CD8⁺ T Cell-Mediated Suppression of Autoimmunity in a Murine Lupus Model of Peptide-Induced Immune Tolerance Depends on Foxp3 Expression

Ram Pyare Singh, Antonio La Cava, Maida Wong, Fanny Ebling and Bevra H. Hahn


http://www.jimmunol.org/content/178/12/7649

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

**References**  This article cites 41 articles, 18 of which you can access for free at: http://www.jimmunol.org/content/178/12/7649.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD8+ T Cell-Mediated Suppression of Autoimmunity in a Murine Lupus Model of Peptide-Induced Immune Tolerance Depends on Foxp3 Expression1

Ram Pyare Singh, Antonio La Cava, Maida Wong, Fanny Ebling, and Bevra H. Hahn2

Systemic lupus erythematosus is an autoimmune disease caused by autoantibodies, including IgG anti-DNA. New Zealand Black/New Zealand White F1 female mice, a model of spontaneous polygenic systemic lupus erythematosus, tolerized with an artificial peptide (pConsensus) based on anti-DNA IgG sequences containing MHC class I and class II T cell determinants, develop regulatory CD4+CD25+ T cells and CD8+ inhibitory T cells (CD8+ Ti), both of which suppress autoantibody production. CD8+ Ti inhibit primarily via secretion of TGF-β. In the present study, we show that the inhibitory function of CD8+ T cells from tolerized mice is sustained for up to 8 wk and at all times depends on expression of Foxp3. Both CD28-positive and CD28-negative CD8+ T cells contain inhibitory cells, but the expression of mRNA for Foxp3 and for TGF-β is higher and lasts longer in the CD28− subset. In vitro addition of TGF-β (in the presence of IL-2) induces Foxp3 expression in a dose-response manner. Gene inhibition or blockade with small interfering RNA of Foxp3 abrogates the ability of the CD8+ Ti to inhibit anti-DNA production and the proliferation of CD4+ Th cells. Moreover, a significant correlation between expression of Foxp3 and ability of CD8+ Ti to secrete TGF-β is observed. Therefore, CD8+ Ti in this system of tolerance are similar to CD4+CD25+ regulatory T cells in their dependence on expression of Foxp3, and there may be a bidirectional Foxp3/TGF-β autocrine loop that determines the ability of the CD8+ T cells to control autoimmunity. The Journal of Immunology, 2007, 178: 7649–7657.

The discovery of T cells that can suppress autoimmunity (1) suggested that induction of such cells might be used to control diseases mediated by pathogenic T cells or by Th cell interactions with B cells that produce pathogenic autoantibodies. Multiple T cell types that mediate suppression have been described. They include CD8+ T cells that are not cytotoxic (2–8), CD4+CD25+ regulatory T cells that suppress Th cells via cell contact (1, 9–12), and CD4+ T cells that suppress via secretion of cytokines, including TGF-β and IL-10 (13–15). The power of these cells to prevent, delay, or suppress established autoimmunity has been demonstrated in many animal models, including experimental allergic encephalomyelitis (3, 14, 15), experimental autoimmune orchitis (4), and murine systemic lupus erythematosus (7, 8, 11–13). In the work described in this study, we studied the characteristics of one group of inhibitory T cells (Ti),3 CD8+ Ti, which we have previously shown are induced in vivo after administration to New Zealand Black/New Zealand White F1 female (BWF1) mice of a peptide based on amino acid sequences within the V\textsubscript{H} region of murine Abs to DNA. That peptide, called pConsensus (pCons), contains MHC class I- and class II-binding T cell determinants (16, 17). Administration of pCons in a tolerogenic regimen to premorbid BWF1 female mice, a model of polygenic spontaneous systemic lupus erythematosus-like disease mediated in part by pathogenic IgG Abs to dsDNA, resulted in significant delay of both autoantibody production and nephritis, and substantially prolonged survival (16). The immune mechanisms elicited by pCons in tolerized BWF1 mice are complex and include induction of hyporesponsiveness in the CD4+ Th cells (11), induction of peptide-binding pCons-reactive CD4+CD25+ T regulatory (Treg) cells that inhibit anti-DNA via cell contact with the effector cells (11), and induction of CD8+ Ti that inhibit anti-DNA production via secretion of TGF-β (8).

