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Many individuals develop a single or a few brief episodes of autoimmunity from which they recover. Mechanisms that quell pathologic autoimmunity following such a breakdown of self-tolerance are not clearly understood. In this study, we show that in nonautoimmune mice, dsDNA-specific autoreactive B cells exist but remain inactive. This state of inactivation in dsDNA-specific B cells could be disrupted by autoreactive Th cells; in this case T cells that react with peptides from the V_H region of anti-DNA Abs (hereafter called anti-V_H T cells). Immunization with anti-DNA mAb, its \( \gamma \)-chain or peptides derived from its V_H region induced anti-V_H Th cells, IgG anti-dsDNA Ab, and proteinuria. The breakdown of B cell tolerance in nonautoimmune mice, however, was short-lived: anti-DNA Ab and nephritis subsided despite subsequent immunizations. The recovery from autoimmunity temporally correlated with the appearance of T cells that inhibited anti-DNA Ab production. Such inhibitory T cells secreted TGF-\( \beta \); the inhibition of anti-DNA Ab production by these cells was partly abolished by anti-TGF-\( \beta \) Ab. Even without immunization, nonautoimmune mice possess T cells that can inhibit autoantibody production. Thus, inhibitory T cells in nonautoimmune mice may normally inhibit T-dependent activation of autoreactive B cells and/or reverse such activation following stimulation by Th cells. The induction of such inhibitory T cells may play a role in protecting nonautoimmune mice from developing chronic autoimmunity.

S
elf-reactive B and Th cells exist in the normal immune repertoire, yet most individuals avoid pathologic autoantibody production. Some otherwise healthy individuals develop a single or a few brief episodes of autoimmunity from which they recover (1), whereas chronic autoantibody production is characteristic of individuals with lupus (2). Some autoantibodies, such as Ab against DNA, cause tissue damage (3); their production is largely dependent on Th cells (4–7).

To understand the mechanisms that prevent autoreactive B cells from producing Ab, several laboratories have generated transgenic mice in which most B cells express Igg of a single specificity. These studies have identified several mechanisms, such as clonal deletion and anergy, that protect mice from breaking self-tolerance in B cells (8–13). Not many studies, however, have addressed the protective mechanisms that come into play once self-tolerance has been broken in normal mice.

Here, we ask: 1) Can B cells in nonautoimmune mice be activated to secrete anti-DNA Ab by exposure to autoreactive Th cells? 2) Will such a breakdown in the state of inactivation in B cells cause persistent anti-DNA Ab production and disease in these mice? 3) Can these mice develop protective mechanisms that contain or reverse the loss of self-tolerance in B cells?

To address these questions, we used a nontransgenic model in which MHC class II-restricted Th cells help B cells produce IgG anti-DNA Ab (4); the (NZB × NZW) F_1 (BWF_1) strain of lupus-prone mice; and two nonautoimmune mice that have MHC class II molecules identical to those in BWF_1 mice. Using this system, we show that nonautoimmune mice, upon persistent exposure to autoreactive Th cells, develop inhibitory T cells that can inhibit autoantibody production and renal disease.

Materials and Methods

Mice

NZB/B1nj, NZW/Lacj, BALB/c, and B10.PL mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and intercrossed to generate BWF_1 (NZB × NZW) F_1, CWF_1 (BALB/c × NZW) F_1, and BPWF_1 (NZB × B10.PL) F_1 mice in the Cincinnati Veterans Affairs Medical Center or University of California, Los Angeles Vivarium. Female mice were used in all experiments. Experiments were performed in accordance with the institutional animal research committee guidelines.

Peptides

15-mer overlapping peptides representing the entire V_HD region sequence of an anti-dsDNA mAb, A6.1, were synthesized using a modified pin synthesis or a macrocrown method (14). Each 15-mer peptide overlapped its neighbor by 10 residues. Several 11- to 15-mer T cell epitopes and control peptides (Table I) were synthesized in large quantities at the Chiron Laboratories (Clayton, Australia), using F-moc chemistry. The synthetic peptides were analyzed for purity by HPLC and by mass spectrometry. Each peptide chromatographed essentially as a sharp single peak. All purified peptides had the expected molecular mass.

Immunization of mice

Young CWF_1 or BWF_1 mice were immunized i.p. with 20 \( \mu \)g of A6.1 (purified from ascites fluid as IgG), emulsified in CFA, and boosted with 10–20 \( \mu \)g of A6.1 in IFA, as described in the figure legends.
Purification of cells
Spleen cells were pooled from 3 to 12 mice and purified as mononuclear cells on Ficoll-Hypaque. Enrichment of CD4+ , CD8+ , and B cells was performed with the Vario MACS magnetic purification system using microbead-coated Ab (Miltenyi Biotec, Auburn, CA). Purity of the cell populations as determined by FACS varied from 92 to 99%.

