Defective Maintenance of T cell Tolerance to a Superantigen in MRL-*lpr/lpr* Mice

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Summary

In normal mice neonatal injection of staphylococcal enterotoxin B (SEB) induces tolerance in T cells that express reactive T cell receptor (TCR) Vβ regions. To determine if a T cell neonatal defect was present in MRL-*lpr/lpr* mice, 20 μg of SEB was injected intraperitoneally every other day into Vβ8.2 TCR transgenic and nontransgenic MRL-+/+ and MRL-*lpr/lpr* mice from birth to 2 wk of age. At 2 wk of age, Vβ8$^+$ T cells were depleted, and SEB reactivity was lost, in spleen, lymph node, and thymus. These effects were equivalent in +/+ and *lpr/lpr* SEB-tolerized mice. However, MRL-*lpr/lpr* mice failed to maintain neonatal tolerance. By 4 wk of age, there was a dramatic increase in T cells expressing Vβ8.2 in the peripheral lymph nodes of MRL-*lpr/lpr* mice but not MRL-+/+ mice. In vitro stimulation with SEB or TCR crosslinking revealed a total loss of neonatal tolerance 2 wk after cessation of SEB treatment in *lpr/lpr* mice, but not +/+ mice. The time-course of recovery of Vβ8$^+$ T cells and reactivity to SEB and TCR crosslinking in the thymus of MRL-*lpr/lpr* mice was similar to that in the lymph node. Thymectomy at 2 wk of age eliminated tolerance loss in lymph nodes of MRL-*lpr/lpr* mice at 4 wk of age, indicating that loss of peripheral tolerance was due to the emigration of untolerized T cells from the thymus. Challenge of neonatally tolerized MRL-*lpr/lpr* mice with SEB (100 μg, i.p.) at 8 wk of age resulted in a dramatic onset of T cell–mediated autoimmune disease characterized by 30% weight loss and 60% morality. This indicated that loss of tolerance to SEB also occurred in vivo. In contrast, neonatally tolerized MRL-+/+ mice remained totally unresponsive to SEB challenge and did not undergo any detectable weight loss. These results suggest that there is normal induction of neonatal tolerance to SEB in *lpr/lpr* mice, but that tolerance is not maintained after the tolerizing antigen is removed. This loss of neonatal tolerance can lead to severe weight loss and death on exposure to the tolerizing antigen later in life.

In MRL-*lpr/lpr* mice, which develop lymphadenopathy and autoimmune disease, extensive studies suggest that abnormal thymic development results in release of increased numbers of T cells from the thymus before tolerance induction, leading to excessive numbers of self-reactive T cells in peripheral lymphoid organs (1). This is supported by the recent finding that the *lpr* gene is a mutation in the Fas antigen, which normally induces apoptosis (2–4). Although autoreactive T cells are thought to play a prominent role in the development of autoimmune disease, it is unclear if the persistence of autoreactive T cells results from a defect in induction of functional elimination either by clonal deletion or anergy induction, or from a defect in the maintenance of anergy. The present experiments were designed to study directly defects in induction and maintenance of T cell tolerance in MRL-*lpr/lpr* mice.

To better understand the mechanism of T cell tolerance loss in autoimmune MRL-*lpr/lpr* mice, Vβ8.2 TCR transgenic and nontransgenic MRL-*lpr/lpr* and MRL-+/+ mice were analyzed during and after neonatal tolerance induction by staphylococcal enterotoxin B (SEB).[1] We have previously shown that TCR transgenic *lpr/lpr* mice do not produce CD4$^-$CD8$^-$B220$^+$ T cells and do not develop lymphadenopathy (5). Nevertheless, these mice develop many of the autoimmune features typical of *lpr/lpr* mice (5, 6). Thus, analysis of these TCR transgenic mice allows the analysis of autoimmunity in *lpr/lpr* mice that have phenotypically normal T cells in the absence of the potential effects of the CD4$^-$CD8$^-$ T cell population. In addition, all the T cells

[1] *Abbreviation used in this paper:* SEB, staphylococcal enterotoxin B.

in Vβ8.2 TCR transgenic mice express the Vβ8.2 gene and therefore are potentially reactive with SEB. Consequently, phenotypic and functional analyses are simplified. Finally, neonatal SEB injection deletes almost all Vβ8$^+$ T cells in nontransgenic normal mice, making it difficult to analyze the SEB responsiveness of the few remaining Vβ8$^+$ T cells (7). Although neonatal injection of SEB into Vβ8.2 TCR transgenic mice also deletes large numbers of Vβ8.2 T cells, sufficient numbers persist to allow analysis of the comparative roles of clonal deletion and anergy induction in neonatal tolerance.

Materials and Methods

Transgenic Mice. Mice expressing the single β TCR transgene derived from the D^b/HY-specific B6.2.16 clone were constructed as previously described (8).