We have previously shown that aging BWF1 mice develop abnormalities in their CD8+ T cell compartment (2), including inability to proliferate and apoptotic rather than activation responses to TCR stimulation. In this study, we show that after tolerization with pCons, BWF1 CD8+ Ti with both CD28+ and CD28− phenotypes can suppress CD4+ T cell proliferation and anti-dsDNA IgG production. Within both the CD28+ and CD28− Ti subsets, expression of the suppression-related transcription factor Foxp3 is increased and remains elevated for at least 3–4 wk. Concomitantly, secretion of TGF-β increases in both subsets of CD8+ Ti. Moreover, the ability of both CD28+ and CD28− Ti to suppress in vitro can be fully inhibited by blockade of Foxp3 via small interfering RNA (siRNA) technology.

The data suggest that these CD8+ Ti are somewhat similar to CD4+CD25+ Treg in that their suppressive capacities are associated with the expression of Foxp3, which can be up-regulated by TGF-β and IL-2.
Materials and Methods

Mice

New Zealand Black (H-2d), New Zealand White (H-2b), and NZB × NZW F1 (H-2b) mice were bred and maintained at the University of California or purchased from The Jackson Laboratory. Mice were treated in accordance with the guidelines of the University of California 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 tolerizing peptide, pCons (FIEWNKLRFRQGLEW), is an artificial peptide designed to contain I-Eα-binding and Kd-binding T cell determinants identified in several different J558 Vh regions of anti-dsDNA Ig of BWF1 mice (16–19). The negative control peptides pNeg and p93 are nonstimulatory and non tolerogenic. pNeg is artificial and designed to contain an I-Eα-binding T cell determinant that is not stimulatory, p93 is a wild peptide, a 15-mer beginning at position 93 in the Vh molecule of a BWF1 mAb anti-DNA. A positive control peptide, p33b, is a wild peptide from Vh of a BWF1 mAb anti-DNA: it binds I-Eα and can induce proliferation in BWF1, Cd4+ T cells. 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 pCons, as reported previously (8, 11). In some experiments, mice were treated with pNeg or p93 as negative controls.

Cell isolation and staining

At various times after administration of peptide, single-cell suspensions of splenocytes were prepared by passing cells through a sterile wire mesh. After lysis of BRC with ACK lysing buffer (Sigma-Aldrich), cells were centrifuged and washed before resuspension in HEL-1 medium (BioWhittaker). In experiments involving whole cell populations, CD4+ B, and CD8+ T cells were isolated by positive selection on an AutoMACS system (Miltenyi Biotec) and found >95% pure by subsequent FACS analysis. In experiments studying CD28+ or CD28− CD8+ T subsets, cells were sorted into these populations by FACS or isolated via magnetic beads. Such cells maintained good viability (85% or better by FACS analysis using 7-aminoactinomycin D staining to identify dead cells) and were at least 90% pure. We have previously shown that positive and negative selection of CD8+ T cells gave similar functional and molecular results (8). 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 as described elsewhere (11). Staining procedures described elsewhere (11). 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.

Intracellular staining

For intracellular staining, cells were first stained for expression of cell surface markers and then fixed, permeabilized, and stained using the Cytofix/Cytoperm kit (BD Pharmingen), according to the manufacturer’s instructions. Intracellular TGF-β and Foxp3 were identified in cells that had been fixed and permeabilized by staining with the appropriate Abs.

Cell cultures

Methods have been described previously (8, 11). In brief, purified CD4+ T cells from young naive BWF1 females and B cells from older naive BWF1 females with high titers of anti-DNA in serum were cultured with or without addition of CD8+ T cells from naive or tolerized mice (harvested at various times from 1 to 8 wk after tolerization) in 24-well microtiter plates at 37°C in complete medium containing antibodies and 10% FCS. To acti- tivate the CD8+ T cells from tolerized mice, pCons peptide was added to cultures to 20 μg/ml concentrations, unless otherwise stated in the figure legends. Ratios used were 10 CD4+ T cells to 1 B cell; CD8+ T cells were added at a ratio of 1 CD8+ to 1 CD4+ T cell. Cells were cocultured for 5 days in experiments measuring anti-DNA production, for 48 h in experiments measuring cytokine production, and for 72 h in studies measuring cell proliferation. In some experiments, CD8+ T cells were cultured with addition of TGF-β (20 ng/ml), and medium was enriched with rIL-2 (10 ng/ml) to study up-regulation of Foxp3.

