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Tolerogenic Treatment of Lupus Mice with Consensus Peptide Induces Foxp3-Expressing, Apoptosis-Resistant, TGFβ-Secreting CD8+ T Cell Suppressors

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Lupus-prone (NZB × NZW)F1 mice spontaneously develop elevated titers of anti-DNA Abs that contain T cell determinants in their VH regions. We have previously shown that tolerization with an artificial peptide based on these T cell determinants (pConsensus (pCons)) can block production of anti-DNA Abs and prolong survival of the mice. In this study, we show that this protection depends in part on the generation of peripheral TGFβ- and Foxp3-expressing inhibitory CD8+ (Ti) cells. These CD8+ Ti cells suppress anti-DNA IgG production both in vitro and in vivo and require up-regulated expression of both Foxp3 and TGFβ to exert their suppressive function, as indicated by microarray analyses, small interfering RNA inhibition studies, and blocking experiments. Additionally, CD8+ Ti cells from pCons-tolerized mice were longer-lived suppressors that up-regulated expression of Bcl-2 and were more resistant to apoptosis than similar cells from naive mice. These data indicate that clinical suppression of autoimmunity after administration of pCons depends in part on the generation of CD8+ Ti cells that suppress secretion of anti-DNA Ig using mechanisms that include Foxp3, TGFβ, and resistance to apoptosis. The Journal of Immunology, 2005, 175: 7728–7737.

Development of autoimmune disease depends upon ability to make immune responses against the self and inability to regulate those responses. For example, in both humans and mice with systemic lupus erythematosus (SLE),1 autoantibodies develop months or years before the onset of clinical disease (1). It is likely that disease develops in genetically prone individuals with inappropriate regulatory response (2). Regulation depends upon many networks, including idiotypic reactions to B and T lymphocytes, complement, clearance of immune complexes and apoptotic materials, and induction of regulatory/suppressor T cells (2–5).

In female (NZB × NZW)F1 (BWF1) lupus-prone mice, subsets of IgG anti-DNA Ab have long been known to cause nephritis (6). We have identified many peptides from the VH regions of Ig anti-DNA that stimulate T cell help, and have described combinations of these wild peptides that, when administered in tolerogenic regimens, delay appearance of autoantibodies and nephritis (7). However, the most effective tolerogen is an artificial synthetic peptide, pConsensus (pCons), based on T cell stimulatory VH sequences found in several BWF1 anti-DNA Ab. When administered i.v. in high doses to either young or diseased mice, pCons was highly effective in prolonging survival of BWF1 mice (8). We also showed that tolerogenic administration of pCons induced anergy in CD4+ T cells, thus contributing to impaired T cell help for IgG anti-DNA production (9). In this study, we report that pCons also significantly expands the number and suppressive phenotype of CD8+ T cells that inhibited IgG anti-DNA production both in vitro and in vivo. The characteristics of those cells could provide clues to some of the molecules contributing to inadequate suppression in lupus, thus increasing our understanding of how to control autoimmune reactivity.

Materials and Methods

Mice

NZB (H-2bd), NZW (H-2sw), and (NZB × NZW)F1 (H-2bw) mice were bred and maintained at the University of California Los Angeles or purchased from The Jackson Laboratory. Mice were treated in accordance with the guidelines of the University of California Los Angeles Animal Research Committee, an institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All experiments were conducted in female mice.

Peptides

The peptides used in this study, their sources, and the MHC molecules they bind are listed in Table I. The tolerizing peptide pCons contains T cell determinants identified in several different J558 VH regions of anti-dsDNA Ig of BWF1 mice (8). The negative control peptide pNeg is nonstimulatory and nontolerogenic. Wild 12-mer or 15-mer peptides from VH of BWF1 anti-DNA Ab that stimulate CD4+ T cells from BWF1 mice include p7, p34, and p58 (6.10). PCDR1 is a wild stimulatory peptide described by Elait et al. (11) from a similar region in the Vh of an anti-DNA Ig. Other nonstimulatory control peptides are phyHEL, derived from the CDR1/FR2 VH region of a murine Ab against hen egg lysozyme, and p11 and p93, which derive from the same VH of the stimulatory wild Ig peptides p7, p34, and p58 (BWF1 anti-DNA Ab A6.1) (12). Peptides were synthesized at Chiron Biochemicals, purified to single peak on HPLC, and analyzed by mass spectroscopy for expected amino acid content.

