Cyclosporin A Markedly Enhances Superantigen-induced Peripheral T Cell Deletion and Inhibits Anergy Induction

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Summary

Cyclosporin A (CsA) is a well-known immunosuppressive agent that modulates immune tolerance in many ways. CsA can give rise to a state of long-term nonimmunosuppressed transplantation tolerance, but it can also aggravate autoimmune diseases, and provoke specific forms of autoimmunity. These effects, which are often paradoxical, remain largely unexplained. In this study, we investigated the effects of CsA on superantigen (superAg)-reactive peripheral T cells. The intravenous injection of either staphylococcal enterotoxin B (SEB), or Mls-1^a cells into Mls-1^b recipients, causes long-term in vitro nonresponsiveness (anergy) and partial elimination of the peripheral T cell receptor (TCR) Vβ8$^+$/CD4$^+$ and -Vβ6$^+$/CD4$^+$ T cell subsets, respectively. We report that CsA markedly enhances the peripheral elimination of SEB- and Mls-1^a-reactive T cells such that up to 90% of the targeted CD4$^+$/Vβ subpopulations are deleted. The degree of deletion depends on the dose and the schedule of CsA administration, and the number of superAg injections. In situations where the extent of deletion is only moderate, we find that the remaining superAg-reactive T cells fail to develop anergy, unlike the T cells of control superAg-immunized mice. Higher doses of CsA are required to enhance T cell deletion ($\geq$25 mg/kg/d, i.p.) than to impair anergy induction ($\geq$6.25 mg/kg/d, i.p.). In view of these results, it appears that the degree of tolerance in CsA/superAg-treated mice depends on the balance between these opposing effects, i.e., enhancement of peripheral elimination versus the abrogation of anergy. The possibility of enhancing or preventing immune tolerance with a drug may have important clinical implications.

Cyclosporin A (CsA)[1] is a potent immunosuppressive agent used clinically to control allograft rejection, GVHD, and some autoimmune diseases (1). It is well accepted that the principal inhibitory action of CsA is the result of impaired lymphokine production by T cells, especially IL-2 (2–4). In rats, a form of long-term nonimmunosuppressed tolerance to allografts can be induced by a short course of CsA treatment (5, 6). The underlying mechanism is not well understood, but may depend on the generation of antigen-specific suppressor cells (5, 7). Paradoxically, in some circumstances, CsA treatment can have detrimental effects, which include the induction of autoimmune diseases and syngeneic GVHD (reviewed in reference 8). It has been suggested that CsA induces syngeneic GVHD by blocking intrathymic clonal deletion (9, 10). However, our results (11) and those of Bryson et al. (12) do not support this interpretation. Alternatively, CsA could affect tolerance by acting on peripheral T cells.

In this study, we analyzed the effects of CsA therapy on the peripheral tolerance induced by superantigens (superAgs). Two superAgs were used, staphylococcal enterotoxin B (SEB) and Mls-1^a (a product of the intrinsic mouse mammary tumor virus 7 [MMTV-7]) (13), which stimulate TCR Vβ8$^+$ and TCR Vβ6$^+$/CD4$^+$ T cells, respectively (14, 15). These superAgs are known to cause both a partial elimination and a long-term in vitro anergy in their targeted T cell subpopulations (16–20). We found that superAg recognition during the course of CsA treatment leads to a considerable enhancement of the peripheral elimination of the reactive T cells. This elimination process is superAg specific. With protocols that provoke an extensive elimination of superAg-reactive T cells, the proliferative responses to these antigens in vitro are very low. In contrast, CsA abrogates the development of anergy, which usually occurs after injection of these superAgs. The latter effect is only apparent when sufficient numbers of undeleted T cells remain after CsA/superAg treatment. Relatively low doses of CsA could inhibit anergy induction, while higher doses were required to enhance T cell deletion. The blockage of anergy by CsA may be of importance in

[1] *Abbreviations used in this paper:* CsA, cyclosporin A; SE, staphylococcal enterotoxin; Tx, thymectomized.

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experimental models where CsA induces autoimmune phenomena. Based on our results, we postulate that CsA can induce a state of tolerance by provoking antigen-specific T cell elimination, but can sometimes paradoxically increase responses by preventing anergy induction.

Materials and Methods

Mice. BALB/c mice (H-2^d, Mls-1^b, Mls-2^a, Lyt-1.2, Lyt-2.2) and DBA/2 mice (H-2^d, Mls-1^a, Mls-2^a, Lyt-1.1, Lyt-2.1) were purchased from Charles River Laboratory (St. Constant, Quebec, Canada), and C3H/HeJ mice (H-2^k, Mls-1^b, Mls-2^a) were from The Jackson Laboratory (Bar Harbor, ME). Adult BALB/c mice (4–6 wk old) were thymectomized in our facility as described (21) and rested for 7 d before the onset of treatments. All the experiments were performed in female mice.

mAbs and Reagents. PE-conjugated anti-CD4, PE-conjugated anti-CD8, anti-Lyt-1.1, anti-Lyt-2.1, and anti-Thy-1.2 were purchased from Cedarlane Laboratories (Hornby, Canada). The following mAbs were produced as culture supernatants and purified by protein G (Pharmacia, Montreal, Quebec, Canada) affinity chromatography: anti-Vβ6 (44-22-1 hybridoma; 22), anti-Vβ8.1-8.2 (KJ16 hybridoma; 23), anti-Vβ8.1-8.2-8.3 (F23.1 hybridoma; 24), anti-Vβ14 (14.2 hybridoma; 25), and anti-TCR-α/β (H57–579 hybridoma; 26). For flow cytometry analysis the purified mAbs were directly conjugated to FITC. CsA was a kind gift of Sandoz (Dorval, Canada). SEA was purchased from Toxin Technology (Sarasota, FL) and SEB from was purchased from Sigma Chemical Co. (St. Louis, MO).

SuperAg Immunization and CsA Treatments. SuperAg immunizations were performed by tail vein injections of either 10^7 Mls-1^{a+} splenocytes (RBC depleted), or 50–100 μg of SEB. Cells or SEB were injected in a volume of 0.25 ml of PBS. The day of superAg injection was considered day 0 of our experiments. CsA was suspended in olive oil (OO) (20 mg/ml) and given intraperitoneally at a dose of 50 mg/kg/d, unless otherwise indicated. CsA was administered daily starting 1 d before superAg immunization (day −1) and continued until the day the mice were killed, unless otherwise indicated. Control groups included untreated mice, CsA-treated mice (no superAg), superAg-immunized mice (no CsA), and superAg- and diluent (OO)-treated mice. In control mice, CsA and/or diluent were administered in the same dose and/or volume as in the experimental groups.