Normal Mice. The original breeding pairs of MRL-*lpr/lpr* and MRL-+/+ mice were obtained from the The Jackson Laboratory (Bar Harbor, ME). The TCR Vβ8.2 transgenic mice were backcrossed with MRL-*lpr/lpr* and MRL-+/+ mice, and the transgene was identified by probing Southern blots of tail DNA as previously described (5). Transgenic mice backcrossed with MRL-*lpr/lpr* mice were unambiguously identified as *lpr/lpr* or *lpr/+* by observation of the transgenic-negative offspring of the third backcross for development of lymphadenopathy and autoimmunity (5). The transgenic mice were backcrossed for at least 10 generations with MRL-*lpr/lpr* or MRL-+/+ mice (>99% MRL) and will be referred to as TCR-β transgenic MRL-*lpr/lpr* or TCR-β transgenic MRL-+/+ mice. All mice were homozygous for H-2^k.

Antibodies and Reagents. Anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.77), anti-CD3 (clone 145–2C11), and a pan-TCR antibody (clone H57-597) were purchased from PharMingen (San Diego, CA). Anti-Vβ8 (clone KJ16.133) was obtained from J. Kappler (National Jewish Hospital, Denver, CO). Highly purified SEB was purchased from Toxin Technologies (Sarasota, FL).

SEB Tolerization and Challenge. Neonatal tolerance to SEB was induced by injection of SEB intraperitoneally (20 μg/50 μl PBS) every other day from the day of birth to 2 wk of age as previously described (7). Challenge with SEB in vivo was carried out in SEB neonatally tolerized Vβ8 TCR transgenic +/+ and *lpr/lpr* mice by injection with SEB intraperitoneally (100 μg/200 μl PBS) at 8 wk of age.

Flow Cytometry Analysis. Single cell suspensions of lymphocytes or thymocytes (10^6/sample) were stained with optimal concentrations of antibodies in PBS with 5% FCS and 0.1% sodium azide for 30 min at 4°C. The first-step reagent was biotin-conjugated anti-CD4. Second-step reagents were a PE-conjugated anti-CD8, a FITC-conjugated anti-pan-TCR, a FITC-conjugated anti-Vβ8-TCR (KJ16.133), or a FITC-conjugated anti-CD3 and a Texas red®–PE tandem fluorochrome conjugated to streptavidin (Southern Biotechnology Associates, Birmingham, AL). Viable cells (10,000/sample) were analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA) with logarithmic scales.

Purification of T Cell Subpopulations. All cell separations were performed by negative selection techniques to avoid T cell activation. Single cell suspensions were prepared from axillary and inguinal lymph nodes, spleen, and thymus. Spleen or lymph node cells (10^8) were depleted of B cells and Ia^k-positive accessory cells using a magnetic activated cell sorter (MACS) (Miltenyi Biotec GmbH, Sunnyvale, CA) column. Purified T cells were analyzed by flow cytometry and verified to be >98% CD3$^+$. For some experiments, MHC class II–positive APC were purified from syngeneic spleen cells by depletion of T cells using anti-Thy-1.2 biotin as the initial antibodies in the column purification procedure.

Stimulation by Crosslinking of TCR or CD3. Serial dilutions from 0.5 μg/ml of either anti-Vβ8 TCR (clone KJ16.133) or anti-CD3 (clone 145.2C11) mAbs were coated on flat-bottomed 96-well plates (Costar, Cambridge, MA) by incubation for 12 h at 4°C. The plates were thoroughly washed using sterile PBS, and purified T cells were added in duplicate cultures for 72 h. During the last 8 h of culture, 1 μCi [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) was added and the amount incorporated measured using a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

SEB Reactivity. MHC class II–positive APC were obtained from syngeneic spleen cells that had been irradiated (3,300 rad) and T cell depleted by a MACS column as described above. In stimulation assays, T cells were purified from the lymph nodes and spleens of different strains of mice and incubated with class II APC with varying concentrations of SEB for 72 h. Proliferation was determined by pulsing the wells with 1 μCi [^{3}H]thymidine (Amersham Corp.) 8 h before harvest.

Assay of IL-2 and IFN-γ. The production of IL-2 was assayed by IL-2-dependent proliferation of HT-2 cells. Briefly, supernates were harvested from antibody-crosslinked or SEB-stimulated T cell cultures at 36 h and were kept frozen at −70°C until use. IL-2 content was determined by [^{3}H]thymidine (Amersham Corp.) incorporation of 5 × 10^3 HT-2 cells during the last 12 h of a 24-h culture with the supernates. IFN-γ production was measured by ELISA. Duplicate samples were assayed in three separate experiments, and the mean was determined. The statistical significance was determined using the Wilcoxon signed-rank nonparametric test.