Treatment of mice

For tolerance induction, 10- to 12-wk-old BWF1 mice received a single i.v. dose of 1 mg of one of the peptides listed in Table I, dissolved in saline.

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus, pCons, pConsensus; AFC, Ab-forming cell; siRNA, small interfering RNA.
as reported previously (8). Controls in most experiments received identical volumes of saline i.v. in some experiments the controls received a single i.v. dose of a different Ig-derived peptide (Table I).

Cell isolation and staining

One week after administration of peptide, single cell suspensions of splenocytes were prepared by passing cells through a sterile wire mesh. After lysis of RBC with ACK lysing buffer (Sigma-Aldrich), cells were centrifuged and washed before resuspension in HL-1 medium (BioWhittaker). In some experiments, CD8+ T cells were isolated by positive selection on an AutoMACS System and found >95% pure by subsequent FACS analysis. Negative selection using mixtures of Abs was also tested to enrich for CD8+ T cells. Negative selection did not differ from positive selection in terms of functional outcome of purified CD8+ cells (data not shown), although cell yield and purity were higher in the positive selection condition. Abs used to analyze the cells included anti-Thy1.2, anti-CD8, and anti-CD28 (all from BD Pharmingen).

FACS analysis

Phenotypic analysis of splenocytes from untreated and pCons-tolerized mice was performed with a FACS Calibur flow cytometer (BD Biosciences) using either CellQuest (BD Biosciences) or FCS Express software (De Novo Software). Staining with multiple combinations of Ab (indicated in the pertinent sections) was performed according to standard procedures described elsewhere (9). Staining with annexin V and with 7-aminoactinomycin D was used to distinguish cells undergoing apoptosis from dead cells. The Ab used were all purchased from BD Pharmingen.

Adoptive transfer experiments

Purified populations of B cells, CD4+ T cells, and CD8+ T cells from treated mice or controls were injected into irradiated recipient mice. Each recipient mouse was irradiated with 600–800 rad before receiving a single i.v. injection of isolated 1 × 10^7 purified B cells, 1 × 10^7 CD4+ T cells, 1 × 10^7 CD8+ T cells, or combinations of the above lymphocyte subsets suspended in saline. After transfer, sera of recipient mice were monitored weekly for anti-dsDNA Ab by ELISA. Early morning urine was monitored at intervals of 2 wk each for presence of proteinuria using Albustix strips.

Cytokine measurement

Cytokine measurement in the supernatant of cultured spleen cells was done using either CellQuest or FCS Express software. Cytokine measurement in the supernatant of cultured spleen cells was done with a FACSCalibur flow cytometer (BD Biosciences) using either CellQuest or FCS Express software (De Novo Software). Staining with multiple combinations of Ab (indicated in the pertinent sections) was performed according to standard procedures described elsewhere (9). Staining with annexin V and with 7-aminoactinomycin D was used to distinguish cells undergoing apoptosis from dead cells. The Ab used were all purchased from BD Pharmingen.

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each group were pooled for each experimental group; usually two such pools were studied from each group simultaneously. For some experiments, CD8<sup>+</sup> and CD8<sup>+</sup> cells were isolated by positive selection using microbeads with Miltenyi AutoMACS as described above. A ribosomal RNA control primer and probe set (Applied Biosystems) were used for normalization purposes. The possibility of genomic DNA contamination was excluded by use of no reverse transcriptase controls in combination with ribosomal primers. GAPDH was used as endogenous control in each experimental set. Normalization was used as indicated in the figures.

**Small interfering RNA (siRNA) transfection**

CD8<sup>+</sup> T cells isolated as described above were plated and cultured in 24-well plates for 24 h in complete medium containing 10% FCS. For transfection, we used the Silencer siRNA Transfection kit from Ambion, which uses lipofection for transfection of siRNA into cells. In some experiments, OptiMEM reduced serum medium (Invitrogen Life Technologies) was used to dilute the siPORT amine. Validated siRNA of Foxp3 and GAPDH was obtained from Ambion, as well as positive and negative siRNA controls. The negative control siRNA was a scrambled sequence that bears no homology to human, mouse, or rat genomes. The transfection agent alone served as another control (siPORT amine). The agent was mixed with siRNA of Foxp3 (50–100 nM) or GAPDH (50–100 nM) or controls in serum-free medium and incubated at room temperature for 30 min. Cells were transfected with siRNA complexes by overlaying siRNA drop-wise onto the cells. After 8–10 h, medium was removed and fresh medium (1–2 ml) added. Viability was assayed with trypan blue staining. After 48 h of culture, transfected CD8<sup>+</sup> T cells were transferred to cultures of fresh BWF1 CD4<sup>+</sup> T cells plus B cells plus pCons for measurement of suppression of anti-DNA Ab production. Some transfected cells were lysed with cell lysing solution (Invitrogen Life Technologies) and RNA isolated for real-time PCR, to validate knock down of the target gene.