Measurement of anti-DNA and cytokines in supernatants of cell cultures

IgG anti-DNA was measured in concentrated cell culture supernatants with BD OptEIA ELISA kits (BD Biosciences). Cytokine measurement in the supernatant of cultured spleen cells was done with BD OptEIA ELISA kits (BD Biosciences) for TGF-β1.

Measurement of cell proliferation

Spleen cells were isolated from BWF1 mice at various times, as described in figure legends, after a single injection of pCons. BRC were removed by RBC lysing solution (Sigma-Aldrich). B220+ B cells, CD4+, and CD8+ T cells were isolated and cultured in triplicate in 96-well plates containing varying amounts of CD8+ Ti, 1 × 105 CD4+, and 2 × 105 irradiated or nonirradiated B cells cultured in medium containing murine rIL-2 for 96 h and pulsed with 65 μg/ml [3H]thymidine during the last 18 h of culture. Incorporation of tritiated thymidine into DNA was assessed by liquid scin- tillation counting in an automated counter (Beckman Coulter). Results are expressed as mean stimulation index or mean cpm ± SE and represent the average cpm of triplicate determinations.

Real-time PCR

Quantitative real-time reverse transcription was performed using TaqMan technology on an ABI Prism 7900 HT Sequence Detection System (Ap- plied Biosystems). TaqMan RT-PCR mix was used for the RT-PCR, fol- lowing the manufacturer’s instructions. Reverse transcription used 50 ng of total RNA. Total RNA was isolated with TRIzol (Invitrogen Life Tech- nologies), per manufacturer’s protocol. The oligonucleotide sequences used for the primers and TaqMan probes are as follows: TGF-β forward, 5′-AACAGGAAGGCGATCGA-A-3′; reverse, 5′-GGACTCTGCGAG- CGTCTCATT-3′; Foxp3 forward, 5′-CGACATCCAGTACCTACTA-3′; reverse, 5′-TCTGGAGAGTTTTTCTGCT-3′; and probe 6FAM, TC CGAACAGCATCTTTTACTCC TAMRA. The amplification primers were at 900 nM, and the probe was at 200 nM. A passive reference dye (ROX) provided an internal standard for normalization of FAM fluorescence, correcting for fluctuations due to volume changes. For relative quantitation, a standard curve was constructed for each primer and probe set, using total RNA. RNA was isolated from spleen cells of 10- to 13-wk-old naive or tolerized mice. Spleen cells from two to three mice in each group were pooled for each experimental group; usually two or more such pools were studied from each group simul- taneously. For some experiments, CD4+ and CD8+ T cells isolated by positive selection 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 for normal- ization. A ribosomal RNA control primer and probe set was used as indicated in the figure legends.

siRNA transfection

CD8+ 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 circee siRNA Transfection kit from Ambion, which uses lipofection for transfection of siRNA into cells. In some ex-periments, OptimEM-reduced serum medium (Invitrogen Life Technolo-gies) was used to dilute the siPORT amine. Validated siRNA of Foxp3, CCR7, p53, 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, p53, CCR7, 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 dropwise onto the cells. After 8–10 h, medium was removed and fresh medium (1–2 ml) was added. Viability was assessed by trypan blue staining. After 48 h of culture, transfected CD8+ T cells were transferred to cultures of fresh BWF1, CD4+ T cells plus B cells plus pCons for measure- ment of suppression of anti-DNA production or for cell proliferation. Some transfected cells were lysed with cell lysing solution (Invitrogen Life
is significantly different from Ad present in all of these cultures. These data suggest that Foxp3 activity is necessary for most of the suppressive capacity of the tCD8 cells.  