Results

Increased Reactivity to SEB in Lymph Node T Cells of SEB Neonatally Tolerized TCR Vβ8 Transgenic MRL-lpr/lpr Mice. At 2 wk of age, purified T cells from axillary and inguinal lymph nodes of either SEB-tolerized Vβ8 TCR transgenic MRL-+/+ or MRL-*lpr/lpr* mice were unable to proliferate in response to SEB (Fig. 1 *A*), whereas purified lymph node T cells from SEB-untolerized control Vβ8 TCR transgenic +/+ and *lpr/lpr* mice were able to proliferate. At 4 wk of age, 2 wk after SEB tolerance induction, purified T cells from lymph node or spleen of Vβ8 TCR transgenic MRL-*lpr/lpr* mice were able to proliferate in a dose-dependent fashion to SEB (Fig. 1 *B*). In contrast, purified T cells from SEB-tolerized Vβ8 TCR transgenic MRL-+/+ mice continued to be unresponsive. The time course of SEB anergy loss was determined using lymph node T cells from SEB-tolerized TCR Vβ8 transgenic MRL-+/+ and MRL-*lpr/lpr* mice (Fig. 1 *C*). During, and up to 1 wk after, neonatal tolerization, there was a significantly reduced proliferative response to SEB (10 μg/ml) by lymph node T cells from both *lpr/lpr* and +/+ mice. However, at 2 and 3 wk after SEB tolerance induction, there was a rapid loss of unresponsiveness leading to hyperresponsiveness to SEB by lymph node T cells from TCR Vβ8 transgenic MRL-*lpr/lpr*, but not -+/+, mice.

Increased IL-2 and IFN-γ Production by Vβ8$^+$ T Cells from Neonatally Tolerized Nontransgenic and TCR Vβ8 Transgenic MRL-lpr/lpr Mice at 4 wk of Age. The loss of T cell tolerance in the LN T cells of 4-wk-old neonatally tolerized *lpr/lpr*

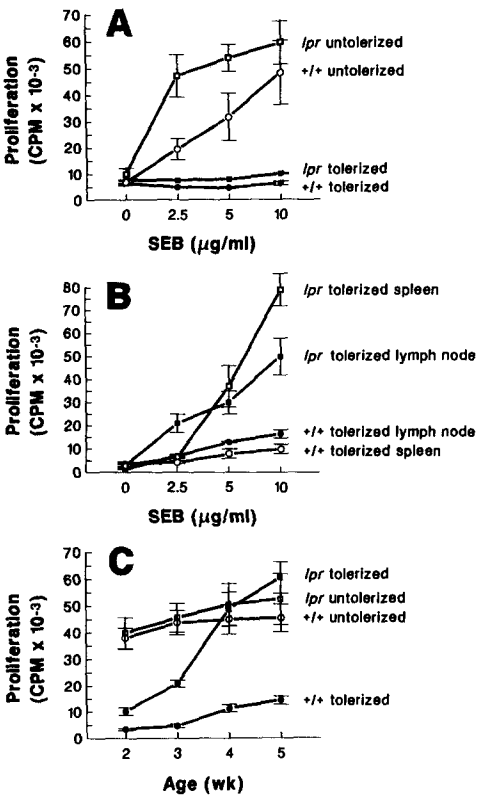

Figure 1. Anergy induction during and after neonatal tolerance induction with SEB. The dose-response to challenge with SEB was determined using (*A*) purified T cells from LN of tolerized and untolerized MRL-*lpr/lpr* and MRL-+/+ 2-wk-old mice, and (*B*) LN or spleen of MRL-*lpr/lpr* and MRL-+/+ 4-wk-old tolerized mice. The time course of the response to SEB challenge (10 μg/ml) was determined for purified LN T cells from MRL-*lpr/lpr* and MRL-+/+ mice, during (2 wk) and after (3–5 wk) neonatal tolerization (*C*). Transgenic and nontransgenic MRL-*lpr/lpr* and MRL-+/+ mice were tolerized with SEB by intraperitoneal injection of 20 μg every other day from day of birth to 2 wk of age (tolerized). 10^5 T cells isolated from the LN and spleen (>98% Thy-1.2$^+$) were stimulated with SEB. The proliferative responses are expressed as cpm of incorporated [^{3}H]thymidine and represent the mean ± SEM of three experiments.