Flow Cytometry Analysis. For two-color flow cytometry analysis, LN cells (pooled cervical, axillary, para-aortic, and mesenteric LN) were stained with either PE-anti-CD4 or PE-anti-CD8, and with one of the FITC-labeled-anti-Vβ mAbs mentioned above. One-color flow cytometry analysis was performed with FITC-labeled mAbs only. Briefly, 5×10^5 LN cells were incubated with PE-conjugated mAb for 15 min at 4°C, then washed three times with PBS containing 1% FCS (Gibco, Burlington, Canada) and then incubated in the same conditions with the second FITC-labeled-anti-Vβ mAb. Propidium iodide (Sigma Chemical Co.) was added after the last wash. Dead cells were excluded based on propidium iodide staining and forward scatter. In each sample, 10^4 cells were analyzed on a FACScan® (Becton Dickinson & Co., Missisauga, Canada) flow cytometer, and results were plotted on a logarithmic scale. In some experiments spleen cells were analyzed. Before staining, spleens cells (RBC depleted) were depleted of B cells by two rounds of sequential J11.D2 mAb and complement treatment, performed as we have previously described (27). After depletion of dead cells on a lymphocyte-M gradient (Cedarlane Laboratories), spleen cells were stained and analyzed as described above for LN cells.

In Vitro Proliferative Assays. LN cells were cultured in 96-well flat-bottomed plates (Gibco Laboratories, Grand Island, NY) in quadruplates at 5×10^5 cells/well with either: (*a*) for SEB stimulation, with 10^6 syngeneic irradiated (2,000 rad) splenocytes of untreated BALB/c mice and 10 μg/ml of SEB, or 1 μg/ml of SEA as a control; or (*b*) for Mls-1^a experiments, with 10^6 irradiated (1,000 rad) DBA/2 splenocytes or irradiated (1,000 rad) allogeneic C3H/HeJ (H-2^k, Mls-1^b) splenocytes. Cells were cultured in RPMI 1640 (Gibco Laboratories) containing 10% FCS, L-glutamine, 5×10^{-5} M 2-ME, and antibiotics. Cells were pulsed with [^{3}H]thymidine (ICN, Montreal, Canada) at day 3 of culture and harvested on day 4, unless otherwise indicated. Days in culture refer to the day that the cells were harvested. In all cases, the cells were harvested after an 18-h incubation with [^{3}H]thymidine. In the case of stimulation with either SEA or SEB, Δcpm = cmp experimental − cpm control (no superAg). In the case of Mls-1^a or MLC stimulation, Δcpm = cpm experimental − cpm with syngeneic stimulator cells.

Results

Enhanced Elimination of superAg-reactive T Cells in CsA/superAg-treated Mice. When injected to an appropriate recipient, Mls-1^{a+} cells and SEB induce a partial elimination of the specific superAg-reactive T cells (19, 20, and Figs. 1 and 2). We find that the elimination of superAg-reactive T cells is markedly augmented by CsA administration. As shown in Table 1 and Figure 1, *a–d*, the frequency of CD4$^+$/Vβ6$^+$ T cells (reactive to Mls-1^a) in Mls-1^b recipients treated with CsA (50 mg/kg/d) decreases to <20% of the original values in BALB/c mice. The CD4$^+$/Vβ8.1$^+$ T cells, another Mls-1^a-reactive subset, may also be affected by CsA/Mls-1^a-treatment, since there was a partial decrease in KJ16$^+$ (Vβ8.1$^+$/Vβ8.2$^+$)/CD4$^+$ T cell population (data not shown). The loss of CD4$^+$/Vβ6$^+$ T cells in CsA/Mls-1^a-treated mice represents a 3.6-fold greater deletion, when compared with the control Mls-1^a-immunized (non-CsA-treated) groups (Table 1). The deletion is specific since the frequency of CD4$^+$/Vβ14$^+$ T cell (which do not react to Mls-1^a antigen) remained relatively unchanged. The enhanced deletion of CD4$^+$/Vβ6$^+$ T cells can be observed in both the spleen and LN of treated mice (Table 1). Generally, the CsA/Mls-1^a-induced elimination is completed by day 7 postimmunization (data not shown). Comparable results were obtained in CsA-treated CBA/CaJ (Mls-1^b) mice injected with CBA/J (Mls-1^a) spleen cells (data not shown).

A CsA-enhanced T cell elimination process was also observed in SEB-immunized mice. A single injection of SEB after the initiation of CsA therapy (50 mg/kg/d) caused a rapid decline in the numbers of the reactive CD4$^+$/Vβ8$^+$ T cells, as demonstrated in Fig. 1, *e–h*, and Table 2, unlike in the SEB-immunized control animals. This early decline was apparent at day 3 (Table 2), and was observed within 36 h post-SEB immunization (data not shown). However, with only one SEB injection, CsA did not greatly enhance the deletion observed at days 12 and 30 (Table 2). When a second injection of SEB (SEBx2) was performed at day 7,