Determination of T cell help for anti-DNA Ab synthesis by ELISPOT and ELISA
Sonicated, nitrocellulose-filtered calf thymus dsDNA (100 μg/ml; Sigma-Aldrich, St. Louis, MO) was coated onto 96-well microtiter plates (catalogue no. 3590; Costar, Cambridge, MA) and incubated overnight at 4°C. After washing with PBS, DNA-coated plates were blocked for 1 h with 10% FCS in PBS at room temperature. Splenic T and B cells in complete medium (DMEM with 10% FBS) and varying concentrations of peptides were added to each well. After 8–12 h of incubation at 37°C, cells were poured off, plates were washed eight times, and plates were then incubated overnight at 4°C with alkaline phosphatase-conjugated antigo IgG or IgA, diluted 1/500 in 10% FCS in PBS. Plates were washed again with Tris/Tween 20, and incubated with a mixture of 5-bromo-4-chloro-3-indolyl phosphate with agarse gel at a final concentration of 0.6%. Anti-dsDNA Ab-forming cells (AFC) were enumerated as blue spots using an inverted microscope (Olympus America, New York, NY) by two of us (F.M.E. and R.R.S.) in a blinded fashion and recorded as number of AFC per 104 B cells. The specificity of anti-dsDNA ELISPOT was confirmed by competitive inhibition studies; the anti-dsDNA ELISPOT was inhibited by DNA but not with cardiolipin, keyhole limpet hemocyanin, or hen egg lysozyme (HEL) (15–17). The anti-dsDNA ELISPOT was inhibited by both dsDNA and ssDNA but it required two times more ssDNA than dsDNA for 50% inhibition (15, 17). This is consistent with observations that some populations of anti-DNA Abs share dsDNA and ssDNA binding specificities.

The results of ELISPOT assays for anti-dsDNA Ab were further confirmed in simultaneous ELISA. Briefly, spleen cells were cultured with or without peptides for 5 days; supernatants were tested for IgG anti-dsDNA Abs, as described previously (3, 4). Briefly, 96-well plates (Costar) were coated with dsDNA (100 μg/ml; Sigma-Aldrich) overnight at 4°C. After blocking with 10% FCS, samples were added. Bound Ab was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Fisher, Pittsburgh, PA). Reactions were developed with p-nitrophenyl phosphate (Sigma-Aldrich) and 405 was measured with an ELISA reader. The results of ELISA and ELISPOT assays correlated well with each other (15).

Establishing T cell lines
Splenic single-cell suspensions from the CWF1 or BWF1 mice immunized with A6H31–45 were fractionated into CD4+ or CD8+ T cells. The fractionated CD4+ and CD8+ T cells were cultured and periodically stimulated with syngeneic APCs (irradiated T cell-depleted spleen cells). A6H31–45 and Con A supernatant, as described previously (4).

B cell hybridomas
Four B cell hybridomas, including a dsDNA-specific (A6.1) and three non-DNA-specific (Jates 375-57 and 375-100) hybridomas, were established from BWF1 mice by fusion of their spleen cells with a nonsecreting murine myeloma cell, as described previously (3, 18).

Cytokine assays by ELISA
Resting T cell lines were cultured with APCs and peptide; supernatants were tested for cytokines by ELISA, as previously described (19). TGFβ was assayed by sandwich ELISA using a TGFβ1-specific kit (G1230; Promega, Madison, WI) that is designed to measure biologically active TGFβ1. To assay for total TGFβ1, the samples were acid treated and neutralized according to the manufacturer’s instructions (www.promega.com). In brief, multwell ELISA plates (Nunc Maxisorp; Nunc, Naperville, IL) were coated with 100 μl of an anti-TGFβ1 mAb in carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed three times with Tris-HCl (pH 7.6) containing 0.05% Tween 20, followed by blocking for 35 min at 37°C. Standards and samples were added, and plates were incubated for 90 min at room temperature. Plates were washed five times before the addition of 100 μl of a second polyclonal anti-TGFβ1 Ab. After 2 h at room temperature, plates were washed and 100 μl of a tertiary HRP-conjugated species-specific Ab was added. The plates were incubated for 2 more hours, then washed six times, and 100 μl of peroxidase substrate/tetramethylbenzidine solution was added. Fifteen minutes later, the reaction was stopped with 1 M phosphoric acid. A405 was measured with a L absystem ELISA reader.

Assessment of clinical disease
Proteinuria and blood urea nitrogen were tested using Alustix and Azostix (range, 0–4+), respectively, and creatinine was estimated by reagents from Stanbio Laboratories (San Antonio, TX), as described previously (4, 20).