mice was assayed by the production of IL-2 and IFN-γ in response to TCR or CD3 signaling induced by different concentrations of plate-bound anti-Vβ8 (KJ16) or anti-CD3 (145.2C11). After TCR Vβ8 crosslinking, there was no difference in the production of IL-2 by T cells from 4-wk-old SEB tolerized nontransgenic MRL-*lpr/lpr* mice compared to untolerized nontransgenic MRL-*lpr/lpr* mice (Fig. 2 *A*). In contrast, IL-2 production was markedly reduced after TCR crosslinking of T cells from lymph nodes of SEB tolerized nontransgenic MRL-+/+ mice compared with untolerized nontransgenic MRL-+/+ mice. There was a linear relationship between the production of IL-2 by lymph node T cells and the concentration of crosslinking antibody. The degree of tolerance could be quantitated by determining the slope of a least-squares linear fit of IL-2 production by T cells from tolerized mice compared with untolerized mice (Fig. 2 *B*). This analysis indicated that at 4 wk of age, there was equivalent IL-2 production by T cells from lymph nodes of tolerized compared to untolerized MRL-*lpr/lpr* mice (slope = 1.20), but a 10-fold reduction in IL-2 production by T cells from lymph nodes of tolerized compared with untolerized MRL-+/+ mice (slope = 0.13) ($p < 0.001$). Tolerance induction in nontransgenic MRL-+/+ mice was specific for the subpopulation of SEB-reactive T cells, since T cell crosslinking with anti-CD3 resulted in IL-2 production in tolerized MRL-+/+ mice (Fig. 2 *C*). This was also indicated by the slopes of a least-squares linear fit of IL-2 production by lymph node T cells from SEB-tolerized compared with untolerized MRL-+/+ (slope = 0.79) and MRL-*lpr/lpr* mice (slope = 0.57) ($p > 0.05$) (Fig. 2 *D*).

Since it was possible that the T cells could be anergic to TCR crosslinking but responsive to CD3 crosslinking, the experiments were also carried out in Vβ8 TCR transgenic mice. In Vβ8 TCR transgenic MRL-+/+ mice, nearly all of the CD3$^+$ T cells also express the Vβ8 TCR. There was a significant decrease in slope of the tolerized vs. untolerized IL-2 production curve by lymph node T cells from Vβ8 TCR transgenic MRL-+/+ mice compared with Vβ8 TCR transgenic MRL-*lpr/lpr* mice after crosslinking with both anti-Vβ8 (slope +/+ = .37, slope *lpr* = .97, $p < 0.01$) and anti-CD3 (slope +/+ = 0.69, slope *lpr* = 2.38; $p < 0.01$) (Table 1). These results indicate that neither signaling through TCR nor CD3 can induce equivalent IL-2 production in tolerized T cells from MRL-+/+ mice compared with MRL-*lpr/lpr* mice. The IL-2 response to SEB stimulation in 4-wk-old SEB-tolerized mice compared with untolerized mice was also significantly reduced in T cells from nontransgenic and Vβ8 TCR transgenic MRL-+/+ mice, compared with MRL-*lpr/lpr* mice (Table 1). Supernates from all assays were also analyzed for IFN-γ, and results were in agreement to those found for IL-2 production (Table 1). These results indicate that loss of tolerance in T cells of *lpr* mice includes both TCR and CD3 signaling, and results in increased production of both IL-2 and IFN-γ.

Neonatal Tolerance to SEB Is Associated with Development of TCR Vβ8^{dull}CD4^{dull} Cells in Lymph Nodes of TCR Vβ8 Transgenic MRL-+/+ and MRL-lpr/lpr Mice. In 2-wk-old untolerized TCR Vβ8 transgenic MRL-+/+ mice, ~80% of lymph node cells expressed high levels of TCR Vβ8 (Fig. 3 *A*). These T cells also expressed high levels of TCR α/β and high levels of CD3, and were CD4$^+$ (57%) or CD8$^+$ (23%) (Fig. 3 *B*). There was no significant difference between 2-wk-old untolerized TCR Vβ8 transgenic MRL-*lpr/lpr* mice and untolerized TCR Vβ8 transgenic MRL-+/+ mice (Fig. 3, *A* and *B*). In contrast, in 2-wk-old SEB-tolerized MRL-+/+ mice, only 30% of lymph node T cells expressed high levels of TCR Vβ8 (Fig. 3 *C*). This decrease in expression of the Vβ8 TCR was preferential, as 60% of peripheral lymph node T cells expressed high levels of TCR α/β and CD3. The TCR Vβ8^{bright} T cells were either CD4$^+$ or CD8$^+$ (Fig. 3 *D*). A distinct subpopulation of TCR Vβ8^{dull} T cells also expressed low levels of CD4 and CD8 (Fig. 3 *D*). There was no significant difference between 2-wk-old SEB-tolerized TCR Vβ8 transgenic MLR-+/+ mice and 2-wk-old SEB-tolerized TCR Vβ8 transgenic MRL-*lpr/lpr* mice.