In contrast, pretreatment of tCD8 with siRNA for Foxp3 (Ag) or a combination of siRNA for Foxp3, p53, and CCR7 (Ab) increased anti-DNA production, indicating significant difference from Ac (p < 0.05 by Tukey’s posttest). In the third group of lanes (c–d), we show the siRNA controls as follows: tCD8 were preincubated with scrambled siRNA, the polyamine transfection medium, and siRNA for GAPDH. Suppression was present in all of these cultures. These data suggest that Foxp3 activity is necessary for most of the suppressive capacity of the tCD8+ T cells. The fourth group (l–n) shows the dose response for pCons added to cultures of nCD4+ T cells. Addition of 20 µg/ml pCons activated the suppressive capacity of the cells (5% of cells failed to exclude trypan blue); 100 µg of pCons was toxic to cells, indicated by >90% of cells failing to exclude trypan blue and low anti-DNA production (n). The fifth row of lanes shows absence of a dose response to a control peptide p33b, which is a wild peptide from the V\textsubscript{v3} of a mAb anti-DNA that binds I\textsuperscript{E\textsubscript{b}} and can induce BWF, T cells to proliferate (A, o–q). There was no suppression of anti-DNA production when tCD8 were incubated with 0 or 20 µg/ml p33b instead of with pCons. Again, 100 µg/ml peptide was toxic to cells. ANOVA analysis of all bars showed highly significant differences, p < 0.001. B. Suppression of anti-DNA by tCD8 T cells is still demonstrable at 3–4 wk after tolerization. Mean ± SEM of five experiments in each group is shown. Presentation is similar to that in A. tCD8 were harvested from spleens of mice treated once 3–4 wk earlier with pCons, and those cells were added to cocultures with naive CD4 and naive B cells (except where nCD8 or no CD8 were added; B, a–d). The first group are controls and show that CD4 plus B made the highest anti-DNA (Be), with addition of nCD8 producing nonsignificant suppression of anti-DNA production. In contrast, addition of tCD8+ T cells activated by pCons in the culture suppressed anti-DNA production significantly (compare Be and BC, p < 0.03 by unpaired Student’s t test). In the second set of lanes are shown the effects of preincubating tCD8 cells with siRNA for various molecules. There is no effect on suppression with siRNA for p53 and CCR7 (B, f and g), but siRNA for Foxp3 abrogates the suppressive capacity of the CD8+ T cells (compare Bh and Bi with Be, p < 0.05 for each). In the last group on the right are shown control siRNA for GAPDH and scrambled sequence of GAPDH (B, j and k), which do not affect suppression (compare with Be; differences not significant). ANOVA analysis of all the lanes shown in this graphic indicates significant differences as follows: p < 0.006.

### Results

**Blockade of Foxp3 abrogates the suppression of anti-DNA IgG production, and this effect lasts several weeks**

Having shown that CD8+ T cells induced by pCons express Foxp3 (8), we asked whether silencing of Foxp3 abrogates the suppression of IgG anti-DNA production that occurs when CD8+ T cells are added to cultures containing naive CD4+ T and B cells, and whether Foxp3 controls the suppressive effects of CD8+ T cell over time. Characteristics of spleen cells harvested 1 wk after tolerization are shown in Fig. 1A, with results grouped. The left group shows baseline measures of anti-DNA production by naive CD4+ T cells (Fig. 1Aa), then by naive B cells (Fig. 1Ab), with addition of naive CD8+ T cells (Fig. 1Ac), and finally suppression by addition of tolerized CD8+ T cells activated by pCons (Fig. 1Ad) (p < 0.05 compared with Fig. 1Aa). None of these controls abrogated the suppression of anti-DNA production by tolerized CD8+ T cells to inhibit anti-DNA production (data not shown). In the third group there are the results of pretreating tCD8+ T cells with siRNA for p53 and CCR7, which did not inhibit the ability of tCD8+ T cells to inhibit anti-DNA production (data not shown). In the third group, these results are presented by different conditions of siRNA and siRNA for GAPDH (Fig. 1A, i, j, and k). None of these controls abrogated the suppression of anti-DNA production by tolerized CD8+ T cells. These data suggest that Foxp3 activity is necessary for most of the suppressive capacity of the tCD8 cells.

**Statistical analyses**

Statistical analyses were performed using Prism 4 software (GraphPad). Comparisons between two groups were performed by one-tailed Student’s 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 of the table were then compared by posttest analysis using Tukey’s multiple comparison test. Values of p < 0.05 were considered significant.