**Statistical analyses**

Statistical analyses were performed using Prism 4 software (GraphPad). Parametric testing between two groups was performed by the paired t test or by the Mann-Whitney U test. Nonparametric testing among more than two groups was performed by one-way ANOVA. Data in each cell were then compared by posttest analysis using Tukey’s multiple comparison test. Values of p < 0.05 were considered significant.

**Results**

Intravenous administration of pCons expands peripheral CD8<sup>+</sup> T cells

As shown in Fig. 1a, CD3<sup>+</sup>CD8<sup>+</sup> T cells harvested 1 wk after injection with pCons expanded from 11 to 17% of total splenocytes in naive vs tolerized mice. This difference was significant (p < 0.01 by the Tukey’s multiple comparison test), detectable for at least 3 wk, and accompanied by an increased number of total CD8<sup>+</sup> T cells (Fig. 1b). In contrast, there was no expansion in the CD3<sup>+</sup>CD4<sup>+</sup> compartment (Fig. 1a and b). As shown in Fig. 1, c and d, the CD8<sup>+</sup> T cell expansion was initially in the CD8<sup>+</sup>CD28<sup>+</sup> compartment (p < 0.01).

**Administration of pCons induces CD8<sup>+</sup> T cells that suppress anti-dsDNA IgG in vitro**

CD8<sup>+</sup> T cells can suppress pathogenic immune responses in several systems. To address whether tolerization with pCons could induce suppressor CD8<sup>+</sup> T cells, CD8<sup>+</sup> T cells isolated from spleens of 10- to 12-wk-old BWF1 mice treated or not with pCons were cocultured for 5 days with helper CD4<sup>+</sup> T cells from young untreated mice and B cells from old nephritic mice. Fig. 2a shows that addition of CD8<sup>+</sup> T cells in numbers from 10<sup>5</sup> to 10<sup>7</sup> significantly reduced the number of anti-DNA AFC (p < 0.001 by the Tukey’s multiple comparison test) when compared with anti-dsDNA AFC formed in cultures of B cells and helper CD4<sup>+</sup> T cells only. In contrast, addition of CD8<sup>+</sup> T cells from naive mice to CD4<sup>+</sup> T cells plus B cells plus pCons did not alter the number of AFC (Fig. 2b). Specificity was suggested by the observation that production of total IgG AFC was similar when CD4<sup>+</sup> and B cells were cocultured with CD8<sup>+</sup> T cells from untreated or tolerized mice (data not shown). We conclude that peripheral CD8<sup>+</sup> T cells...
FIGURE 2. Administration of pCons induces CD8− T cells that suppress IgG anti-dsDNA Ig production in vitro. Groups of five to seven 10- to 14-wk-old BWF1 mice were treated with 1 mg of pCons or saline i.v. One week later, purified CD8− T cells were cocultured with naive helper CD4+ T cells and B cells (10:1:1 ratio) for 5 days with or without pCons (20 μg/ml). At the end of culture, anti-DNA Ig were measured by ELISPOT. Results are presented as mean numbers ± SE (from four experiments) of AFC per 10^6 B cells. a, Effect of addition of increasing numbers of CD8− T cells from pCons-tolerized mice (x-axis) to cultures containing CD4+ T cells, B cells, and pCons (p < 0.001 for column 1 vs columns 2, 3, and 4 by Tukey’s test; p < 0.01 between 10^5 and 10^7 cells (columns 2 and 4)). b, Addition of CD8− T cells from untreated mice (nCD8) to CD4+ T cells plus B cells plus pCons does not change the number of AFC as compared with CD4− T cells plus B cells only. Means of three to six experiments. c, CD8− Ti cells are present in both the CD8−CD28+ and CD8−CD28− subsets, as Ti cells from both subsets suppressed anti-DNA Ab production (p < 0.002 by ANOVA for the comparisons of columns 2 and 3 to column 1). Means of three experiments. d, Ability of different peptides to activate Ti cells in vitro. Mean anti-DNA AFC per 10^6 B cells on the y-axis is shown in pairs. All donors of tolerated CD8− T cells (tCD8) were inoculated with pCons 1 wk before harvest of spleen cells. The black-filled columns represent cultures containing naive CD8− T cells (nCD8) and peptides as indicated; the gray-filled column to the right of each black column represent cultures containing tCD8 plus peptide. Addition of pCons or p7 (columns 3 and 5) activated Ti cells from tolerized mice to suppress anti-DNA Ig production; p < 0.001 for both compared with columns 2 and 4 by Tukey’s test. In contrast, addition of pCDR1 and p33b did not activate suppression. pHyHel and p93 are Ig V_H peptides that are not stimulatory for T cells in BWF1 mice and are negative controls. Means of three experiments. e, Ability of peptides other than pCons to induce CD8− Ti cells in vivo. BWF1 mice were inoculated with p7, p34, or p93 1 wk before harvest of spleen cells. Peptide p7 can induce CD8− Ti cells (compare black-filled to gray-filled columns as in d). Differences between columns 3 and 4 are significant, p < 0.001 by Tukey’s test). In contrast, p34—which is stimulatory for BWF1 CD4+ T cells—did not induce detectable CD8− Ti cells. p93 is not stimulatory for BWF1 T cells and is a negative control. Means of four experiments.