Statistical analysis
Student’s t test was performed to compare the test and control groups. A Shapiro-Wilk test of normality was performed to determine whether the distribution of the effect of CWF1-derived T cell lines on anti-DNA Ab production (see Fig. 4C) matches a normal distribution.

Results
F1 mouse strains that express identical MHC class II molecules to those of BWF1 do not develop anti-DNA Ab and proteinuria
The BWF1 mice, which are the F1 intercross of NZB (H-2b) and NZW (H-2d) mice, develop an autoantibody-mediated disease that shares many features with human lupus (2, 4). The autoantibody production in these mice is mostly dependent on MHC class II-restricted TCROβ+ cells (2–4). To determine whether F1 mouse strains that express MHC class II molecules (H-2k–k) identical to those of BWF1 mice develop a lupus-like disease, we crossed BALB/c (H-2d) with NZW mice to generate CWF1 mice. Since NZW class II molecules have an identical sequence to the H-2k–k haplotype (21), we crossed NZB with B10.PL (H-2u–u) to generate another F1 strain, BPF1, that expresses H-2k–k also. Twenty BWF1, 20 CWF1, and 32 BPF1 female mice were monitored for serum IgG anti-DNA Ab and proteinuria up to 1 year of age. All BWF1 mice had anti-DNA Ab by 15 wk of age, proteinuria by 28 wk, and died by 42 wk. In contrast, all CWF1 and BPF1 mice appeared to be healthy during the study period; <10% had anti-DNA Ab, but none had proteinuria. A similar observation in BPF1 mice has been reported by others (21).

Auto-reactive B and Th cells are inactive or tolerant in nonautoimmune mice
To confirm the absence of IgG anti-DNA Ab-secreting B cells in naive nonautoimmune mice, we cultured spleen cells from CWF1, BPF1, or BWF1 mice for 8–12 h in microtiter plates coated with DNA and estimated the numbers of IgG anti-DNA AFC by ELISPOT. Less than 10 AFC per 106 B cells were detected in CWF1 or BPF1 mice, while BWF1 cultures had 180 AFC (Fig. 1, T plus B). Similar results were obtained when spleen cells from these mice were cultured for 5 days and supernatants were tested for anti-DNA Ab in an ELISA.

Auto-reactive, anti-VH Th cells that recognize peptides produced from the VH of anti-DNA Ig molecules (4, 20, 22–27) arise spontaneously in humans and mice with systemic lupus erythematosus (6, 20, 28) and modulate the development and progression of disease in lupus mice (4, 6, 20, 29, 30). These MHC class II-restricted, anti-VH Th cells increase anti-DNA Ab production by BWF1 B cells (15). To determine whether such anti-VH Th cells exist in nonautoimmune mice, splenic T and B cells from naive CWF1, BPF1, or BWF1 mice were cultured with VH peptides (Table I), and IgG anti-DNA Ab production was estimated by ELISPOT (Fig. 1). In the presence of T cell stimulatory peptides, the anti-DNA AFC significantly increased in BWF1, but not in CWF1 or BPF1 mice. Thus, in contrast to BWF1 mice, MHC class II-identical CWF1 and BPF1 mice do not exhibit spontaneous activation of DNA-specific B and anti-VH Th cells. These cells may either be absent, ignorant, or tolerant in these mice.
FIGURE 1. B cells from nonautoimmune mice that express identical MHC molecules to those of lupus-prone BWF1 mice do not spontaneously secrete IgG anti-DNA Ab. Splenic B cells pooled from 15- to 20-wk-old, naive nonautoimmune (CWF1 and BPF1) or lupus-prone BWF1 mice (n = 3–5) were cultured with autologous T cells and without (T plus B) or with V_{H}-derived T cell determinant (A6H34–45, A6H58–69, and A6H84–95) or control (A6H93–107) peptides (Table I). IgG anti-DNA AFC were enumerated by ELISPOT assay. Results are expressed as mean AFC number per 10^6 B cells in triplicate wells; deviations from the mean were <12%. Anti-DNA AFC in cultures containing B cells alone, with or without added peptides ranged from 20 to 50, 0, and <5 per 10^6 B cells in BWF1, CWF1, and BPF1, respectively (data not shown). Results from one of four similar experiments are shown here.

Th cells disrupt B cell self-tolerance (or inactive state) in nonautoimmune mice

In four separate experiments, CWF1 or BPF1 B cells were cultured with BWF1 T cells and vice versa. Strikingly, CWF1 and BPF1 B cells were induced to produce IgG anti-DNA Ab in the presence of BWF1 T cells (Fig. 2A, left panel). In contrast, T cells from the nonautoimmune mice did not promote anti-DNA Ab production by BWF1 B cells (Fig. 2A, right panel). This indicates that DNA-specific B cells exist in nonautoimmune mice and can be activated to secrete IgG anti-DNA Ab by autoimmune Th cells in vitro.