Technologies), and RNA was isolated for real-time PCR, to validate knockdown of the target gene. We have previously shown that this method knocks down all expression of Foxp3 (8).
suppressive capacity of tCD8⁺ T cells. In the last group of lanes are shown the peptide dose response and the specificity data. When no peptide was in the culture (Fig. 1A, l and o), there was no suppression. At 20 μg/ml (Fig. 1Am), the peptide pCons induced suppression by the CD8⁺ T cells (p < 0.007 by Student’s t test, Fig. 1A, comparing l with m). At 100 μg/ml pCons, there was toxicity to the cells (indicated by trypan blue stain with >90% of the cells dead), and production of anti-DNA was baseline (Fig. 1Ar, compare with Fig. 1Aa). Finally, the p-Cons-induced tCD8⁺ T cells could not suppress anti-DNA production when stimulated by p33b at 20 μg/ml (Fig. 1Ap). p33b is a control wild VH peptide that binds I-Ek and can induce T cell activation in BWF1 mice. At 100 μg/ml, the peptide p33b was toxic to cells (Fig. 1Aq).

In Fig. 1B, a similar experiment is performed with CD8⁺ T cells harvested 3–4 wk after tolerization with pCons. The bars are grouped as in Fig. 1A. Suppression is present when tCD8⁺ T cells activated by pCons are added (Fig. 1B, e compared with c, p < 0.03). Suppression is again abrogated by silencing of Foxp3 (Fig.
CD8+ Ti suppress proliferation of CD4+ Th cells, and this effect needs the expression of Foxp3

Next, we asked whether CD8+ Ti could suppress proliferation of naive CD4+ T cells, the cells usually considered to be the major target of CD8+ Ti. CD8+ T cells from naive or tolerized BWF1 mice (harvested 1 wk after tolerization) were added to cultures of naive CD4+ T cells plus naive irradiated B cells (as APCs) from young naive BWF1 females, and proliferation was measured 72 h later. In Fig. 2A, we show the effect of adding CD8+ T cells to naive (n) cells, with nCD4 plus B cells showing the highest proliferation. The middle lanes show the effect of adding CD8+ T cells, with no significant inhibition of proliferation if cells are from naive mice (Fig. 2Bc), but significant suppression if cells are from tolerized mice activated in vitro with pCons (Fig. 2Bd, p < 0.001 compared with Fig. 2Be by unpaired Student’s t test). No suppression was observed if the tolerized CD8+ T cells were incubated with pNeg or p93 (Fig. 2Be, data combined for the two control peptides). In the final lane of this group (f), the suppression of proliferation by tCD8 is abrogated by inhibition of Foxp3. In the third group, we show the effect of tCD8+ cells on CD4+ T cells and B cells from tolerized mice. The tCD8+ T cells in combination with T cells (Fig. 2Bp) show proliferation similar to that of naive CD4+ T cells plus B cells (Fig. 2Bb). Addition of tolerized CD8+ T cells activated by pCons suppresses proliferation (Fig. 2Bh, p < 0.05 compared with Fig. 2Bg). The ability of tCD8+ T cells to suppress proliferation in tolerized CD8+ cells is abrogated by preincubation of tCD8+ cells with siRNA for Foxp3 (Fig. 2Bc).

Fig. 2C shows the ability of tCD8+ T cells isolated into CD28+ or CD28− subsets to suppress proliferation of naive CD4+ plus irradiated naive B cells. The left lanes show the controls, again indicating the ability of tCD8+ activated by pCons to suppress proliferation (Fig. 2C, compare b with a). If the CD8+ are isolated into CD28+ cells from naive mice, they do not suppress proliferation (Fig. 2Cc). If the CD28+ CD8+ T cells are from tolerized mice, they suppress (Fig. 2Cd, p < 0.05 compared with Fig. 2Ca). Naive CD28− CD8+ T cells can suppress (Fig. 2Ce, p < 0.01 compared with Fig. 2Ca), as can CD28− CD8+ T cells from tolerized mice (Fig. 2Cf, p < 0.05 compared with Fig. 2Ca).

We then addressed the question of inhibition of proliferation being an effect of tCD8+ cells on nCD4+ T cells, because CD8+ T have some proliferative capacity, and CD8− T were not irradiated in previous experiments. CD4+ T cells were treated with CFSE, then added to cultures containing naive B and tCD8+ T cells or naive B and naive CD8− T cells, which were cocultured for 72 h. Results are shown in Fig. 2D. Dilution of the CFSE (CD4 proliferation) was prevented by addition of tCD8+ T cells, but not naive CD8 (Fig. 2D). We conclude from all these data that CD4+ T cells are one target of the CD8+ T cells from tolerized mice, with their proliferation impaired in the presence of tCD8+ T cells in vitro.