harvested 1 wk after administration of pCons contain suppressors of anti-DNA Ig production in vitro.

Because we had found expansion of CD8−CD28− T cells after tolerization with pCons and cells with such markers can work as suppressors, we tested whether differences existed in terms of suppression of anti-DNA Ig between CD8−CD28+ and CD8−CD28− T cells from pCons-tolerized mice. As shown in Fig. 2c, suppression was not significantly different in CD8− T cells that were either positive or negative for CD28 surface expression.

Moreover, suppressive CD8− T cells could be activated by addition of pCons to the culture, but not by other Ig-derived anti-DNA V_H peptides (pCDR1, p33b, and p93) and the peptide pHy-Hel from a non-DNA binding mAb (Fig. 2d). An exception to this was p7, which is derived from a wild BWF1 anti-DNA Ab (12). To
further investigate this finding, we inoculated 10- to 12-wk-old BWF1 mice with one of three peptides from V_{α} regions of Ig molecules, two of which (p7 and p34) can stimulate proliferation of BWF1 T cells (7). As shown in Fig. 2e, CD^{8^{+}} T cells from mice inoculated with p7 suppressed anti-DNA Ab production, whereas other peptides (p34 and p93) did not have this property. Thus, only a limited number of artificial and wild peptides have the capacity to induce CD^{8^{+}} T cells in this system.

**Administration of pCons induces CD^{8^{+}} T cells that suppress anti-dsDNA Ab in vivo**

For these experiments, CD^{8^{+}} T cells from untreated or pCons-treated 10- to 12-wk-old BWF1 mice were injected i.v. into irradiated 16-wk-old BWF1 mice that also received B cells from old nephritic BWF1 females, with or without CD^{4^{+}} T cells from young mice. In Fig. 3a, the results from two independent experiments are combined. There were 4–10 mice in each recipient group in each experiment. IgG Abs to dsDNA were measured in the serum by standard ELISA 1 and 2 wk after cell transfer. Results were similar at the two time points and, therefore, were combined; the mean for each animal is shown in Fig. 3a. By Tukey’s multiple comparison test, recipients of B cells alone did not have increased anti-DNA Ab compared with mice that were untreated. In contrast, recipients of B cells plus CD^{4^{+}} T cells or of B cells plus CD^{4^{+}} T cells plus CD^{8^{+}} T cells from untreated naïve donors (nCD8) had anti-DNA Ab ranging from 0.10 to 1.1 (normal range <0.10 in our colonies); these were significantly higher than mice receiving B cells alone (p < 0.05), showing the importance of CD^{4^{+}} T cell help in this system. In contrast, serum anti-DNA Ig levels were significantly lower in mice receiving B cells plus CD^{4^{+}} T cells plus CD^{8^{+}} T cells from pCons-treated donors (tCD8). Anti-DNA Ig levels in those mice ranged from 0.08 to 0.36. The differences between these groups (nCD8 vs tCD8) was significant (p < 0.01 by Tukey’s test; p < 0.0007 by the Mann-Whitney U test). These data indicate that the suppressive effects induced by pCons on CD^{8^{+}} T cells not only occur in vitro but can also be transferred in vivo.