Next, we determined whether autoreactive Th cells break tolerance in DNA-specific B cells in vivo and induce lupus-like disease in nonautoimmune mice. First, immunization with a mAb anti-DNA, A6.1, or its γ-chain or V_{H} epitopes (listed in Table I) in adjuvant induced V_{H} peptide-specific T cell proliferative or Ab responses in CWF1 mice (data not shown), suggesting that anti-V_{H} Th cell repertoire exists in nonautoimmune mice. To determine whether these in vivo-primed anti-V_{H} Th cells can promote anti-DNA Ab production, 20-wk-old CWF1 mice (n = 12) were immunized with A6.1 in CFA and boosted twice at 2-wk intervals with A6.1/JFA. Splenic T cells from these mice were cultured with syngeneic B cells without or with 23 overlapping 15-mer peptides representing the entire V_{H} sequence of the A6.1 (Fig. 2B). Results show that three peptides (peptides 7–9) increased anti-DNA AFC 4- to 5-fold above the background. Importantly, all immunized CWF1 mice developed high levels of serum anti-DNA Ab (Fig. 2C) and most developed renal involvement. Three of 20 mice had a severe nephritis (proteinuria, 300 mg/dl with increased blood urea nitrogen (80 mg/dl) and creatinine (1.8 mg/dl)); 14 developed a mild disease (proteinuria, 100 mg/dl with normal blood urea nitrogen (<20 mg/dl) and creatinine (<0.6 mg/dl); and 3 remained normal. Injection of a control, nonautoreactive mAb, MOPC-21, did not induce IgG anti-DNA Ab or proteinuria (Ref. 3 and data not shown). This suggests that potentially autoreactive B cells in nonautoimmune mice can be activated to secrete pathogenic IgG anti-DNA Ab by appropriate Th cells.

To exclude the possibility that serum anti-DNA titers in A6.1-immunized mice (Fig. 2C) were simply coming from the injected IgG2a mAb, we showed that the IgG anti-DNA Abs of all IgG isotypes were detected in the immunized CWF1 mice (data not shown).

Breakdown of self-tolerance in B cells in nonautoimmune mice is transitory in nature

The induction of serum IgG anti-DNA Ab and mild proteinuria upon immunizations with A6.1 prompted us to test whether persistent anti-DNA response and severe nephritis would be induced following chronic immunization of CWF1 mice. Serum anti-DNA Ab levels did increase between immunizations two and four, and 75% (8 of 12) of the mice developed proteinuria (100–300 mg/dl) with normal to mildly elevated plasma creatinine (mean ± SD, 0.9 ± 0.3). Thereafter, serum anti-DNA levels decreased (Fig. 3A). All immunized mice slowly recovered from disease by the sixth immunization, at which time all had ≤30 mg/dl proteinuria and blood urea nitrogen and creatinine in the normal range. Subsequent immunizations did not induce a vigorous anti-DNA Ab response (Fig. 3A) or proteinuria. This suggests that, although CWF1 mice are susceptible to breakdown of B cell tolerance, this loss of tolerance is self-limited.

We then demonstrate that the in vivo decrease in anti-DNA Ab temporally correlates with the disappearance of anti-V_{H} Th responses ex vivo. At different time points after immunizations, CWF1 spleen cells were cultured with 23 overlapping A6.1 V_{H} peptides. After the second and third immunizations, culture with several peptides increased anti-DNA Ab ≥2-fold of the background. Thereafter, none of the peptides elicited increases in anti-DNA production (Fig. 3B).

Activation of inhibitory T cells is involved in inhibiting autoantibody response

Multiple mechanisms may contribute to the decrease in anti-DNA Ab production after repeated immunizations: 1) induction of inhibitory or regulatory T cells or cytokines; 2) induction of anergy or deletion in anti-V_{H} Th cells; 3) induction of regulatory antiidiotypic Ab; and 4) development of Ab against A6.1 and formation of A6.1-anti-A6.1 immune complexes that could alter Ag processing and presentation. Although we have not excluded the relative contribution of each of these possibilities, we demonstrate several lines of evidence supporting the activation of inhibitory T cells that down-regulate anti-DNA Ab production.