The suppression mediated by CD8+ Ti is long lasting

The suppression of proliferation by CD8+ T cells from tolerized mice to suppress proliferation of naive T plus B cells from syngeneic mice over time.

Because the suppressive capacity of all CD8+ Ti seems to depend on Foxp3 expression in our system, we examined the expression of Foxp3 in CD8− Ti and CD8+ Ti subsets of CD8+ T cells studied ex vivo from spleens of tolerized mice and we explored regulation of Foxp3 by TGF-β. Results are shown in Fig. 4. In Fig. 4A, each bar shows the mean of 3–12 experiments, comparing fold changes of mRNA for Foxp3 in cell subsets from tolerized mice with the same subsets from naive mice. Mean fold changes varied from 1 to 7, with elevations being higher in CD8− CD8+ cells compared with CD8+ CD8+ cells at early time points. Foxp3 was elevated from 1–4 wk after a single treatment with pCons in CD8− CD8+ T cells (Fig. 4A, lanes 2 and 4), and the increases returned to baseline by 6–8 wk (lane 6, p < 0.01 compared with lane 2). In CD8+ CD8+ T cells, elevations of Foxp3 were apparent at 1–2 wk (lane 1), close to normal at 3–4 wk (lane 3), and normal at 6–8 wk (lane 5). At all times, Foxp3 levels were elevated; they were higher in CD8+ than in CD8+ cells. In Fig. 4B, the mean ratio of relative value units for both Foxp3 and TGF-β, comparing tolerized with naive mice, is shown over time for CD8− and CD8+ subsets of CD8+ T cells (reflecting splenic CD8+ T cells obtained ex vivo 1 wk after tolerization). Levels of TGF-β rose 9-fold in CD8− T cells (left panel) and 16-fold in CD8+ T cells (right panel) by 1–2 wk after tolerization, then fell toward normal, although they did not reach baseline in CD8− and CD8+ subsets of CD8+ T cells before returning to baseline at 6–8 wk, when all returned to baseline.

In Fig. 4C, dose response to TGF-β is shown in cultures containing naive B, naive CD4+, and tolerized CD8+ T cells (medium enriched with rIL-2) plus pCons. Results in the cultures without
with TGF-β mRNA was increased 3-fold after incubation of the naive cells /H11001
Relative value units (RVU) were calculated as ratio of Foxp3 to the GAPDH housekeeping gene. Differences between CD28+/H11001
+t test.

Bp

significantly different at both 1–2 wk (p = 0.009 by Mann-Whitney U test) and 3–4 wk (p < 0.05). Differences are significant between CD28+/H11001
T cells at 1–2 wk and at 6–8 wk (p = 0.05) and between CD28+ CD8+ T cells at 2–3 wk and at 6 wk. Mean of 3–12 experiments in each group is shown.

B, The time course of expression of mRNA encoding Foxp3 and TGF-β over 8 wk after tolerization is shown. The panel on the left shows mRNA for TGF-β and Foxp3 over time in CD28+/H11001
T cells from tolerized mice. The panel on the right shows the same for CD28+ CD8+ T cells from tolerized mice. Cells were isolated from spleen at the times specified on the x-axis and studied for mRNA expression without additional culture. Note that both TGF-β and Foxp3 expression were higher in CD28+ than CD28- CD8+ cells, but that they were elevated at 1–2 wk compared with baseline in both sets of cells. Data points are mean of two to three experiments at each time point, comparing fold increases in mRNA expression in tolerized with those in naive freshly isolated peripheral CD8+ T cells.

C, TGF-β induces Foxp3 expression in a dose-response manner in cultures containing CD8+ T cells from mice tolerized 1 wk earlier coincubated with naive T cells and naive B cells. CD8+ T cells (1 x 10^6) were cultured for 3 days with and without rTGF-β (20–40 ng/ml, as indicated on the x-axis) in complete medium with 10% FCS and rIL-2 (10 ng/ml) and with antibiotics. After 3 days, RNA was isolated for real-time PCR analysis. Cells were pooled together from three spleens. Foxp3 mRNA values were normalized to housekeeping gene GAPDH. Data are representative of one of three experiments with similar results.

D, TGF-β induces Foxp3 expression in CD8+ T cells cultured without other cells. Culture conditions as in A. Data are representative of one of three experiments with similar results. The two lanes on the left show increase of Foxp3 mRNA expression when tolerized CD8+ T cells are cultured with TGF-β. The two lanes on the right show the small increase in Foxp3 mRNA when tolerized CD8+ T cells are from naive mice. Lane 1 vs 2, p < 0.05 by unpaired Student’s t test.