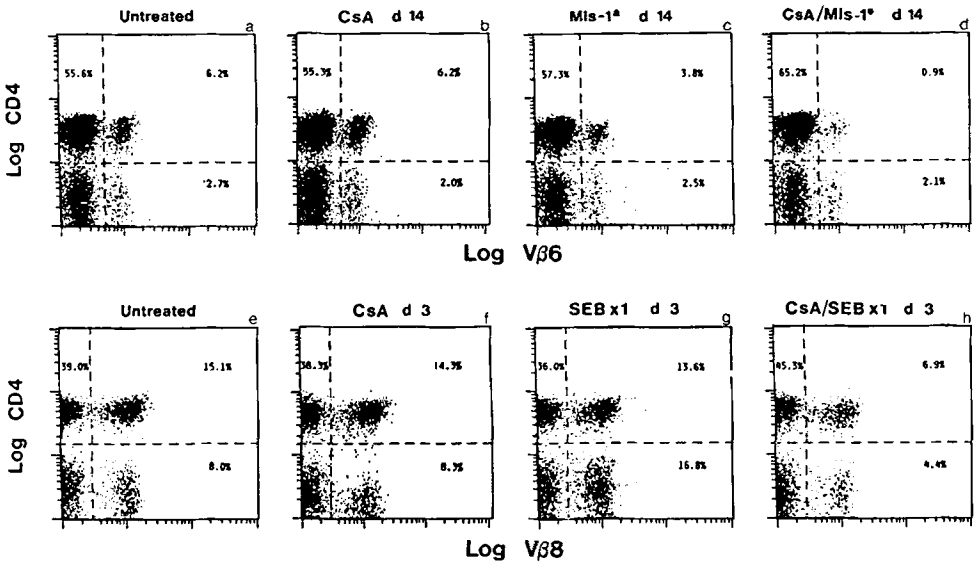

Figure 1. Enhanced elimination of superAg-reactive T cells in CsA/superAg-treated mice. Day 0 refers to the day of superAg (Mls-1^a or SEB) injection. CsA (50 mg/kg/d) was administered from day −1 of the experiments until either day 3 or 14 as indicated. Histograms show two-color flow cytometry analysis of BALB/c LN cells of untreated mice (*a* and *e*); CsA-treated control mice (*b* and *f*); Mls-1^a-immunized mice (*c*); CsA-treated and Mls-1^a-immunized (CsA/Mls-1^a) mice (*d*); SEB-immunized mice (*g*); and CsA-treated SEB-immunized (CsA/SEB) mice (*h*). Staining was performed with CD4-PE and either anti-Vβ6-FITC (44-22-1 mAb) or anti-Vβ8-FITC (F23.1, anti-Vβ8.1, 8.2, 8.3 mAb). 10,000 cells were analyzed in each histogram and results are plotted on a logarithmic scale.

Table 1. *CsA Enhances Elimination of Mls-1^a-reactive CD4$^+$/Vβ6$^+$ T Cells in Mls-1^a-treated Mice*

Mls-1^a donor	Mls-1^b recipient	CsA treatment	Vβ6 among CD4* day 0‡	Vβ6 among CD4* day 3	Vβ6 among CD4* day 14	Vβ6 among CD4* day 28	Vβ6 among CD8 day 0‡	Vβ6 among CD8 day 28
DBA/2	BALB/c	−	9.9 ± 0.5 (10.3 ± 0.7)	5.2 ± 0.6	5.3 ± 0.3 (7.1 ± 0.8)	6.2 ± 0.8	11.9 ± 1.2	11.3 ± 1.3
		+	10.2 ± 0.5 (10.4 ± 0.2)	4.3 ± 0.2	1.7 ± 0.1 (2.4 ± 0.9)	1.7 ± 0.2	11.0 ± 0.9	10.7 ± 1.4
		+§ (interrupted)			3.3 ± 0.2			
			Vβ14 among CD4 day 0‡	Vβ14 among CD4 day 3	Vβ14 among CD4 day 14	Vβ14 among CD4 day 28	Vβ14 among CD8 day 0‡	Vβ14 among CD8 day 28
DBA/2	BALB/c	−	9.4 ± 0.2 (9.8 ± 0.1)	9.5 ± 0.3	10.3 ± 0.4 (9.9 ± 0.2)	9.9 ± 0.7	4.0 ± 0.3	4.5 ± 0.4
		+	8.9 ± 0.5 (9.9 ± 0.0)	9.9 ± 0.2	10.4 ± 0.2 (10.9 ± 0.1)	10.0 ± 0.5	4.0 ± 0.5	4.8 ± 0.2
		+§ (interrupted)			10.7 ± 0.0			

* Day 0 represents the day of superAg immunization. Mice received either no CsA (−) or a daily injection of 50 mg/kg of CsA beginning on day −1, until either day 3, 14, or 28 as indicated. LN cells were analyzed by two-color flow cytometry (see Fig. 1). Values in parentheses represent analysis of B cell–depleted spleen cells.

‡ These mice were not injected with Mls-1^a cells. T cell frequencies for CsA-treated control mice are expressed as the mean for all CsA-treated control groups (i.e., mice were injected with CsA from day −1 to either day 3, 14, or 28). Vβ6$^+$ and Vβ14$^+$ T cell frequencies were closely similar in these three groups.

§ CsA was administered on day −1 and days 2–14 (i.e., a 2-d interruption of CsA treatment).

Table 2. *CsA Enhances Elimination of $V_\beta8^+$ T Cells in SEB-treated Mice*

BALB/c SEB injected	CsA treatment	SEB-reactive $V_\beta8$ subset*					
		$V_\beta8$ among CD4				$V_\beta8$ among CD8	
		day 0‡	day 3	day 12	day 30	day 0‡	day 30
x1	–	23.7 ± 0.5	24.8 ± 0.9	13.2 ± 0.3	13.4 ± 0.5	41.6 ± 1.6	35.0 ± 1.4
	+	24.2 ± 0.9	11.9 ± 0.8	11.6 ± 0.6	8.9 ± 0.9	40.1 ± 2.0	16.3 ± 2.5
	+ (stopped day 20)§	22.9 ± 1.1			8.5 ± 0.3		15.9 ± 1.8
x2	–			12.4 ± 0.2	12.7 ± 0.3		18.3 ± 0.3
	+			6.5 ± 0.3	5.2 ± 0.2		14.2 ± 0.6
		Control $V_\beta6$ subset					
		$V_\beta6$ among CD4				$V_\beta6$ among CD8	
		day 0‡	day 3	day 12	day 30	day 0‡	day 30
x1	–	9.9 ± 0.6	9.5 ± 0.4	12.2 ± 0.2	11.9 ± 1.1	11.5 ± 1.1	13.1 ± 0.7
	+	10.2 ± 0.2	13.0 ± 1.2	13.8 ± 0.6	11.5 ± 0.3	11.6 ± 0.6	14.9 ± 0.8
	+ (stopped day 20)§	9.8 ± 0.4			10.5 ± 0.6		15.0 ± 0.2
x2	–			12.6 ± 0.1	12.1 ± 0.9		14.4 ± 1.1
	+			13.7 ± 1.0	14.3 ± 1.0		15.2 ± 2.1

* Day 0 represents the day of the first super Ag immunization. Mice received one (x1) or two (x2) injections of 100 μg of SEB. The second injection of SEB was performed at day 7. Mice received either no CsA (–) or 50 mg/kg of CsA daily from day –1, until either day 3, 12, or 30 as indicated. LN cells were analyzed by two-color flow cytometry (see Fig. 1). $V_\beta8^+$ T cells were detected with the F23.1 mAb (anti-$V_\beta8.1$, 8.2, 8.3).
‡ These mice were not injected with SEB. T cell frequencies for CsA-treated control (non-SEB-injected) mice represent the mean of the three groups injected with CsA from day –1 to either day 3, 12, or 30. $V_\beta8^+$ and $V_\beta6^+$ T cell frequencies were closely similar in these three groups.
§ These mice received CsA from day –1 to day 20.