Increase in Vβ8$^+$ T Cells in Lymph Nodes after Cessation

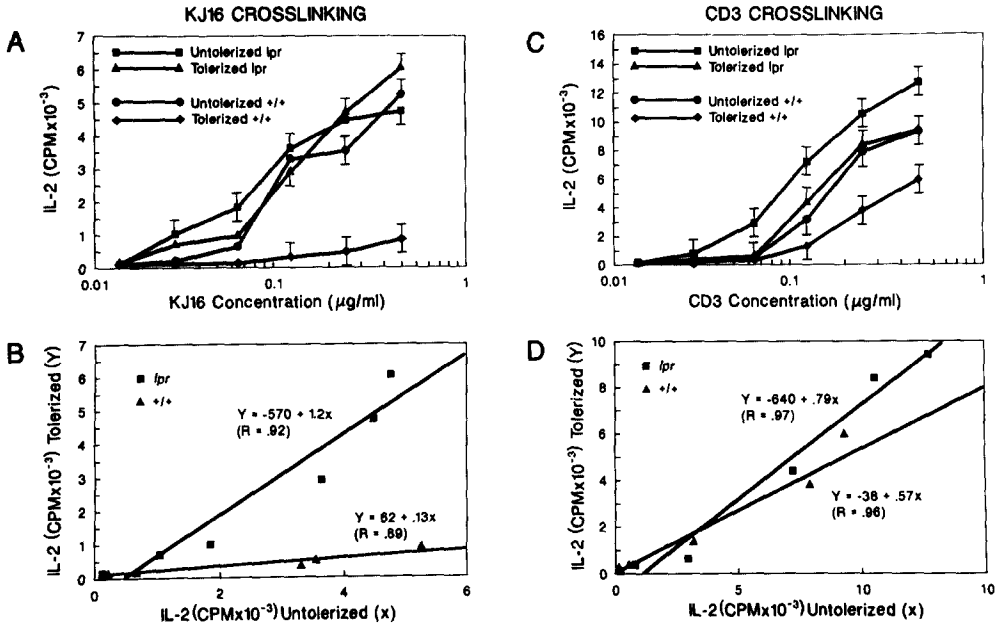

Figure 2. IL-2 production by LN T cells from SEB-tolerized nontransgenic MRL-*lpr/lpr* and MRL-+/+ mice. Purified LN T cells from tolerized and untolerized 4-wk-old MRL-*lpr/lpr* and MRL-+/+ mice were stimulated by crosslinking using plate-bound anti-TCR (KJ16.133) (*A*) and anti-CD3 (clone 145.2C11) (*C*) mAbs. IL-2 production was assayed in supernatants collected after 48 h. The activity is expressed as the cpm of [^{3}H]thymidine incorporated into the HT-2 IL-2-dependent cell line, and represents the mean ± SEM of five mice assayed separately in triplicate culture. The IL-2 response to crosslinking with anti-TCR (*B*) and anti-CD3 (*D*) is compared between tolerized and untolerized mice by least squares fit analysis. The calculated slope values after anti-TCR crosslinking are 1.20 for MRL-*lpr/lpr* mice (●) and 0.13 for MRL-+/+ mice (▲).

of SEB Tolerization in MRL-lpr/lpr Mice. In 4-wk-old mice, 2 wk after SEB tolerization had been completed, there continued to be nearly equal numbers of Vβ8 TCR^{dull} and Vβ8 TCR^{bright} T cells in Vβ8 TCR transgenic MRL-+/+ mice (Fig. 4 and Table 2). The TCR^{dull} lymph node T cells were predominantly CD4^{dull}CD8^{dull} (Fig. 4 *A*), whereas the TCR^{bright} lymph node T cells were predominantly either CD4$^+$ or CD8$^+$ (Fig. 4 *B*). The TCR Vβ8^{dull} T cells exhibited a low forward light scatter intensity, suggesting they were small, resting T cells (Fig. 4 *C*). In contrast, 2 wk after SEB tolerization in TCR Vβ8 transgenic MRL-*lpr/lpr* mice, there was a large increase of TCR Vβ8^{bright} T cells in the lymph node (Fig. 4). These T cells were either CD4$^+$ or CD8$^+$ (Fig. 4 *B*), and most exhibited a high forward light scatter, suggesting that these cells were activated. There was also a small population of TCR^{dull} lymph node T cells that exhibited low forward light scatter consistent with small resting T cells (Fig. 4 *C*).

Reduction of Total Lymphocyte Population during and after SEB Tolerization. Compared with untreated mice, there was a

Table 1. *Production Ratio of IL-2 and IFN-γ in 4-wk-old SEB-tolerized Compared with Untolerized Mice*

Strain	Vβ8 TCR transgenic	Anti-Vβ8 (KJ16)		Anti-CD3		SEB	
		IL-2	IFN-γ	IL-2	IFN-γ	IL-2	IFN-γ
MRL-*lpr/lpr*	–	1.20* (r = 0.92)	4.44* (r = 0.90)	0.79 (r = 0.97)	1.02 (r = 0.99)	1.19* (r = 0.89)	ND
MRL-+/+	–	0.13 (r = 0.89)	0.26 (r = 0.92)	0.57 (r = 0.97)	0.71 (r = 0.48)	0.33 (r = 0.74)	ND
MRL-*lpr/lpr*	+	0.97* (r = 0.75)	0.39* (r = 0.86)	2.38* (r = 0.92)	0.61* (r = 0.77)	0.93* (r = 0.93)	0.62* (r = 0.90)
MRL-+/+	+	0.37 (r = 0.78)	0.037 (r = 0.89)	0.69 (r = 0.99)	0.25 (r = 0.87)	0.11 (r = 0.86)	0.25 (r = 0.83)