The transfer of CD^{8^{+}} T cells from tolerized mice also affected lupus-like nephritis. In Fig. 3, b and c, the development of proteinuria and survival, respectively, are shown in a comparison of transfer of CD^{8^{+}} T cells from naïve mice vs CD^{8^{+}} T cells from tolerized mice (plus CD^{4^{+}} T cells plus B cells in both cases). pCons-induced CD^{8^{+}} T cells delayed onset of proteinuria and increased survival of the mice (p < 0.02 by χ^{2} analysis).

**Cytokine production by CD^{8^{+}} Ti cells from pCons-treated mice**

As shown in Fig. 4a, cytokine secretion in CD^{8^{+}} T cells obtained 1 wk after administration of pCons was significantly altered. Cells were cultured with pCons for 48 h and secretion of cytokines into the supernatants was measured by ELISA. Secretion of TGFβ and IFN-γ were significantly increased, whereas secretion of IL-10 was significantly decreased. This was of interest because all three cytokines have been reported to mediate suppression by CD^{8^{+}} T cells (13). As shown in Fig. 4b, mRNA for TGFβ and for IL-2 were significantly increased in CD^{8^{+}} T cells from tolerized compared with naïve mice. In contrast, mRNA for IFN-γ and IL-12-p40 were not significantly different in tolerized vs naïve cells, and mRNA for IL-10 was intermediate. We tested whether the expression of TGFβ mRNA could be increased by additional activation of CD^{8^{+}} T cells from tolerized mice. Results are shown in Fig. 4c, where the ratio of mRNA for TGFβ to GAPDH in naïve CD^{8^{+}} T cells was set at 1. Activation of tolerized cells with pCons resulted in 3.5-fold increase in the message for TGFβ, and activation with anti-CD3 mAb plus anti-CD28 mAb increased the message to
4.5-fold. These differences (between columns 4 and 2 and their controls containing naive CD8\textsuperscript{+} T cells) were significantly different (p < 0.05 by Tukey’s test).

To determine the effects of CD8\textsuperscript{+} Ti cells on cytokine secretion in cultures secreting anti-DNA Ig, the CD8\textsuperscript{+} T cells from pCons-treated mice were cocultured with naive CD4\textsuperscript{+} T cells and naive B cells as in the experiments shown in Fig. 2. Fig. 4, d and e, indicate that activation of CD8\textsuperscript{+} T cells by addition of pCons to the culture significantly decreased secretion of IFN-γ. Mean levels were 700–800 pg/ml in cultures with no added peptide or the control peptide pHyHel, and <200 pg/ml in the cultures containing pCons. High levels of IFN-γ production are characteristic of cultures containing BWF1 CD4\textsuperscript{+} T cells plus B cells; the main source is the CD4\textsuperscript{+} T cells, and the production of anti-DNA Ig and disease is dependent to a large extent on this cytokine (8, 14). The fact that CD8\textsuperscript{+} Ti cells were not suppressive of all cytokines was underscored by increased secretion of IL-2 in the cultures activated by addition of pCons. Proliferation of naive CD4\textsuperscript{+} T cells labeled with CFSE was dramatically reduced by addition of tCD8 but not nCD8 to cultures (data not shown).

**Characteristics of CD8\textsuperscript{+} Ti cells induced by pCons**

The experiments in Fig. 4, a–c, suggested that TGFβ might be the most important of the cytokines tested in mediating suppression.
However, we also wondered whether CD8+ Ti cells were cytotoxic for CD4+ T cells or B cell targets. CD8+ T cells from tolerized or naive mice were cocultured at various concentrations for 24 h with naive CD4+ T cells or naive B cells, and cytolysis of target cells was measured. There were no differences in cytolysis in either target cell when coincubated with tolerized CD8+ Ti cells (data not shown). Furthermore, the ability of tCD8+ T cells to suppress IFN-γ production by CD4+ T cells was detectable whether or not the cells were separated by a membrane (data not shown). These data suggested that suppression was mediated by soluble factors released by CD8+ Ti, and because of the data shown in Fig. 4, TGFβ was a likely candidate. To test this possibility, CD8+ T cells isolated 1 wk after treatment with pCons were added to cultures containing CD4+ T cells, B cells, pCons, and various anti-cytokine mAb at different concentrations (Fig. 5). ELISPOT experiments indicated that specific suppressive effects of tCD8 were evident at a concentration of 20 μg/ml anti-TGFβ Ab, which abrogated suppression completely (column 8), restoring anti-DNA Ig production to the baseline level shown in column 2. Column 8 differs from column 3 at p < 0.001 by Tukey’s test. In contrast, mAb to IFN-γ, IL-10, and IL-2 did not abrogate suppression. We concluded that the primary soluble effector of CD8+ Ti cells from pCons-treated mice is secreted TGFβ1.