the long-term deletion of $CD4^+/V\beta8^+$ T cells became clearly more pronounced in CsA-treated than in non-CsA-treated mice (Table 2). Multiple additional injections of SEB in CsA-treated mice greatly enhanced the elimination of $CD4^+/V\beta8^+$ T cells, such that their frequency decreased to 1.8% (Fig. 2). This represents <10% of the frequency of $CD4^+/V\beta8^+$ T cells normally found in BALB/c mice. In mice that received a single injection of SEB, CsA provoked the loss of approximately half of the $CD8^+/V\beta8^+$ T cells, while there was only a small reduction in the numbers of these cells in control mice (Table 2). Unlike $CD4^+/V\beta8^+$ T cells, the elimination of $CD8^+/V\beta8^+$ T cells was not further augmented by multiple injections of SEB (Table 2 and data not shown). The CsA/SEB elimination process is superAg specific, as shown by the unaltered (or slightly increased) frequency of $CD4^+/V\beta6^+$ and $CD8^+/V\beta6^+$ T cells (Table 2).

Effect of CsA Dose on T Cell Deletion. SuperAg-induced T cell deletion was enhanced in mice injected with CsA at doses of 25 mg/kg/d, and even more so at doses of 50 mg/kg/d, but not in mice injected with lower CsA doses or diluent (OO) (Fig. 3). While CsA enhanced T cell deletion, it inhibited the development of anergy that was observed in control mice (see below under in vitro stimulation assays).

CsA/superAg-induced Elimination in Thymectomized (Tx) Mice and the Effects of CSA Treatment Termination. To determine if the T cell deletion was a peripheral event, adult BALB/c mice were Tx 7 d before CsA treatment and/or Mls-1^a im-

Table 3. *Enhanced $CD4^+/V_\beta6^+$ T Cell Elimination by CsA Treatment in the LN of Thymectomized Mls-1^a-immunized BALB/c*

CsA treatment*	$V_\beta6$ among $CD4^+$ cells			
	day 0‡	day 3	day 14	day 28
–	10.6 ± 0.2	6.4 ± 0.3	6.5 ± 0.0	6.5 ± 0.5
Continuous	10.3 ± 0.4	6.4 ± 0.1	2.2 ± 0.1	2.4 ± 0.2
Stopped at day 20§	10.2 ± 0.2	–	–	2.2 ± 0.4

* Mice received either no CsA (–) or 50 mg/kg/d of CsA from day –1, up until either day 3, 14, or 28 as indicated.
‡ Mice not immunized with Mls-1^a cells.
§ These mice received CsA from day –1 to day 20.

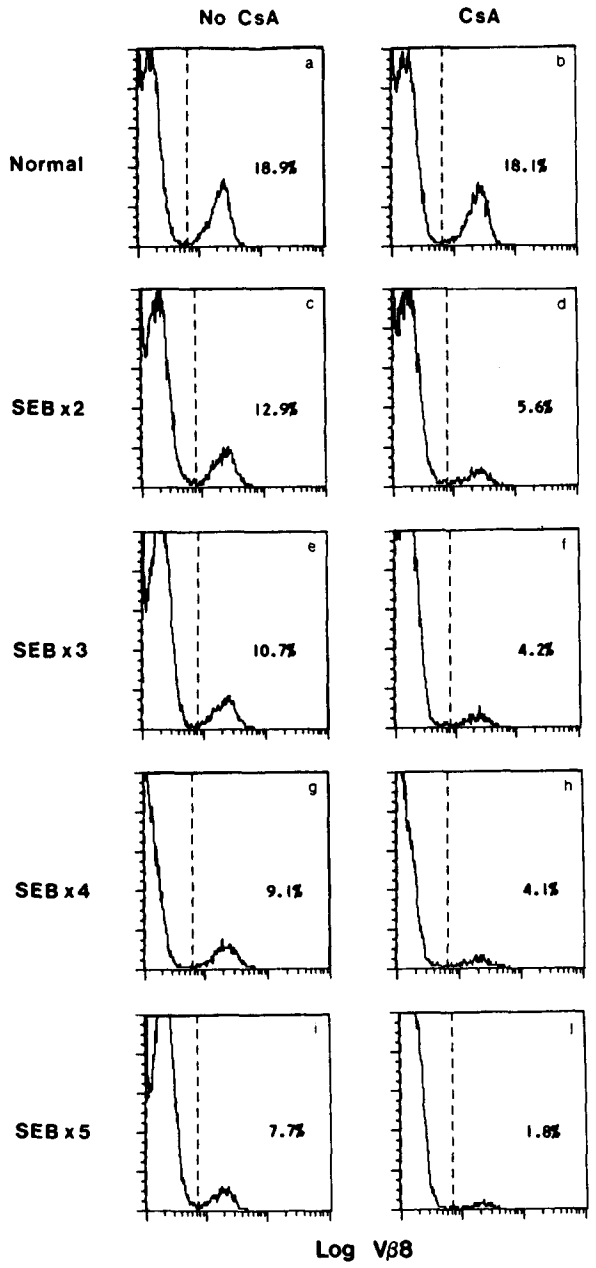

Figure 2. Marked enhancement of $CD4^+/V\beta8^+$ T cell deletion with multiple injections of SEB in CsA-treated mice. Single-color histograms represent the relative numbers of gated LN $CD4^+$ cells (*y*-axis) stained with FITC-conjugated anti-$V\beta8.1$–8.2 (KJ16) mAb (*x*-axis). SEB (50 μg) was injected intravenously either once (SEBx1) or every 4 d up to five times (SEBx5) and the mice were killed 4 d after the last injection. The histograms on the left are from non-CsA-treated mice, while those on the right are from CsA-treated mice. CsA treatment (50 mg/kg/d) was begun 1 d before the first SEB injection and continued until the day at which the mice were killed. Representative results are shown.