E, Protein expression of Foxp3 is increased in tolerized vs naive CD8+ T cells after culture with TGF-β. The mean fluorescence intensity is shown for Foxp3 in CD8+ T cells isolated from tolerized mice (line on the right) or CD8+ T cells from naive mice (line on the left).

TGF-β are set to 1.0. RNA harvested from this cell combination showed up-regulation of Foxp3 expression after in vitro addition of TGF-β in a dose-response manner. In experiments shown in Fig. 4D, CD8+ T cells from tolerized mice were cultured with pCons in IL-2-containing medium, with or without TGF-β. Ratio of Foxp3 to GAPDH in the cells without TGF-β was set at 1.0. The addition of TGF-β increased expression of Foxp3 mRNA 15-fold in CD8+ T cells from tolerized mice (lane 2). The effects of TGF-β on the expression of Foxp3 mRNA in naive CD8+ T cells were also measured before and after incubation with TGF-β. Relative expression of Foxp3 in naive cells was 0.4 compared with tolerized cells (compare lane 3 with lane 1). Expression of Foxp3 mRNA was increased 3-fold after incubation of the naive cells with TGF-β. Thus, TGF-β increases Foxp3 expression in both tolerized and naive cells, but more dramatically in the tolerized CD8+ T cells. Finally, data shown in Fig. 4E show that the protein expression of Foxp3 is up-regulated in cells in which mRNA is up-regulated. In this study, we show the mean fluorescence intensity for labeled Foxp3 expression in cells studied by FACS, gated on CD8. Overall, the data in Fig. 4 show that TGF-β can increase the already up-regulated expression of Foxp3 mRNA in either tolerized CD8+ T cells, in both CD28+ and CD28- subsets of CD8+ T cells, or in the mRNA from combinations of those cells with other lymphocytes.

Previously, we have shown that the suppression of anti-DNA production by CD8+ T cells from tolerized mice is associated with increased expression of Foxp3, which is induced by TGF-β. In this study, we show that Foxp3 expression is enhanced in cells cultured with TGF-β, and we extend these observations to naive CD8+ T cells. Finally, data shown in Fig. 4E show that the protein expression of Foxp3 is up-regulated in cells in which mRNA is up-regulated. In this study, we show the mean fluorescence intensity for labeled Foxp3 expression in cells studied by FACS, gated on CD8. Overall, the data in Fig. 4 show that TGF-β can increase the already up-regulated expression of Foxp3 mRNA in either tolerized CD8+ T cells, in both CD28+ and CD28- subsets of CD8+ T cells, or in the mRNA from combinations of those cells with other lymphocytes.

Previously, we have shown that the suppression of anti-DNA production by CD8+ T cells from tolerized mice is associated with increased expression of Foxp3, which is induced by TGF-β. In this study, we show that Foxp3 expression is enhanced in cells cultured with TGF-β, and we extend these observations to naive CD8+ T cells. Finally, data shown in Fig. 4E show that the protein expression of Foxp3 is up-regulated in cells in which mRNA is up-regulated. In this study, we show the mean fluorescence intensity for labeled Foxp3 expression in cells studied by FACS, gated on CD8. Overall, the data in Fig. 4 show that TGF-β can increase the already up-regulated expression of Foxp3 mRNA in either tolerized CD8+ T cells, in both CD28+ and CD28- subsets of CD8+ T cells, or in the mRNA from combinations of those cells with other lymphocytes.
asked whether Foxp3 inhibition by siRNA influenced the ability of CD8$^+$ T cells to secrete TGF-β. In Fig. 5A, cells were cocultured as indicated on the x-axis for 48 h, and the secretion of TGF-β into the culture medium was measured by ELISA. Naïve BWF1 CD4$^+$ T cells (Fig. 5Ab) secreted TGF-β at a mean level of ~1,000 pg/ml. There was little secretion by CD8$^+$ T cells from naïve mice (Fig. 5Ac), but high secretion by CD8$^+$ T cells from tolerized mice (Fig. 5Ae, d compared with c, p < 0.009 by one-tailed Student’s t test). In the group of C, CD8$^+$ T cells were cocultured with CD4$^+$ T and B cells from naïve mice, then assayed 48 h later for TGF-β secretion. Secretion was high by cultures containing either nCD8 or tCD8 (Fig. 5A, e and f); however, when cultures were treated with siRNA for Foxp3 (Fig. 5Ag) secretion of TGF-β was significantly reduced (Fig. 5A, compare g with f, p < 0.009). In contrast, transfection with siRNA for p53 did not suppress TGF-β secretion (Fig. 5A, h, not significantly different from e or f). These data strongly suggest regulation of TGF-β by Foxp3 in the CD8$^+$ T cells generated in our tolerance model. To confirm that the relation between TGF-β production and Foxp3 applied to CD8$^+$ T cells in isolation from the other cells, CD8$^+$ T cells from tolerized or naïve mice were isolated from spleens 1 wk after tolerization, and their ability to secrete TGF-β was determined after 48-h culture. The tolerized CD8$^+$ T cells (Fig. 5Bb) secreted ~600 pg/ml TGF-β, but that secretion was suppressed significantly by pretreatment of the cells with siRNA for Foxp3 before addition to culture (Fig. 5Bc, p < 0.01 compared with Fig. 5Bb). In contrast, pretreatment of CD8$^+$ cells with siRNA for p53, GAPDH, or scrambled sequence did not impair TGF-β production (Fig. 5B, d–f). These data confirm a link between expression of Foxp3 and the ability of tCD8$^+$ T cells to secrete TGF-β.