CD8+ T cells from tolerized mice express Foxp3, which contributes to their suppressive functions

Microarray analysis comparing gene expression in splenic CD8+ T cells harvested 1 wk after tolerization with pCons compared with naive mice showed differential expression of 60 genes (data not shown). Real-time PCR analysis confirmed increased expression of Foxp3 in the CD8+ T cells from tolerized mice (Fig. 6a). In particular, mRNA for Foxp3 was increased ~2.5-fold in tolerized CD8+ T cells compared with cells from naïve BWF1 mice. Flow cytometry analysis reported in Fig. 6b confirmed that CD8+ T cells from tolerized mice had increased intracellular expression of Foxp3 on a per-cell basis compared with cells from naïve mice. Because of these findings, we tested the possibility that isolation of CD8+ T cells might activate them and induce Foxp3 expression. As shown in Fig. 6c, a strong activation signal (anti-CD8 mAb plus anti-CD28 mAb) increased Foxp3 expression in tolerized cells to 15-fold compared with cells from naïve mice also activated by this method. Therefore, Foxp3 expression is increased in tolerized CD8+ Ti cells but can be increased further by cell activation.

To test the importance of Foxp3 in the mechanisms of suppression by CD8+ Ti cells in our system, this gene was inhibited by specific siRNA. Inhibition of Foxp3 resulted in partial abrogation of the ability of tCD8+ cells to suppress anti-DNA Ig production in vitro, and after transfection with siRNA inhibiting Foxp3, anti-DNA Ig synthesis increased significantly (Fig. 6, d and e; p < 0.02 by t test comparing column 4 to column 3 in e), indicating that the Ti cell function in tolerized CD8+ that hyperexpress Foxp3 was influenced by expression of Foxp3.

CD8+ T cells from tolerized mice are protected from apoptosis

An additional gene that we found modulated in tolerized CD8+ Ti cells by microarray analysis was Bcl-2 (Fig. 6a), suggesting the possibility that these Ti cells might be relatively resistant to apoptosis. Staining by annexin V was significantly decreased in CD8+ T cells from pCons-treated mice as compared with those cells from naïve mice (Fig. 7a). Importantly, 1 wk after inoculation of various peptides, the percents of annexin-positive CD8+ T cells were significantly lower in mice receiving pCons or p7 than in mice receiving the VH peptides p11, 34, 58, or pNeg, indicating that the two peptides that were most effective in inducing CD8+ Ti cells also protected those cells from apoptosis.

