37) showed that T cells able to respond to an ovarian self-antigen were present in neonatal mice. Tolerance to the antigen developed in the first week of life in females, but males that did not express the antigen did not develop tolerance. We previously hypothesized that B cell–deficient mice might not develop SAT if the antigen that initiates activation of autoreactive T cells for SAT had to be presented by B cells. In the absence of B cells, presentation of antigen by other APCs might induce unresponsiveness so autoreactive T cells would be unable to respond even if antigen-presenting B cells were subsequently provided. To test this hypothesis, we used B cell–deficient mice to ask if SAT would develop if T reg cells were depleted. The results suggest that T reg cells may be preferentially activated in SAT-resistant B cell–deficient NOD.H-2h4 mice. When T reg cells are depleted using anti-CD25 mAb or by day 3 Tx, effector T cells are activated and B cell–deficient mice develop SAT (Tables I and IV). Similar mechanisms are apparently operative in mice with Ig nonsecreting B cells specific for the NP hapten (Table II).

To our knowledge, these results are the first to demonstrate that resistance of B cell–deficient mice to autoimmune diseases can be due to the activity of T reg cells. Consistent with our results, Olson et al. (38) recently showed that B cells expanding in mice developing intestinal inflammation in a mouse model of Crohn's disease could block T reg cell activity, resulting in exacerbation of disease. B cells in their model expressed increased mRNA for GITR-L compared with B cells from control mice that did not block T reg cell activity. GITR-L, expressed by APCs including dendritic cells, B cells, and macrophages, interacts with GITR, a molecule expressed by both T reg and activated T effector cells (38–42). When GITR is engaged with its ligand, suppression is presumably "off," whereas when GITR and its ligand are not engaged, suppression is "on" (40). Our results could be explained if B cells in WT NOD.H-2h4 mice expressed more GITR-L (or another molecule able to interfere with T reg cell activity) than B cells from NP Tg mice or non–B cell APCs in NP Tg or B cell–deficient mice. Although further studies are needed to fully address this possibility, preliminary results indicate that splenocytes from WT, B cell–deficient, and NP Tg mice express similar levels of GITR-L mRNA (not depicted).

Another unexpected and interesting observation in this study was that depletion of T reg cells in NP Tg mice resulted in very severe SAT with many B220$^+$ B cells in thyroids (Fig. 3). These results indicate that B cells can traffic to sites of inflammation even if they have no apparent specificity for thyroid antigens and are unable to secrete autoantibodies. The role played by presumably antigen-nonspecific B cells in the thyroids of NP Tg mice is unknown, although they may be able to present thyroid antigens to effector T cells in sites where the antigen is abundant, as in the thyroid. Antigen-nonspecific B cells also migrate to the pancreas in NP Tg B cell–deficient nonobese diabetic (NOD) mice, but the incidence of diabetes was very low compared with that in WT NOD mice (43). In a model of autoimmune arthritis with a requirement for B cells for development of disease, B cells from NP Tg mice functioned as APCs for activation of T cells only if autoantigen was specifically targeted to B cells to increase antigen uptake (20). This suggests that conjugating MTg, the putative autoantigen for SAT, to NP might result in increased uptake of MTg by NP-specific B cells, and NP Tg mice might then develop SAT. Studies to address this possibility are in progress. In addition to the difference in antigen specificities of the B cells in NP Tg and WT mice, mice lacking serum IgM reportedly have more marginal zone B cells and fewer follicular B cells (44), and differences in B cell subsets could also influence whether T reg or T effector cells would be preferentially activated.

Several studies have shown that CD4$^+$CD25$^+$ T cells can regulate experimental autoimmune thyroiditis in mice (45–49). In some cases, T reg cells were presumably induced by MTg presented peripherally on particular subsets of dendritic cells (47, 48) or by MTg presented under tolerogenic conditions (45). In contrast, the T reg cells described here presumably belong to the class of "natural" T reg cells because their function is lost after day 3 Tx (21, 22). These natural T reg cells might be activated by Tg expressed in the thymus during T cell development (50, 51). Further studies will be required to determine if T reg cells that inhibit SAT in B cell–deficient and NP Tg mice are specifically activated by MTg or another thyroid antigen, and whether they are activated in the thymus or in peripheral lymphoid organs (52).

MATERIALS AND METHODS

Mice. NOD.H-2h4 mice, originally provided by L. Wicker (University of Cambridge, Cambridge, UK), are I-E negative and express I-A^k, H-2K^k, and D^d on the NOD background (1–4). B cell–deficient NOD.H-2h4 mice were derived as described previously (5). NOD mice expressing Tg B cells specific for the NP hapten and unable to secrete Ig (12, 43) were obtained from S. Wong (Yale University, New Haven, CT). They were crossed with B cell–deficient NOD.H-2h4 mice to generate NP Tg mice on the NOD.H-2h4 B cell–deficient background. The mice are serum Ig negative and have normal numbers of peripheral B220$^+$ B cells that express the NP transgene (12, 20). All mice were bred and maintained under specific pathogen-free conditions in our animal facilities at the University of Missouri. All animal protocols were approved by the University of Missouri and the VA Animal Care and Use Committees. Both male and female mice were used, and mice were age- and sex-matched for each experiment. All mice received 0.05% NaI in their drinking water beginning at 7–8 wk of age.