First, we demonstrate that the freshly isolated T cells from the “recovered” CWF1 mice decrease anti-DNA Ab production by

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Source (Ref. 3)</th>
<th>Sequence (Refs. 3 and 15)</th>
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<td>A6.1 V_{H}</td>
<td>GYFMNWVKQSHGKSL</td>
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<tr>
<td>HYHEL34–48</td>
<td>HYHEL3–V_{H}</td>
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</tr>
<tr>
<td>HEL106–116</td>
<td>HEL</td>
<td>NAWVAVRRCK</td>
</tr>
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*These peptides are derived from the V_{H} region of a BWF1-derived anti-DNA mAb, A6.1 (3); they stimulate spontaneous T cell proliferation and help for anti-DNA Ab in unimmunized BWF1 mice (14, 15, 20).

* Controls used were: nonstimulatory peptides from the same V_{H} (A6.1), such as A6H93–107; peptides, such as HYHEL31–45, from a non-autoreactive mAb, HYHEL-5 that uses the same V_{H} gene family as does A6.1 (15); and a foreign peptide, HEL106–116. The control peptides do not induce spontaneous activation of BWF1 T cells but can elicit immune response when injected in vivo with adjuvant (4). Additionally, the control peptides do not increase anti-DNA Ab formation by BWF1 or CWF1 spleen cells.
B cell self-tolerance can be disrupted in nonautoimmune mice, as demonstrated in vitro (A), ex vivo (B), and in vivo (C). A, Autoimmune Th cells activate nonautoimmune mouse B cells in vitro. T and B cells were separately pooled from 20-wk-old mice (n = 3–12) of different strains. BWF1 T cells were incubated with B cells from CWF1 or BPF1 mice (left panel) and vice versa (right panel). T cell determinant or control peptides (Table I) were added to the cultures. Results are expressed as the mean triplicate IgG anti-DNA AFC number per 10^6 B cells without (○) or with 23 overlapping peptides from the A6.1 VH of A6.1 mAb for 5 days. Supernatants were tested for IgG anti-DNA Ab. Results are shown as the mean ± SD of OD at 1/25 serum dilution. After the second and fourth immunizations, the immunized mice had increased anti-DNA Ab compared with the controls (▲, p < 0.05; ●, p < 0.001). However, anti-DNA Ab in 8 of 10 mice returned to the background level when tested after the sixth immunization (●, p > 0.01 compared with anti-DNA Ab level after the fourth immunization) and remained low despite subsequent immunizations (●, p < 0.05). B, Fifteen young (12-wk-old) CWF1 mice were immunized with A6.1/IFA and boosted every 2–4 wk with A6.1/IFA. Five mice each were sacrificed after the second, third, and fifth immunizations. Their pooled splenic T and B cells were cultured with 23 overlapping peptides from the V_H of A6.1 mAb for 5 days. Supernatants were tested for IgG anti-DNA Ab. Results are expressed as the mean triplicate OD from one of two similar experiments.

BWF1 spleen cells in a peptide-specific manner (Fig. 4A). This decrease in AFC was not due to a changed proportion of T plus B cells or crowding, since addition of the same numbers of BWF1 T cells or CWF1-derived, HEL106–116-reactive T cells to BWF1 T plus B cell cultures did not affect anti-DNA AFC numbers (Fig. 4A and data not shown).

Second, we asked whether a similar immunization regimen would activate inhibitory T cells in lupus mice (Fig. 4B). In two independent experiments, 8–10 short-term T cell lines were established from pooled spleen cells of 5 each of CWF1 or BWF1 mice that were immunized with A6H31–45 (stimulatory peptide, no. 7 in Fig. 2B) five times at 2-wk intervals. Three sets of independent, resting short-term T cell lines (in varying numbers from 10^2 to 10^3) were cocultured with 10^5 B cells from 26- to 32-wk-old unimmunized BWF1 mice. Results show that while CWF1-derived T cell lines decreased anti-DNA production by BWF1 B cells in a dose (T cell number)-dependent manner, BWF1-derived T cell lines increased anti-DNA Ab production (Fig. 4B). This suggests that the development of inhibitory T cells may be inadequate or defective in BWF1 mice.

Third, 42 long-term lines or clones were established by subcloning CWF1-derived short-term T cell lines and tested for the ability to influence anti-DNA Ab production by BWF1 B cells (Fig. 4C). Although only 2 (5%) T cell lines increased anti-DNA AFC numbers by 2-fold, 13 (31%) T cell lines decreased the AFC numbers to <50% of the background. The remaining T cell lines had no or <2-fold effect on anti-DNA Ab production (Fig. 4C). This distribution of the effect of T cell lines on anti-DNA Ab production did not match a normal distribution (p = 0.003, Shapiro-Wilk test). Thus, both inhibitory T and Th cells coexist in CWF1 mice.