Discussion

In this study, we report that an IgG-VH-region peptide-based tolerizing regimen that delays autoantibody production and nephritis in a murine model of spontaneous lupus-like disease induces inhibitory CD8+ T cells that decrease autoantibody production both in vitro and in vivo. The action of these Ti cells is probably on helper CD4+ T cells, as Ti cells from tolerized mice suppressed IFN-γ production in cocultures of Ti cells plus CD4+ T cells plus B cells but did not have direct effects on B cells unless CD4+ T cells were present in the culture. The tolerized Ti cells secreted large amounts of TGFβ and blockade of this cytokine abrogated suppression of anti-DNA Ab production, suggesting a major role of this cytokine in the mechanisms of suppression elicited by tolerization with pCons. The data are consistent with our previous data showing that immunization with p7 (from the VH of an anti-DNA Ig H chain) induced CD8+ Ti cells in normal mice that mediated their suppressive effects primarily via secretion of TGFβ (10). The data are also in agreement with the findings by Kang et al. (14) in which low s.c. doses of nucleosomel peptides in lupus prone (SWR × NZB)F1 mice decreased anti-DNA Ab production and nephritis and prolonged survival by inducing CD4+ CD25+ Foxp3 and CD8+ Ti cells, which secreted TGFβ and suppressed IFN-γ production by CD4+ Th cells in a contact-independent manner. Also, Sela et al. (15) described a central role for TGFβ in the mechanisms by which another Ig VH peptide protects from lupus induced in BALB/c mice by immunization with human 16/6 Id1 mAb. The relevance of our current data lies in the observation that human T cells can also recognize Ig VH peptides, and patients with SLE are more likely than healthy individuals to have peripheral T cells that are activated to release cytokines upon exposure to certain peptides from human anti-DNA VH.
FIGURE 6. Foxp3 in CD8\(^+\) T cells from tolerized mice. a, Differences in mRNA expression in CD8\(^+\) T cells from mice treated with pCons compared with those from naive mice normalized to GAPDH mRNA. Arbitrary units were set at 1 in CD8\(^+\) T cells from naive mice. Shown are the mean ± SD for three experiments comparing tCD8 to nCD8. The reduced levels of Bcl-2 expression in CD4\(^+\) T cells from the same spleen pools are shown as a control. b, Intracellular staining of Foxp3 in purified CD8\(^+\) T cells from naive mice (1) and pCons-treated mice (2). c, Comparison of mRNA for Foxp3 in CD8\(^+\) T cells from naive or tolerized mice (columns 1 and 2) with or without activation by anti-CD3 mAb plus anti-CD28 mAb (columns 3 and 4) normalized to GAPDH, which is set at 1.0 in naive mice as in a. Foxp3 is significantly increased in tolerized CD8\(^+\) T cells (column 2 vs column 1, \(p < 0.005\) by Mann-Whitney; column 4 vs column 3, \(p < 0.02\)). d, Effect of siRNA silencing Foxp3 in CD8\(^+\) T cells from naive mice. Preincubation of nCD8 cells with siRNA for Foxp3 (column 4) did not change anti-DNA Ig secretion as compared with cultures containing nCD8 plus CD4\(^+\) T cells plus B cells plus pCons (column 3). Foxp3 siRNA is indicated as fox+; Foxp3− indicates cultures with addition of transfection vehicle without vector. e, Silencing of Foxp3 in tolerized CD8\(^+\) T cells partially abrogates their suppressive function. As internal control for efficiency of transfection, we silenced GAPDH according to the manufacturer’s instructions. Results shown are the mean of four experiments. Mean levels of anti-DNA Ig (four experiments) ± SD are shown for CD8\(^+\) T cells from tolerized mice added to CD4\(^+\) T cells plus B cells plus pCons. Note the suppressive effect of iCD8 (column 3 compared with column 2, \(p < 0.05\) by Tukey’s test). This suppression is partially abrogated by incubation of iCD8 with siRNA for Foxp3 before addition of cells to the cultures of CD4\(^+\) T cells plus B cells (column 4, differs from column 3, \(p < 0.05\)). In contrast, preincubation of iCD8 cells with the transfection vehicle (fox−) or with vector encoding GAPDH (gap+) or with empty vector (gap−) did not abrogate suppression.

FIGURE 7. CD8\(^+\) T cells from mice tolerized with pCons or p7 are relatively protected from apoptosis compared with cells from mice treated with other Ig V\(_h\) peptides. Apoptosis was measured as cell surface staining for annexin V. Whole spleen cells were obtained from mice 1 wk after injection of 1 mg of the peptides indicated. The cells were maintained in medium at room temperature for 6–8 h, then stained for annexin V and 7-aminoactinomycin D and analyzed by FACS for costaining with CD8. a, Annexin V staining of CD8\(^+\) T cells from mice receiving pCons (1) compared with naive mice (2). Data shown are representative of six experiments with similar results. b, Apoptosis in CD8\(^+\) T cells after administration of several different V\(_h\) peptides. Results are presented as mean percentage of CD8\(^+\) T cells staining for annexin V (±SD) in two to seven experiments with each peptide. Note that the injection of peptides known to activate CD8\(^+\) T cells to suppress anti-DNA production (pCons and p7, columns 2 and 4, respectively), induces CD8\(^+\) T cells with significantly lower proportions undergoing apoptosis. Compared with naive cells (column 1), percent of cells staining for annexin V is significantly lower in mice treated with pCons (\(p < 0.001\) by Tukey’s test) or with p7 (\(p < 0.001\)). pCons is also significantly lower than p11, p34, p58, and pNeg (\(p < 0.05\) or better), p7 is significantly lower than p11 and pNeg (\(p < 0.05\) or better).
In our system, microarray analysis of gene expression in CD8+ Ti cells from naïve compared with tolerized BWF1 mice indicated that among the ~60 genes that are differentially regulated, Foxp3 and Bcl-2 had a major functional role in the tolerized Ti cells. Indeed, siRNA blockade of Foxp3 in Ti cells partly abrogated their ability to suppress anti-DNA Ab production, whereas Bcl-2 expression correlated with increased survival of Ti cells in the tolerized mice.