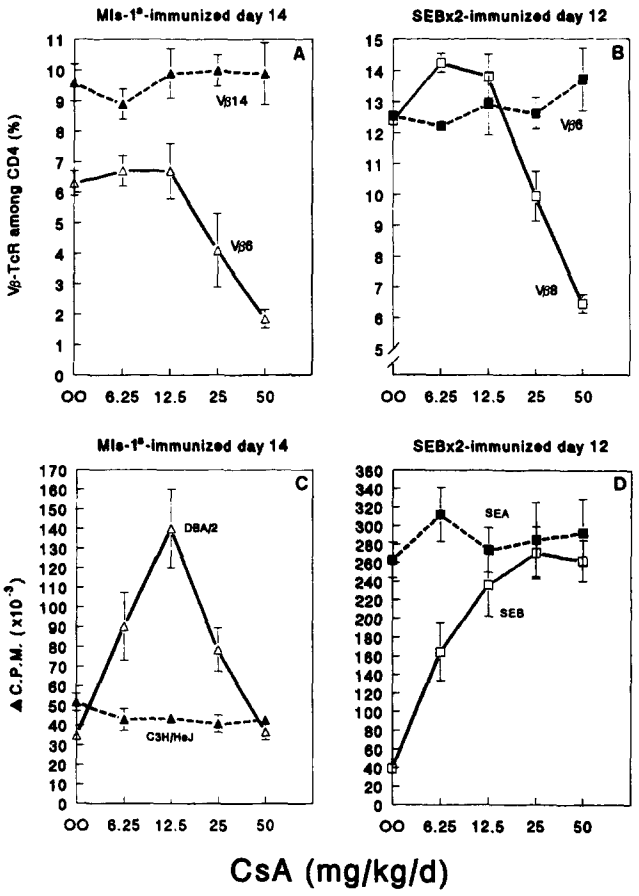

Figure 3. Effects of CsA dose on LN T cell numbers and in vitro responses to superAgs. BALB/c mice were injected with either Mls-1^a cells or SEBx2. Immunizations and analyses were performed as described in Tables 1 and 2. Mice were treated with OO, or various doses of CsA, from day −1 to either day 12 (SEB-immunized mice) or to day 14 (Mls-1^a-injected mice). (*A*) $V\beta6^+$ (△) and $V\beta14^+$ (▲) T cell numbers in Mls-1^a-immunized mice; (*B*) $V\beta8^+$ (□) and $V\beta6^+$ (■) T cell numbers in SEB-injected mice; (*C*) proliferative responses of LN cells to either DBA/2(H-2^d, Mls-1^a) (△) or C3H/HeJ (H-2^k, Mls-1^b) (▲) irradiated stimulator cells, in Mls-1^a-immunized mice; (*D*) proliferative responses of LN cells to either SEB (□) or SEA (■), in SEB-immunized mice. Results represent either percent T cell numbers ± SEM, among $CD4^+$ cells (*A* and *B*), or Δ cpm ± SEM (*C* and *D*). Three to five mice were analyzed in each group.

munization. As shown in Table 3, the extent of elimination of $CD4^+/V\beta6^+$ T cells in CsA/Mls-1^a-treated Tx mice was equivalent to that observed in the euthymic groups.

When CsA treatment was stopped for 8 d in CsA/Mls-1^a-treated Tx mice (Table 3) or 10 d in euthymic CsA/SEB-treated mice (Table 2), we observed no change in superAg-reactive T cell frequency. Thus, withdrawal of CsA does not result in a rapid increase of superAg-reactive T cell numbers.

T Cell Marker Analysis of LN and Spleen Cells. The reduction of superAg-reactive T cells that we observed could have been secondary to either deletion or to downregulation of the TCR. In addition, in the Mls-1^a-injected groups (i.e., Mls-1^a alone or CsA/Mls-1^a-treated mice), there might have been an expansion of donor T cells, which would reduce the relative frequency of the host Vβ subpopulations. To answer these questions, we analyzed the expression of several T cell markers in these mice. We found that the LN of CsA/Mls-

Table 4. *T Cell Marker Analysis of LN and Spleen Cells in CsA/Mls-1^a-treated BALB/c Mice at Day 14*

Group*	$V_\beta6^+/CD4^+$‡	$V_\beta14^+/CD4^+$‡	TCR-α/β^+§	$CD4^+/CD8^+$‡	Thy-1.2$^+$‡	Lyt-1.1$^+$§	Lyt-2.1$^+$§
Untreated	6.0 ± 0.1	5.2 ± 0.3	74.4 ± 0.2	73.6 ± 0.3	75.2 ± 0.7	0.0 ± 0.1	0.0 ± 0.0
						(0.0 ± 0.0)	(0.0 ± 0.0)
CsA	5.9 ± 0.2	4.8 ± 0.1	73.7 ± 0.4	71.8 ± 0.8	72.9 ± 1.0	0.0 ± 0.0	0.0 ± 0.0
						(0.2 ± 0.2)	(0.2 ± 0.1)
Mls-1^a	3.4 ± 0.3	5.6 ± 0.6	75.3 ± 0.5	75.8 ± 0.1	74.7 ± 0.9	0.0 ± 0.1	0.0 ± 0.0
						(0.0 ± 0.0)	(0.1 ± 0.1)
CsA/Mls-1^a	0.7 ± 0.2	5.2 ± 0.3	67.9 ± 0.4	66.4 ± 0.5	65.8 ± 1.2	0.0 ± 0.3	0.1 ± 0.3
						(0.0 ± 0.0)	(0.1 ± 0.2)

* The groups are the same at those described in Fig. 1.
‡ $V_\beta6^+/CD4^+$ cells and $V_\beta14^+/CD4^+$ cells were enumerated by two-color flow cytometry with CD4-PE and either anti Vβ6-FITC or anti-Vβ14 FITC. TCR-α/β^+ and Thy-1.2$^+$ cells were analyzed by one-color staining. $CD4^+/CD8^+$ represents the sum of CD4$^+$ and CD8$^+$ T cells analyzed in two colors. Positive cells are expressed as a percent of total LN cell numbers ± SEM.
§ DBA/2 (donor) cells are Lyt-1.1$^+$ and Lyt-2.1$^+$. BALB/c (recipient) cells are Lyt-1.2$^+$ and Lyt-2.2$^+$. In DBA/2 spleens a mean of 27.5% of cells were Lyt-1.1$^+$, and a mean of 13.5% of cells were Lyt-2.1$^+$ (data not shown). Values in parentheses represent cell numbers in B cell–depleted BALB/c (recipient) spleen cells.

1^a-treated mice had closely similar frequencies of cells expressing either Thy-1, TCR-α/β, or CD4 + CD8 (Table 4). Thus, it seems unlikely that there were significant numbers of T cells bearing conventional T cell markers but that they were negative for the TCR due to downregulation. Moreover, in both Mls-1^a- and SEB-treated groups, no variation in TCR intensity was detected in the superAg-reactive T cell populations (Figs. 1 and 2).