FIGURE 3. The breakdown of B cell self-tolerance in nonautoimmune mice is transitory: Nonautoimmune mice lose ability to make IgG anti-DNA Ab despite multiple immunizations. A, Ten 10-wk-old CWF1 mice were immunized with A6.1 in CFA and boosted every 2–4 wk with A6.1/IFA. These (●) and 10 age-matched unimmunized controls (□) were monitored for serum anti-DNA Ab. Results are shown as the mean ± SD of OD at 1/25 serum dilution. After the second and fourth immunizations, the immunized mice had increased anti-DNA Ab compared with the controls (▲, p < 0.05; ●, p < 0.001). However, anti-DNA Ab in 8 of 10 mice returned to the background level when tested after the sixth immunization (●, p > 0.01 compared with anti-DNA Ab level after the fourth immunization) and remained low despite subsequent immunizations (●, p < 0.05). B, Fifteen young (12-wk-old) CWF1 mice were immunized with A6.1/IFA and boosted every 2–4 wk with A6.1/IFA. Five mice each were sacrificed after the second, third, and fifth immunizations. Their pooled splenic T and B cells were cultured with 23 overlapping peptides from the V_H of A6.1 mAb for 5 days. Supernatants were tested for IgG anti-DNA Ab.
Inhibition of autoantibody production by some inhibitory T cells is partly dependent on TGFβ

TGFβ is an immunoregulatory cytokine that can inhibit autoantibody production (31, 32). To determine whether inhibitory T cells selectively secrete this cytokine, we cultured APCs with peptide and eight T cell lines each from CWF1 and BWF1 mice and measured TGFβ in culture supernatants (Fig. 5A). Collectively, CWF1-derived T cell lines secreted higher levels of TGFβ than BWF1-derived T cell lines ($p < 0.05$, Student’s $t$ test). Essentially similar results were obtained with another set of T cell lines. Interestingly, all CD8$^+$ inhibitory T cell lines tested secreted 2- to 10-fold higher amounts of TGFβ when compared with all CD4$^+$ T cell lines from CWF1 or BWF1 mice.

We then determined whether TGFβ secreted by CWF1-derived T cells contributes to the inhibition of autoantibody production. First, we show that splenic T cells from the hyperimmunized CWF1 mice when cocultured with BWF1 T plus B cells decreased the IgG anti-DNA AFC number. The addition of an anti-TGFβ

**FIGURE 4.** Nonautoimmune mice develop T cells that down-regulate anti-DNA Ab production. A, BWF1 spleen cells were cultured with a T cell epitope (A6H31−45, peptide 7 in Fig. 2B) or control (A6H110−121, peptide 23 in Fig. 2B) peptide. To these cultures were added $10^5$ freshly isolated T cells from CWF1 mice that were immunized five to eight times with A6.1 or from unimmunized BWF1 mice (control; []). Results are expressed as the mean triplicate IgG anti-DNA AFC per $10^6$ B cells. The addition of CWF1 T cells to BWF1 spleen cell cultures decreased anti-DNA AFC 2-fold ($p < 0.001$), but the AFC numbers decreased below the background level when T cells from similarly immunized BWF1 mice () promote anti-DNA Ab production. Results are expressed as the mean triplicate IgG anti-DNA AFC per $10^6$ B cells from one of two similar experiments; deviations from the mean were $<10%$. Similar results were obtained when using another two sets of short-term T cell lines from CWF1 and BWF1 mice. C, Although most long-term T cell lines and clones from CWF1 mice inhibit anti-DNA Ab production, some promote autoantibody production. The resting T cell lines or clones that were established by subcloning short-term lines (shown in B) were cocultured with A6H31−45 and BWF1 splenic T plus B cells. Results are expressed as the mean IgG anti-DNA AFC per $10^6$ B cells. The first bar on the left (BWF1 T plus B cells without any added peptide) represents the background AFC number. The horizontal dashed line crossing this figure denotes one-half of the background AFC number. We arbitrarily used this cut off to label the T cell lines as inhibitory ( ), which included four CD4$^+$ and nine CD8$^+$ T cell lines. Two T cell lines, both CD4$^+$, increased anti-DNA AFC 2-fold above the background (labeled arbitrarily as helper T cell lines). The remaining T cell lines that included 15 CD4$^+$ and 12 CD8$^+$ T cell lines had no or $<2$-fold effect on AFC ( ). Data are representative of two separate experiments.