T cells isolated from the LN of SEB-tolerized or untolerized mice were stimulated by plate-bound anti-TCR (KJ16) or anti-CD3 as in Fig. 2, or SEB as in Fig. 1. The production of IL-2 and IFN-γ was assayed, and a least squares linear fit of the results from tolerized and untolerized mice carried out, as described in Fig. 2. The ratio of the production rate in the tolerized compared with the untolerized mice is presented with the *r* value in parenthesis. A slope value of 1 indicates that the responses from tolerized mice have a dose-response similar to untolerized mice (i.e., failure to induce or maintain tolerance).

* Value in MRL-*lpr/lpr* mice is significantly different from MRL-+/+ mice.

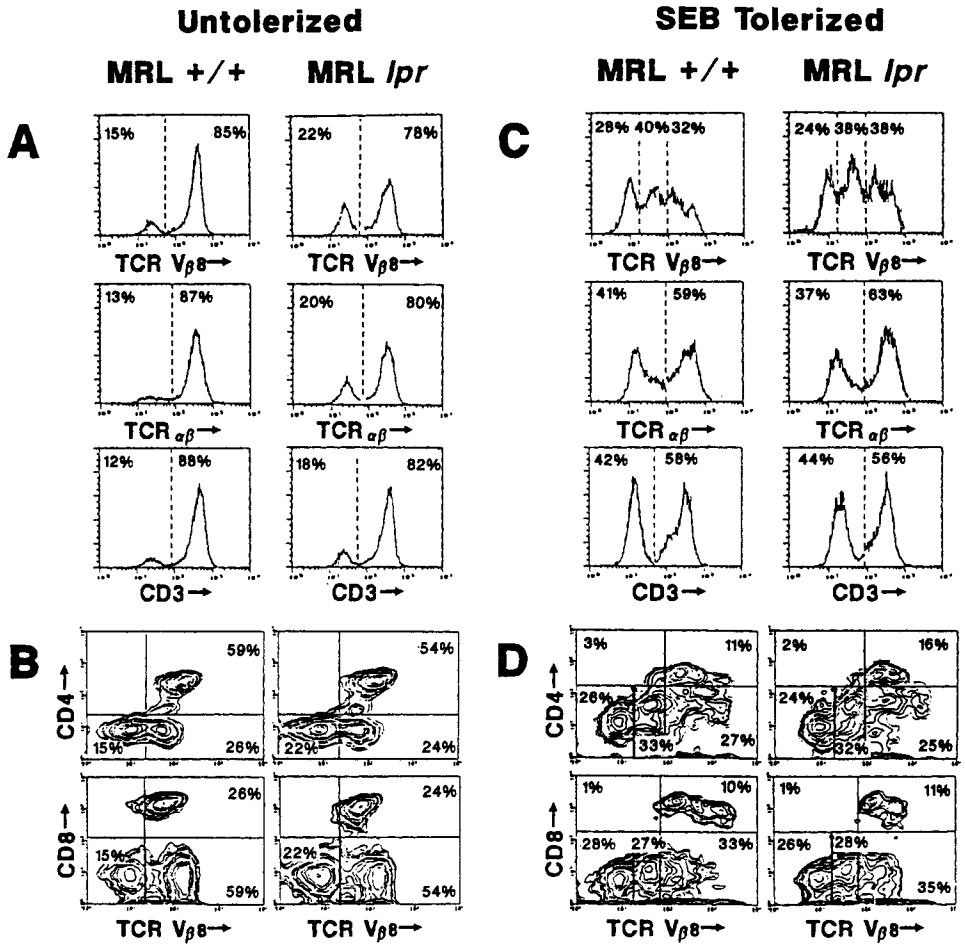

Figure 3. Expression of TCR Vβ8, TCR-α/β, CD3, CD4, and CD8 during induction of neonatal tolerance to SEB in transgenic mice. LN cells from untolerized 2-wk-old transgenic mice were stained with anti-Vβ8 TCR (KJ16.133), anti-pan-TCR α/β, and anti-CD3 (clone 145.2C11) (*A*), and compared with mice undergoing tolerization (*C*) by FACS® analysis. Horizontal axes illustrate the log of fluorescence, and vertical axes illustrate relative cell numbers. Cells were also triple stained with biotin-conjugated anti-CD4, then PE-conjugated anti-CD8, FITC-conjugated anti-Vβ8 TCR, and a Texas red®PE tandem fluorochrome conjugated to streptavidin. The coexpression of TCR Vβ8 and CD4 and CD8 is shown for untolerized (*B*) and tolerized (*D*) mice. The fluorescence histograms and contour plots are representative of four different experiments, and the percent of cells in each quadrant is noted in the figure.