In Mls-1^a-treated BALB/c mice the analysis of Lyt-1 and Lyt-2 alleles in the spleen (depleted of B cells by J11D2 and complement treatment) or the LN revealed that virtually all T lymphocytes were of host origin (Table 4). Consequently, non-Vβ6 expressing donor T cells could not have been replacing or diluting host T cells.

In Vitro Stimulation Assays. Intravenous injection of Mls-1^{a+} cells or SEB results in a state of anergy (proliferative non-

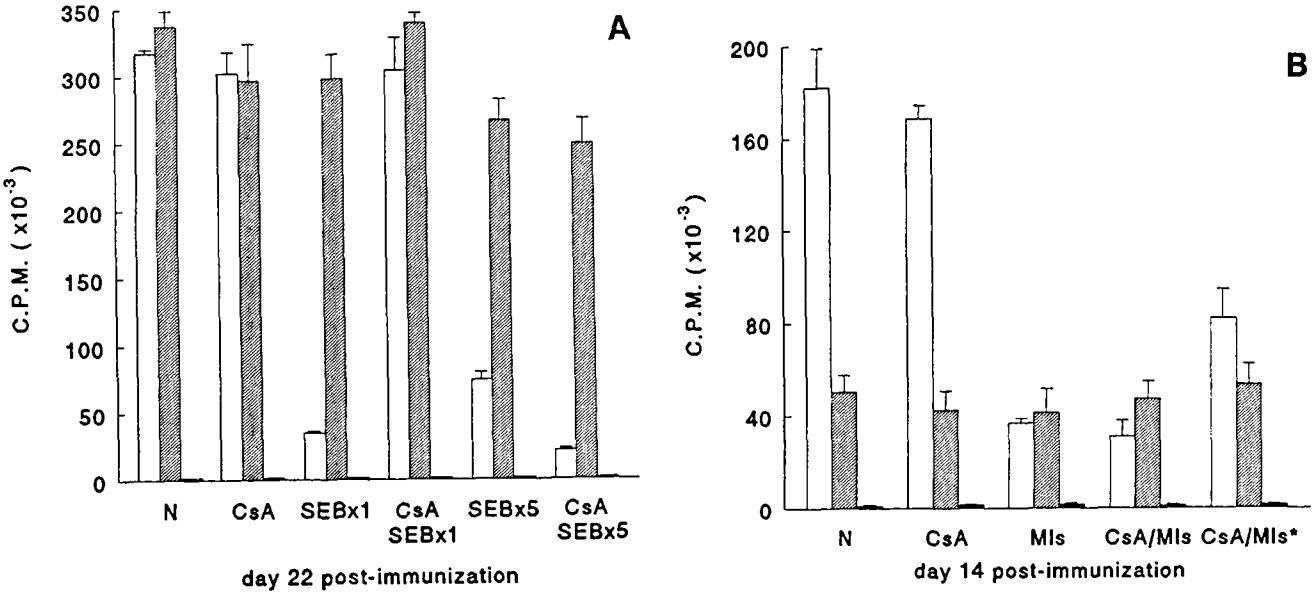

Figure 4. Inhibition of SuperAg-induced clonal anergy by CsA. (*A*) The proliferative responses of the LN cells of BALB/c mice with SEB (*open bars*) or SEA (*hatched bars*) in vitro stimulation (cells harvested at day 4 of culture). Mice were treated with CsA and SEB as explained in the legend to Fig. 2. Results represent the mean cpm ± SEM (four to five mice in each group). (*B*) The in vitro proliferative responses of the LN cells of BALB/c mice treated with CsA and Mls-1^a cell injections as explained in the legend to Fig. 1. LN cells were stimulated either with irradiated DBA/2 (H-2^d, Mls-1^a) spleen cells (*open bars*) or irradiated C3H/HeJ (H-2^k, Mls-1^b) spleen cells (*hatched bars*). The results respresent the mean cpm (cells harvested at day 4 of culture) ± SEM (5–15 mice in each group). Stimulation with syngeneic cells resulted in less than 1,700 cpm in all cases (*filled bars*).

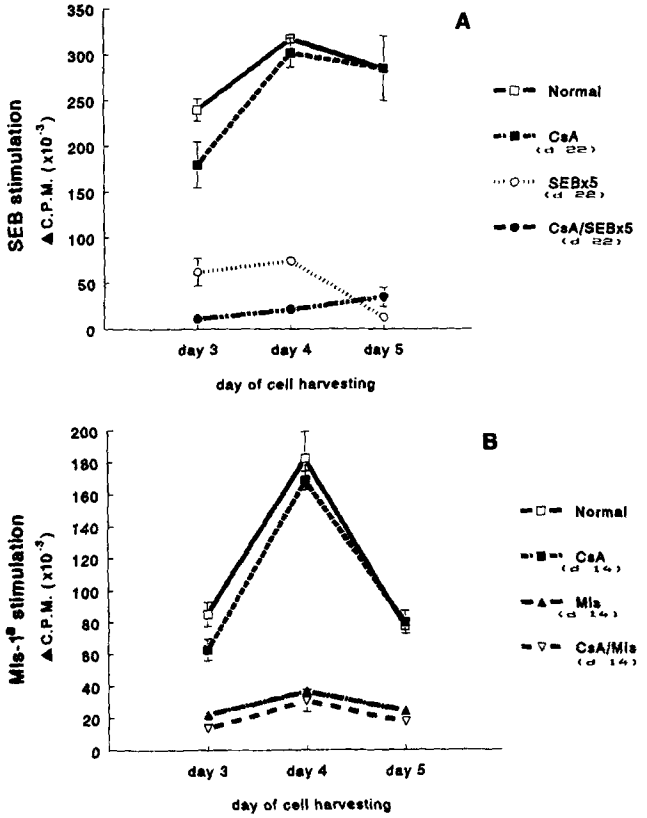

Figure 5. Kinetics of the peak proliferative responses in mice immunized with superAg and/or CsA treated. The groups of mice presented here are the same as in Fig. 4. (*A*) LN cells of BALB/c mice were stimulated with SEB in vitro. The results represent the mean Δcpm ± SEM. (*B*) LN cells of BALB/c (H-2^d, Mls-1^b) mice (treated as indicated) were stimulated with DBA/2 (H-2^d, Mls-1^a) irradiated spleen cells. The results represent the mean Δcpm ± SEM. In both panels the peak proliferative response was observed at day 4 of culture in all groups studied, except in the CSA/SEBx5 group, where responses were maximal at day 5 (the latter responses were very low). Stimulation with syngeneic stimulator cells (no superAg) yielded less than 1,500 cpm in all cases.

responsiveness) when T cells are challenged with the same antigen in vitro (16–18). As shown in Fig. 4 *A*, the T cells of mice injected with SEB once (SEBx1) or five times (SEBx5) are strongly anergic, while responses to SEA are unchanged. The development of anergy was not observed when CsA (50 mg/kg/d) was given in SEBx1-immunized mice (Fig. 4 *A*). The proliferative capacity to SEB remained comparable with untreated mice (Fig. 4 *A*). However, the elimination of SEB-reactive T cells was much more extensive in CsA/SEBx5-treated mice, and in that case the SEB-stimulated proliferation was drastically reduced (Fig. 4 *A*). Presumably, the latter low responses were due to the extensive T cell elimination.