**FIGURE 5.** TGFβ1 may partly mediate the inhibition of anti-DNA Ab production by inhibitory T cells. A, Some inhibitory T cells secrete TGFβ1. Resting T cell lines ($n = 8$ each) from CWF1 or BWF1 mice were cultured with APC and A6H31−45. Results are expressed as the mean total TGFβ1 (picograms per milliliter) in culture supernatants. CWF1-derived CD8$^+$ inhibitory T cell lines (CWF1 #1 to CWF1 #4) secrete 2- to 10-fold more TGFβ than syngeneic CD4$^+$ inhibitory T cell lines (CWF1 #5−CWF1 #8) or CD4$^+$ and CD8$^+$ T cell lines (BWF1 #9−16) from BWF1 mice. B, Inhibition of anti-DNA Ab production by CWF1-derived T cells is abolished by anti-TGFβ Ab. Splenic B plus T cells from six BWF1 mice were cultured in microtiter plates coated with DNA ( ). Results are shown as the mean ± SE of IgG anti-DNA AFC per $10^6$ B cells from representative one of two independent experiments. Addition of peptide A6H31−45 increased anti-DNA AFC ($p < 0.001$), but the AFC numbers decreased below the background level when T cells from eight times immunized CWF1 mice ($n = 5$) were added to these cultures ( , $p < 0.001$). This inhibition of anti-DNA Ab production by CWF1 T cells was abolished by the addition of an anti-TGFβ mAb (1 μg/ml 1.D.11.16, , $*$, $p < 0.001$ as compared with the or (an isotype-matched control rat IgG1)).
mAb (1.D.11.16; Celltrix, Santa Clara, CA), but not an isotype-matched control Ig, to these cultures abolished the inhibition of anti-DNA Ab (Fig. 5B). The TGFβ effect on the inhibition of IgG anti-DNA Ab production was not associated with the isotype skewing of anti-DNA Ab toward IgA (data not shown).

Naive CWF1 mice also have T cells that inhibit anti-DNA Ab production by BWF1 spleen cells

So far, we have shown that primed T cells from CWF1 mice suppress anti-DNA Ab production. To determine whether inhibitory T cells exist in naive mice, we cultured BWF1 spleen cells for 5 days and tested supernatant for anti-DNA Ab by ELISA. To these cultures, addition of T cells from 10-wk-old, naive CWF1 mice significantly decreased anti-DNA Ab; this inhibition by naive T cells was also partially abrogated in the presence of an anti-TGFβ mAb (Fig. 6).

Similar findings were observed in a separate experiment where T cells from 35- to 45-wk-old naive or 8 to 12 times immunized CWF1 mice were cocultured with T plus B cells from 30- to 40-wk-old BWF1 mice (n = 12 each). BWF1 T plus B cell cultures had 240 ± 117 (mean ± SD) anti-DNA AFC per 10⁶ B cells; this number was decreased in the presence of naive (98 ± 34, p < 0.01) or primed (24 ± 11, p < 0.0001) CWF1 T cells. Thus, naive CWF1 T cells also inhibit, albeit to a smaller extent than primed T cells (p < 0.01, naive vs primed), anti-DNA Ab production.

Nonautoimmune CWF1 mice have more active TGFβ than lupus-prone BWF1 mice

We hypothesized that the tendency to develop TGFβ-secreting inhibitory T cells is due to the intrinsic ability of nonautoimmune mice to make more active TGFβ in vivo. To test this, we determined serum levels of active and total TGFβ. Serum-active TGFβ1 was significantly more in young CWF1 mice than in BWF1 mice (Fig. 7). Total TGFβ1 levels, however, were similar in the two strains; thus, the mean ratio of active:total TGFβ1 was significantly higher in CWF1 (0.11 ± 0.016) than in BWF1 (0.05 ± 0.009) mice (p = 0.02, n = 6 each).

Discussion

In this article, we show that in nonautoimmune mice, DNA-specific B cells can be activated in vitro by coculture with anti-VH Th cells or in vivo by immunization with an anti-DNA mAb. Such in vivo breakdown of tolerance caused a self-limited autoimmune disease in these mice. T cells from the recovered mice inhibited anti-DNA Ab production and secreted TGFβ. Addition of anti-TGFβ Ab to the cultures partly abrogated the ability of regulatory T cells to inhibit anti-DNA Ab production.

In nonautoimmune mice bearing transgenes that encode anti-DNA Ig, DNA-specific B cells accumulate at the T-B interface of the splenic follicle, whereas in lupus-prone MRL/lpr mice the anti-DNA B cells are present in the follicle, thus allowing T-B cell interaction (10-12). Anti-DNA B cells from nonautoimmune transgenic mice proliferate in response to CD40 ligand and IL-4 (13), suggesting that B cell self-tolerance is due primarily to lack of availability of T cell help. Indeed, that is the case in our non-transgenic system: B cells from nonautoimmune mice could readily make IgG anti-DNA Ab when exposed to autoreactive Th cells from lupus mice or syngeneic Th cells that were activated following immunization with an anti-DNA mAb (Fig. 2). Such B cell activation induced a strong anti-DNA Ab response in vivo (Fig. 2C) and caused renal disease. Thus, potentially pathogenic autoreactive B cells that may have escaped clonal deletion exist in nonautoimmune mice.