three- to fourfold reduction of total lymphocytes in SEB-treated Vβ8 TCR transgenic MRL-+/+ and MRL-*lpr/lpr* mice at 2 wk of age (Table 2). At 3 wk of age, 1 wk after stopping SEB, the total number of lymph node cells in TCR transgenic Vβ8 transgenic MRL-*lpr/lpr* mice had returned to normal, whereas the number of lymph node cells in TCR

Table 2. *Total Number of Lymph Node Cells in Untolerized and SEB-tolerized Mice*

			2 wk old			3 wk old			4 wk old		
Strain	Vβ8 TCR transgenic	SEB tolerized	Total	Vβ8^{bright} total	Percent	Total	Vβ8^{bright} total	Percent	Total	Vβ8^{bright} total	Percent
MRL-*lpr/lpr*	+	+	2.8 ± .6	1.1	38	28.3 ± 2.1	13.9	49	32.8 ± 5.1	19.0	58
MRL-+/+	+	+	2.2 ± .8	0.70	32	7.1 ± 0.8	0.64	9	17.3 ± 3.5	3.6	21
MRL-*lpr/lpr*	−	+	9.9 ± 1.2	0.0	0	28.3 ± 1.7	1.7	6	31.2 ± 2.9	3.7	12
MRL-+/+	−	+	6.5 ± .8	0.0	0	28.1 ± 1.9	0.0	0	29.2 ± 2.2	0.29	1
MRL-*lpr/lpr*	+	−	8.9 ± 1.2	5.7	64	31.5 ± 2.6	23.9	76	32.2 ± 4.1	21.6	67
MRL-+/+	+	−	8.6 ± 1.1	6.5	75	13.7 ± 1.2	11.1	81	20.4 ± 3.9	14.7	72
MRL-*lpr/lpr*	−	−	8.3 ± .9	1.2	14	27.5 ± 2.1	4.7	17	44.5 ± 3.8	6.7	15
MRL-+/+	−	−	11.2 ± 1.3	2.1	19	13.2 ± 1.1	2.2	17	18.3 ± 1.2	2.9	16

FACS® analysis was used to determine the percentages of Vβ8^{bright} cells, as in Figs. 3 and 4. Values for total LN cells are presented as the mean ± SEM ($\times 10^{-6}$) of three experiments with five mice per group.

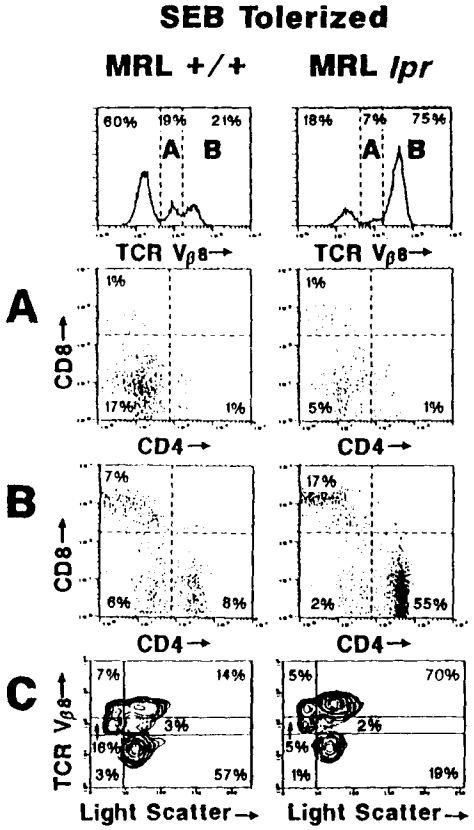

Figure 4. Expression of TCR Vβ8, CD4, and CD8 in relation to LN T cell size in neonatally tolerized transgenic mice. LN region cells from 4-wk-old mice, 2 wk after stopping SEB treatment, were stained as described in Fig. 3 and FACS® gated into TCR Vβ8 (*top*) dull (region *A*) and TCR Vβ8 bright (region *B*) populations, and analyzed for expression of CD4 and CD8. A contour plot of cell size (estimated by light scatter) vs. the expression of TCR Vβ8 is shown in *C*. In this plot, the regions of dull, intermediate, and bright expression of TCR Vβ8 are the same as defined in the TCR Vβ8 histograms. The histograms and contour plots are representative of four different experiments with five mice per group.