The inhibition of anergy induction was apparent at CsA doses as low as 6.25 mg/kg/d, although higher doses were more effective (Fig. 3). It is noteworthy that mice injected with 6.25 or 12.5 mg/kg/d of CsA did not have enhanced T cell deletion (Fig. 3). In mice injected with 50 mg/kg/d of CsA, the blockage of anergy induction after Mls-1^a treatment was difficult to assess, because these mice had low numbers of CD4$^+$/Vβ6$^+$ cells (Table 1, Fig. 3). The low proliferative responses observed with Mls-1^a stimulation (Fig. 4 *b*) could be secondary to the deletion event as well as to delayed onset of anergy. However, when high-dose CsA treatment was temporarily interrupted, e.g., at days 0 and 1, mice had an intermediate extent of deletion of CD4$^+$/Vβ6$^+$ T cells (Table 1) and stronger in vitro proliferative responses to Mls-1^a stimulation (Fig. 4 *b*, *CsA/Mls**). It appears that in the latter case CsA had prevented anergy, while causing insufficient deletion to abrogate the proliferative responses.

Webb et al. (20) have reported that early after Mls-1^a immunization, the CD4$^+$/Vβ6$^+$ T cells had a strong proliferative response to Mls-1^a, but the peak response was seen at day 3 of in vitro cultures rather than day 4 for normal (untreated) mice. However, this form of accelerated kinetics is not observed at day 14 postimmunization (Fig. 5 *B*, and reference 20), at which time the maximal proliferative response is weak. Accordingly, in CsA- and non-CsA-treated groups, we find that the maximal proliferative responses at day 14 post-Mls-1^a immunization and at day 22 post-SEB immunization occur as with usual proliferative kinetics (day 4 peak) (Fig. 5). SuperAg-immunized mice (non-CsA treated) had low peak responses despite the presence of substantial numbers of potentially Mls-1^a-reactive (CD4$^+$/Vβ6$^+$) and SEB-reactive (CD4$^+$/Vβ8$^+$) T cells, and these T cells can be markedly reduced in numbers by CsA treatment (Tables 1 and 2).

Discussion

This study was prompted by the numerous reports showing that CsA can alter immunologic tolerance (1, 8). These effects are poorly understood and often contradictory, since in some cases a short course of CsA treatment has induced tolerance to allografts, while in other experimental models this drug has provoked autoimmune phenomena (8). Enhanced tolerance has often been attributed to the action of suppressor cells (5, 7), while the paradoxical induction of autoimmune diseases has been imputed to defective thymocytic differentiation (9, 10). Nevertheless, for a variety of reasons, we hypothesized that CsA could also act by affecting peripheral T cell tolerance mechanisms (i.e., peripheral deletion and anergy).

Our study shows that CsA treatment can markedly enhance the deletion of superAg-reactive T cells in either Mls-1^a- or SEB-immunized mice. Without CsA treatment, the partial T cell elimination observed in superAg-immunized mice is limited and preceded by an expansion of the targeted T cells (19, 20, and our unpublished observations). Not surprisingly, CsA prevents this expansion phase. Nonetheless, the CsA/superAg-induced deletion occurs earlier and is more extensive than with superAg immunization alone. Under optimal conditions, the extent of T cell elimination in CsA/superAg-treated mice can vary from three- to fourfold greater than in non-CsA-treated superAg-immunized mice. In mice receiving CsA, up to 90% of reactive CD4$^+$ T cells

can be eliminated. Thus, with both superAgs, CsA treatment results in an earlier and more extensive deletion process, seen at the time when clonal expansion is often apparent in control superAg-immunized mice.

A combined CsA and superAg treatment had no effect on effect on nonreactive T cell Vβ subpopulations, other than a slight increase in their frequencies, which likely resulted from the loss of the targeted subsets. Thus, CsA appears to enhance the deletion of only those T cells that are responding to superAgs. No sign of downmodulation of the TCR was detected, and the cessation of CsA administration did not result in a rapid increase in the frequency of reactive T cells. In addition, in the Mls-1^a-injected groups (i.e., Mls-1^a alone or CsA/Mls-1^a), we found no detectable levels of donor T cells in the lymph nodes or the spleen. This excludes the possibility of a massive expansion of non-Vβ6$^+$ donor T cells that could reduce the relative frequency of the host T cell Vβ$^+$ subpopulations. In view of these results, we believe that the disappearance of superAg-reactive T cells in CsA/superAg-treated mice results primarily from a deletion event rather than an alternative process.

The deletion observed in Tx mice treated with CsA and Mls-1^{a+} cells was similar to that of euthymic mice. Therefore, the CsA/superAg-induced T cell deletion is a peripheral event, as reported by others in non-CsA-treated superAg-immunized mice (19, 20).

The T cells of superAg-immunized mice usually display a markedly reduced in vitro proliferative response to the same superAg (16–18). This condition is generally referred to as anergy. Recently, some authors have questioned the anergic status of Vβ6$^+$ T cells in Mls-1^a-immunized mice (20). They showed that early after immunization with Mls-1^{a+} cells, the low responses to Mls-1^a were due to the different kinetics of responses in these mice (i.e., previously activated T cells had an earlier peak proliferative response). At day 14 postimmunization, they observed (as we do) low proliferative responses in Mls-1^{a+} cells with no accelerated kinetics effect. They attributed the loss of strong in vitro proliferative responses to the partial elimination of superAg-reactive T cells. However, our results do not support this conclusion, since there were substantial numbers of undeleted CD4$^+$/Vβ6$^+$ T cells at day 14 post-Mls-1^a immunization (without CsA) that could be markedly reduced by CsA treatment. Therefore, we conclude that most of the unresponsive CD4$^+$/Vβ6$^+$ T cells of Mls-1^a-immunized mice (non-CsA treated) must be potentially Mls-1^a reactive since their deletion is specifically provoked by CsA, as well as that these T cells are anergic.