Although Foxp3 is best known for its association with the regulatory phenotype of CD4+CD25+ T cells, up-regulation of Foxp3 in inhibitory CD8+ T cells has also been described both in rodents and in humans (17–20). We find that up-regulation of Foxp3 in CD8+ Ti cells in our system can be further increased by TCR-induced T cell activation of the cells. Nevertheless, tolerized BWF1 mice express higher amounts of Foxp3 as compared with naïve controls, and blockade of expression of this gene reduces the suppressive capacity of the cells.

We are tempted to hypothesize that increased TGFB expression can be partly responsible for the observed up-regulation of Foxp3 in the Ti cells, because, in CD4+ T cells, exposure to TGFB1 induced expression of Foxp3 and acquisition of suppressor function (21–24). In contrast, although TGFB1 seems not to affect molecules associated with suppressive function such as glucocorticoid-induced TNFR family-regulated gene (25), we found that expression of glucocorticoid-induced TNFR family-regulated gene increased on CD8+ cells of pCons-tolerized mice (12.51 ± 0.28 vs 8.86 ± 1.95 in untreated mice, p < 0.028). Thus, TGFB1 may only be one of the several factors that contribute to the mechanisms of induction of CD8+ suppressors after tolerization with pCons. Ongoing experiments in our laboratory are trying to clarify this issue.

Regarding the observation of Bcl-2-associated increase of survival of Ti cells in the tolerized mice, we may envision a possible teleological advantage in having Ti cells surviving longer to confer longer-lasting suppressive function. Such a mechanism would be similar to the resistance to autoimmunity conferred by CD8+ T cells in normal mice hyperimmunized with anti-DNA V_H or p7 (10). In this context, it is of interest that the two peptides that are most effective in suppressing autoimmune production and clinical disease in BWF1 mice, pCons and p7 (7–8, 10), are the only two of several tested that reduce apoptosis in CD8 T cells. We have previously shown that CD8+ T cells in untreated BWF1 mice have limited expansion as mice age and disease begins—in contrast to CD4+ T cells and B cells that expand 6- to 10-fold during the same time (26). Furthermore, a significantly higher proportion of BWF1 CD8+ T cells undergo apoptosis after activation in older BWF1 mice compared with young BWF1 or normal MHC-matched old CFW1 mice. In view of that, one of the mechanisms by which pCons administration could protect BWF1 mice from autoimmunity might therefore be the increased survival of these Ti cells.

Our data contribute to a better understanding of previous lines of evidence that had suggested the importance of CD8+ T cells in the prevention or inhibition of lupus-like autoimmunity. For example, CD8+ T cell depletion in (NZW × BXSB)F1 mice accelerated the lupus-like disease and decreased survival (27). Prevention or amelioration of lupus-like disease induced by immunization with 16/6 Id in normal mice was observed after adoptive transfer of a 16/6 Id-specific CD8+ suppressor line (28). Also, Fan and Singh (29) induced CD8+ T cells by immunization with DNA encoding MHC class-I-binding peptides from p7; the resulting Ti cells suppressed production of anti-DNA Ig by B cells with DNA-binding surface receptors.

In conclusion, the mechanisms of suppression of autoimmune disease in our model are multiple and complex but occur early, as they appear by 1 wk after the initial tolerizing injection. We hypothesize that in the CD8+ Ti cells, secretion of TGFB, expression of Foxp3, and reduced apoptosis may likely be linked. This aspect, as well as manipulation of the suppressive mechanisms lost or impaired over time, are the subjects of current work, with the ultimate goal of possible translation utility for people with lupus-like autoimmune disorders. Because patients with SLE are more likely than healthy individuals to have peripheral T cells activated by Ig VH peptides similar to those tested in this study, it is reasonable to speculate that the lessons learned in this mouse model might inform future strategies to treat the human disease.

Disclosures
The authors have no financial conflict of interest.

References