Although anti-DNA Ab production by CWF1 mice was readily induced by immunizations that activated Th cells (Fig. 2B), this effect was short-lived, as anti-DNA Ab decreased despite further immunizations (Fig. 3). It is possible to explain this decrease in anti-DNA Ab response in nonautoimmune mice by the emergence of T cells that decreased anti-DNA Ab in these mice. Remarkably, adoptive transfer of such inhibitory T cells into BWF1 mice that are genetically prone to develop lupus resulted in decreased serum anti-DNA Ab levels, delayed onset of nephritis, and improved survival (our unpublished data).

Although the mechanisms by which inhibitory T cells arise and inhibit autoimmunity are not well defined, our studies offer some interesting clues. Most Th and inhibitory T cells appear to recognize the same peptides (Figs. 2B and 4C). Analogous findings have been reported in other models (33-35). For instance, the same self-MHC-reactive T cells helped as well as suppressed B cell Ig production under different conditions (33). The different conditions, in our case, could be created by differences in the in vivo TGFβ levels between nonautoimmune CWF1 and lupus-prone BWF1 mice. Naive, young CWF1 mice that exhibit a dominant inhibitory T cell response had more active TGFβ than BWF1 mice that develop a mostly Th response (Figs. 4B and 7). Such a cytokine milieu might contribute to the development of inhibitory T cells in vivo. An exposure of CD8⁺ T cells to TGFβ can condition
these cells to become suppressors of Ab production (36). TGFβ selectively activates CD8+ T cells to proliferate and augments maturation of naïve to memory T cells (37). The regulatory role of TGFβ (33, 36, 38) was confirmed in our experiments in which anti-TGFβ Ab abrogated, at least in part, the T cell-mediated inhibition of anti-DNA Ab production (Fig. 5B).

Although, TGFβ-mediated suppression appears to be a major mechanism in our model (Fig. 5), it does not represent the sole mechanism of regulation of autoantibody production. Although the inhibition of anti-DNA Ab production by CD8+ inhibitory T cell lines was markedly inhibited by an anti-TGFβ mAb (Fig. 5B), there was no or minimal effect of anti-TGFβ mAb on the inhibition of autoantibody production by CD4+ inhibitory T cell lines (data not shown).

Most Th and inhibitory T cells recognized a peptide, A6H31–45 that contains at least two epitopes: one epitope binds an MHC class II molecule, I-Eβ, and activates CD4+ Th cells that promote anti-DNA Ab production in BWF1 mice (4), and the other epitope binds an MHC class I molecule, Kd, and activates CD8+ T cells that inhibit anti-DNA Ab production.4 The 15-mer peptide, A6H31–45, activated Th cells in both BWF1 (Fig. 4B) and CWF1 mice (Figs. 2B, 3B, and 4C), but activated inhibitory T cells only in CWF1 mice (Figs. 4A, C–E, and 5B). A shorter version of this peptide, a 12-mer A6H34–45 was that used in initial experiments (Figs. 1 and 2A), also contains the same helper and inhibitory epitopes. It remains to be determined whether a differential processing of the Vβ4 molecule into the helper vs inhibitory epitopes are responsible for the predominant helper response in BWF1 mice vs prominent inhibitory response in CWF1 mice, respectively, shown in Fig. 4B.

Numerous examples illustrate that inhibitory T cells recognize molecules involved in Ag recognition (33, 39–41). TCR-reactive T cells arise during recovery from experimental autoimmune encephalomyelitis and suppress it (39); MHC-reactive T cells protect rats from autoantibody-mediated disease (40); and Ig-reactive T cells confer resistance to induction of autoimmune keratitis (41). In our experiments, Ig peptide-reactive inhibitory T cells protect mice from developing persistent autoantibody-mediated disease (Fig. 3A and data not shown).

Finally, the development of inhibitory T cells did not appear to be an artifact of multiple immunizations, as even without immunization splenic T cells from CWF1 mice inhibit autoantibody production, albeit to a much smaller extent than T cells from immunized CWF1 mice (Fig. 6). Thus, it appears that the normal immune repertoire is composed of potentially autoreactive Th as well as inhibitory T cells (Fig. 4C) (35, 42, 43) and inhibitory T cells expand whenever there is an impending danger of pathologic autoimmunity.

In conclusion, inhibitory T cells may normally prevent activation of autoreactive B cells and/or reverse their activation following stimulation by Th cells. We suggest that the ability to develop inhibitory T cells may be one of many checkpoints that protect nonautoimmune mice from developing clinical autoimmunity. Such inhibitory T cells may be responsible for spontaneous regression or waning and waning course of autoimmune diseases, while defective regulation may contribute to sustained autoimmunity and its consequences such as systemic lupus erythematosus.

### References