Discussion

Induction of immune tolerance in vivo is a complex phenomenon that involves multiple different cells and soluble factors. Peripheral tolerance to autoantigens can result from several mechanisms, including ignorance, deletion of T or B cells, anergy of T cells, cytokine shift, BCR editing, and, last but not least, induction of regulatory/suppressor cells. In our system of tolerization with pCons, anergy of Th cells and induction of regulatory/Ti appear to occur concomitantly (8, 11).

This work analyzes some characteristics of the noncytotoxic CD8$^+$ T cells that are induced by pCons, and shows that the transcription factor Foxp3 may regulate the production of TGF-β in these CD8$^+$ Ti. Furthermore, the increased secretion of TGF-β in the CD8$^+$ Ti can be abrogated by disabling Foxp3 via siRNA technology. We also show that these CD8$^+$ Ti can directly suppress the proliferation of CD4$^+$ T cells and subsequent production of anti-DNA by B cells, and that this suppressive capacity of the CD8$^+$ Ti is long lasting (up to 8 wk in some mice).

Several investigators working in other systems, including human CD8$^+$ T cells, have found that CD8$^+$ Ti with a suppressive function can be preferentially associated with a CD28$^-$ phenotype (5, 6, 24). In our system, when we separated the CD8$^+$ Ti into CD28$^+$ and CD28$^-$ cells, we observed similar suppressive capacity and similar production of TGF-β in both the CD28$^+$ and CD28$^-$ subpopulations (Figs. 2C and 4, A and B). Of note, however, Foxp3 mRNA expression was increased to higher levels in the CD28$^-$ CD8$^+$ T cell compartment and persisted for longer periods of time than in the CD28$^+$ CD8$^+$ T cells (Fig. 4B). Furthermore, these kinetics were similar to those for production of TGF-β (Fig. 4B). This suggests the possibility of an influence of TGF-β on Foxp3 expression (similar to that observed in the case of Treg cells) that was higher and sustained longer in the CD28$^-$ T cells than in the CD28$^+$ T cells in our system.

For many years it has been known that oral tolerance depends in part on the induction of CD4$^+$ T cells that secrete TGF-β (25). In many systems, TGF-β, either secreted or membrane bound, is a key molecule for the ability of Treg or CD8$^+$ Ti to regulate organ-specific or systemic autoimmunity (26–28). It has also been known that TGF-β, in the presence of IL-2, can induce Foxp3 expression and can convert nonregulatory CD4$^+$CD25$^-$ T cells to Treg (21–23). Moreover, in peripheral CD4$^+$CD25$^-$ T cells, exposure to IL-4 and IL-13 can also generate Ag-specific Foxp3$^+$
cells with different surface and cytoplasmic phenotypes. Major cells. There are molecular themes that characterize suppressor whether Foxp3 may create an autocrine loop for the regulation production of TGF-$\beta$.

The authors have no financial conflict of interest.

References