Similarly, our results provide strong evidence that most of the nonresponsive Vβ8$^+$ T cells of SEB-immunized mice are in fact TCR-SEB specific, since they can be specifically deleted with CsA treatment. Moreover, mice treated with CsA (50 mg/kg/d) and injected with SEB twice have a twofold greater deletion of Vβ8$^+$ T cells vs. non-CsA-treated mice, but much higher in vitro proliferative responses to this superAg. Thus, the undeleted cells are superAg reactive, but fail to develop anergy in CsA-treated mice. The potential superAg reactivity of the majority of unresponsive Vβ6$^+$ T cells (Mls-1^a-immunized mice) and unresponsive Vβ8$^+$ T cells (SEB-immunized mice) has not been previously demonstrated. In addition, it is not unlikely that some of the residual (undeleted) T cells are not superAg specific. However, if this is the case, such nonspecific cells would represent <10% of all CD4$^+$/Vβ6$^+$ and CD4$^+$/Vβ8$^+$ T cells, based on the percent of cells that resist deletion. In accordance with our results, Rellahan et al. (18) found that the T cells of SEB-immunized mice proliferated only weakly when stimulated with an anti-Vβ8-mAb; i.e., there was no evidence of large numbers of nonanergic Vβ8$^+$ T cells.

CsA clearly prevents the development of anergy in SEB-treated mice. However, this is only apparent in mice that have moderate deletion of CD4$^+$/Vβ8$^+$ T cells, as in mice injected with SEB only once. In mice with severe deletion of CD4$^+$/Vβ8$^+$ T cells, e.g., mice injected five times with SEB, the in vitro proliferative responses to this superAg are very low. This indicates that extensive deletion of reactive T cells can counteract the strong in vitro proliferative responses due to the blockage of anergy by CsA. This is also true in the case of Mls-1^a-immunized mice. Our results show that lower doses of CsA are required to prevent anergy induction than to enhance T cell deletion. This explains why mice injected with low doses of CsA had consistently higher in vitro proliferative responses to superAg than control mice (superAg immunized).

The mechanism(s) by which CsA can prevent the induction of anergy is not clear. CsA is known to block a Ca^{2+}-mediated T cell activation pathway (28–30). Interestingly, Mueller et al. and Jenkins et al. (31, 32) have shown that the induction of anergy in CD4$^+$/Th1-type T cell clones depends on a Ca^{2+}-mediated signal. CsA could prevent the induction of anergy in these clones (33). This raises the possibility that CsA may inhibit anergy induction in vivo by altering a Ca^{2+}-dependent signal. However, the mechanisms of anergy induction in superAg-immunized mice are poorly understood, and our study did not address how CsA acts in this process.

The work of Shi et al. (34) demonstrates that CsA blocks anti-CD3-induced programmed cell death (apoptosis) in the thymus. However, CsA does not prevent radiation-induced apoptosis in mature T cells (35). Nevertheless, it was surprising to find that CsA could enhance peripheral T cell deletion. One possibility is that peripheral T cells are not eliminated by apoptosis, but the recent studies of Kawabe and Ochi (19) suggest that SEB may induce peripheral T cell apoptosis. Interestingly, we found that the stimulation of T cells in vitro with matrix-bound anti-Vβ8 antibodies in the presence of CsA induces specific death (within 72 h) of these cells, apparently by apoptosis (our unpublished observation). These preliminary data suggest that CsA may enhance apoptosis in mature antigen-reactive T cells.

We can only speculate on the mechanism by which CsA enhances peripheral deletion, but Duke and Cohen (36) have shown that IL-2 deprivation of activated T cells results in death by apoptosis. Since CsA blocks IL-2 production (2–4)

and interferes with anergy induction (as presented in this article), the T cells activated by superAgs may be left with no source of IL-2. This could possibly result in apoptosis and loss of reactive T cells in CsA/superAg-treated mice.

CsA/antigen-induced elimination of T cells may represent a mechanism of tolerance to allografts. CsA has been shown to induce long-term nonimmunosuppressed tolerance to allografts in many species (37). This occurs, for example, in rats pretreated with CsA and donor-specific blood transfusion before allograft transplantation (6). Tolerance to allografts has usually been attributed to antigen-specific suppressor cells (5, 7). However, in accordance with our findings with superAg, a recent study with limiting dilution analysis suggests that CsA may induce tolerance to allografts by provoking the elimination of graft-reactive T cells (38). On the other hand, since CsA blocks anergy it is possible that if the conditions of treatment used do not favor an extensive T cell deletion, then CsA may actually enhance immune responses and cause adverse effects. In fact, CsA was shown to aggravate some autoimmune diseases, and to sometimes induce specific form of autoimmunity (reviewed in reference 8). CsA-induced syngeneic (or autologous) GVHD, in particular, has been extensively studied (39–43). This condition occurs after withdrawal of CsA, and is clearly an autoimmune disease that can be adoptively transferred with T cells. Some investigators have suggested that this disease is due to a blockage of intrathymic clonal deletion in CsA-treated mice (9, 10), resulting in the production of autoaggressive T cells. However, we (11) and others (12) did not detect elevated numbers of peripheral forbidden (superAg-reactive) T cells in mice with CsA-induced syngeneic GVHD. In fact, based in our current study, it is possible that even if such T cell clones were not deleted in the thymus, they would be at least partially deleted in the periphery under the influence of CsA. However, as shown by our results, CsA does not necessarily induce a complete deletion of superAg-reactive T cells. For example, CsA-treated mice receiving only one injection of SEB had a moderate deletion of $CD4^+/V\beta8^+$ T cells, and had enhanced in vitro responses to SEB compared with non-CsA-treated controls. Several SEB injections were required to induce a maximal T cell deletion. Similarly, low doses of CsA blocked anergy induction, but did not enhance clonal deletion. Conceivably, since CsA blocks anergy, this drug could induce autoimmune states or aggravate autoimmune diseases in cases where there is only a low or moderate deletion of autoaggressive T cells and a blockage of anergy induction. In CsA-induced syngeneic GVHD, the effector T cells may consist of T cells that are normally tolerized primarily by anergy rather than intrathymic deletion. This hypothesis is consistent with the observation that in some mouse strains CsA treatment induces this disease, despite apparently normal intrathymic clonal deletion (12).

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