Antibody treatment, Tx, and experimental design. Mice were given 3 weekly 0.5-mg injections of rat anti–mouse CD25 mAb PC61 (American Type Culture Collection [ATCC]) beginning in most experiments 10–11 d after birth (23). In some experiments, antibody treatments began later or mice were given rat anti–mouse IL-10 mAb JESS 2A5 (ATCC) as indicated in Results. Anti-CD25 and anti–IL-10 were purified from cell culture supernatants using Protein G columns (Kierkegaard and Perry). At 8 wk of age, treated and control mice were given 0.05% NaI water and thyroids were removed 8–9 wk later (1, 5). For some experiments, mice were thymectomized 3 d after birth as described previously (29). In brief, 3-d-old mice were anesthetized on ice, chests were opened, and thymic lobes were

removed by suction. Mice were given 0.05% NaI water at 8 wk of age, and thyroids were removed 8–9 wk later. At the time of thyroid removal, the thoracic cavity was carefully inspected and mice with obvious thymic remnants were excluded from the experiments.

Assessment of thyroiditis. Thyroids were collected and one thyroid lobe from each mouse was fixed in formalin, sectioned, and stained with hematoxylin and eosin as described previously (1, 5). The other thyroid lobe from each mouse was snap frozen in liquid nitrogen and stored at −70°C for use in immunohistochemical staining. Thyroid histopathology was scored for the extent of thyroid follicle destruction using a scale of 0 to 5+ as described previously (1, 4, 5). All slides were coded and read by two individuals, one of whom had no knowledge of the experimental groups. In brief, a score of 0 indicates a normal thyroid, and 0+ indicates a few inflammatory cells infiltrating the thyroids and/or mild follicular changes. A 1+ severity score is defined as an infiltrate of at least 125 cells in one or several foci, and a 2+ score represents 10–20 foci of cellular infiltration, each the size of several follicles, with destruction of up to one fourth of the gland. A 3+ score indicates that one fourth to one half of the thyroid follicles are destroyed or replaced by infiltrating inflammatory cells, whereas a 4+ score indicates that greater than one half of the gland is destroyed. A few thyroids had almost no remaining intact follicles, and these were given a score of 5+. As described previously (1), thyroid lesions in NOD.H-2h4 mice reach maximal severity 7–9 wk after mice are given NaI water beginning at 2 mo of age; lesions are chronic and remain relatively unchanged in severity for several months (1, 4).

Autoantibody determination. MTg-specific autoantibodies were assessed by ELISA using serum from individual mice as described previously (1, 5).

Flow cytometry. Spleen cells or PBLs were analyzed for expression of CD4, CD8 and CD25 by flow cytometry as described previously (5). FITC-conjugated antibodies were obtained from Caltag Laboratories. PE-conjugated anti-CD25 was obtained from SouthernBiotech (7D4) or Caltag Laboratories (PC 61). Flow cytometry for Foxp3 was performed using splenocytes from 8-wk-old NOD.H-2h4 WT or B cell–deficient mice using a Foxp3 staining kit according to the manufacturer's protocol (eBioscience). Antibodies used were FITC-conjugated anti-CD4 (Caltag Laboratories), PECy5-conjugated CD25 (eBioscience), and PE-conjugated Foxp3 (eBioscience). Cells were analyzed using a FACScan (BD Biosciences).

Separation of $CD4^+CD25^+$ and $CD4^+$CD25-depleted splenocytes. Spleen cells from 6–8-wk-old naive WT and B cell–deficient mice were separated into $CD4^+CD25^+$ and $CD4^+$CD25-depleted populations as follows. Mice were given anti-CD8 mAb 3 d before use to deplete splenic $CD8^+$ T cells. Spleen cells were depleted of most B cells and macrophages by passage over nylon wool columns. Nylon wool nonadherent WT cells typically expressed no detectable $CD8^+$ T cells and <15% B cells, and cells from B cell–deficient mice were >90% $CD4^+$ when analyzed by flow cytometry. $CD4^+CD25^+$ cells were positively selected by labeling with PE-conjugated PC61 and separated into $CD25^+$ and CD25-depleted populations using Easy Sep magnetic beads (StemCell Technologies Inc.). Cells recovered in the $CD25^+$ fraction comprised <10% of the total $CD4^+$ T cells, and ~85% were $CD4^+CD25^+$ as determined by flow cytometry. The CD25-depleted population comprised >90% of the $CD4^+$ T cells and always had some residual $CD25^+CD4^+$ cells as determined by flow cytometry. Cells were counted, pelleted, and snap frozen in liquid nitrogen at $2–4 \times 10^6$ cells per tube.

Quantitative PCR. RNA was isolated using TRIzol (Invitrogen) and cDNA was synthesized as described previously in detail (1). Quantitative PCR was performed using ABsolute QPCR SYBR Green ROX Mix (ABgene) in an ABI PRISM 7000 sequence detection system (Applied Biosystems). A series of five standards with defined values was included in each reaction and a standard curve was obtained to calculate the amount of gene amplified. A dissociation curve was generated to verify the amplification of a single product. The primer sequences for Foxp3 and the housekeeping gene HPRT were described previously (1, 53). The level of HPRT expression for each sample was used for data normalization.

Immunohistochemical staining. Immunohistochemical staining of frozen thyroid sections was performed as described previously (4, 24) using the following primary antibodies: anti-CD4 (GK 1.5; ATCC), anti-CD8 (53.67; ATCC), anti-CD11b (CRL 1969; ATCC), or anti-B220 (Caltag Laboratories). Biotinylated goat anti–rat IgG (Caltag Laboratories) was used as secondary antibody, and 0.3% hydrogen peroxide was used to block endogenous peroxidase. Sections were incubated with Vectastain Elite ABC kit (Vector Laboratories), and peroxidase activity was visualized using the Nova-Red substrate (Vector Laboratories). Slides were counterstained with hematoxylin. Negative controls used IgG isotype controls as primary antibody, with the remaining steps performed as described above. These controls were always negative.

Student's *t* test. Statistical analysis was performed using an unpaired two-tailed Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

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