Vβ8 transgenic MRL-+/+ mice were reduced compared with untolerized control mice. At 4 wk of age, the number of lymph node T cells in tolerized MRL-+/+ mice was equivalent to untolerized control mice. A more accurate indication of the effects of SEB tolerization on the lymph node T cells was obtained by comparison of the number of Vβ8 TCR^{bright} T cells. The value was obtained by multiplying the total number of lymph node cells by the percent of Vβ8 TCR^{bright} T cells determined by flow cytometry analysis. At 2 wk of age, there was five- to sixfold reduction of Vβ8 TCR^{bright} T cells in SEB-tolerized Vβ8 TCR transgenic MRL-+/+ and MRL-*lpr/lpr* mice compared with untolerized control mice. At 3 wk of age, there was no increase in Vβ8 TCR^{bright} T cells in SEB-tolerized Vβ8 TCR transgenic MRL-+/+ mice, but there was a return of Vβ8^{bright} T cells in Vβ8 TCR transgenic MRL-*lpr/lpr* mice to 60% of that found in untolerized controls. At 4 wk of age, there was a partial recovery of the number of Vβ8^{bright} T cells in tolerized Vβ8 TCR transgenic MRL-+/+ mice. A similar pattern of recovery of Vβ8^{bright} T cells was observed in nontransgenic SEB tolerized MRL-*lpr/lpr* compared to MRL-+/+ mice (Table 2).

Effect of SEB Tolerization on Thymocytes. The thymus of Vβ8 TCR transgenic mice undergoing SEB tolerance induction was analyzed by flow cytometry to determine if a defect in clonal deletion of Vβ8^{+} T cells exists in *lpr* mice. There was efficient depletion of Vβ8^{bright} T cells in the thymus of both Vβ8 TCR transgenic MRL-+/+ and *lpr/lpr* mice just after SEB tolerance induction at 2 wk of age (Fig. 5). This resulted in a near total loss of mature CD4^{bright} and CD8^{bright} thymocytes. In contrast to the phenotypic difference of lymph node T cells from 4-wk-old SEB-tolerized TCR Vβ8 transgenic MRL-+/+ mice compared with MRL-*lpr/lpr* mice, there was no difference in the phenotype of thymocytes in 4-wk-old TCR transgenic MRL-+/+ mice compared to TCR transgenic MRL-*lpr/lpr* mice (Fig. 5 *C*). However, there was a more rapid recovery of total thymocytes in 4-wk-old SEB-tolerized TCR Vβ8 transgenic MRL-*lpr/lpr* ($110 \pm 4.5 \times 10^6$) compared with TCR Vβ8 transgenic MRL-+/+ ($67.5 \pm 7 \times 10^6$) mice.

Increased SEB Reactivity of Thymocytes from lpr/lpr Mice. Neonatal tolerance induction with SEB reduced thymocyte proliferation in response to SEB and KJ16 crosslinking at 2 wk of age in both TCR Vβ8 transgenic MRL-+/+ and MRL-*lpr/lpr* mice (Fig. 5, *D* and *E*). Despite the similar phenotype, there was a rapid increase in SEB- and KJ16-stimulated responsiveness of thymocytes from TCR Vβ8 transgenic *lpr/lpr*, but not TCR Vβ8 transgenic +/+, mice at 3, 4, and 5 wk of age. The proliferative response of thymocytes to SEB or KJ16 crosslinking at 4 and 5 wk of age from TCR Vβ8 transgenic MRL-*lpr/lpr* was greater than the response in untolerized 4–5-wk-old mice, indicating SEB induces hyperresponsiveness to thymocytes from MRL-*lpr/lpr* mice.

Loss of Neonatal Tolerance in MRL-lpr/lpr Mice Originates in the Thymus. Loss of SEB-induced neonatal tolerance occurs between 1 and 2 wk after neonatal tolerance induction in both the thymus and lymph node. Peripheral tolerance loss could be due to either production of new untolerized thymocytes or due to spontaneous loss of anergy by lymph node T cells, or a combined loss of anergy in the thymus and lymph node. To distinguish among these possibilities, SEB neonatal tolerized Vβ8 TCR transgenic MRL-+/+ and MRL-*lpr/lpr* mice were either thymectomized or sham thymectomized at 2 wk of age, just after induction of tolerance. The reactivity of lymph node T cells to SEB was then analyzed at 4 wk of age. As expected, lymph node T cells from SEB-tolerized sham-thymectomized, Vβ8 TCR transgenic MRL-*lpr/lpr* mice were highly responsive to SEB, whereas SEB tolerized sham-thymectomized MRL-+/+ mice remained tolerant (Fig. 6 *A*). In contrast, lymph node T cells from thymectomized, SEB-tolerized Vβ8 TCR transgenic MRL-*lpr/lpr* mice remained tolerant to SEB. This can also be seen by plotting the ratio of the proliferative response of lymph node T cells from SEB-tolerized MRL-*lpr/lpr* mice compared with SEB-tolerized MRL-+/+ mice for different concentrations of SEB (Fig. 6 *B*). Lymph node T cells from sham-thymectomized

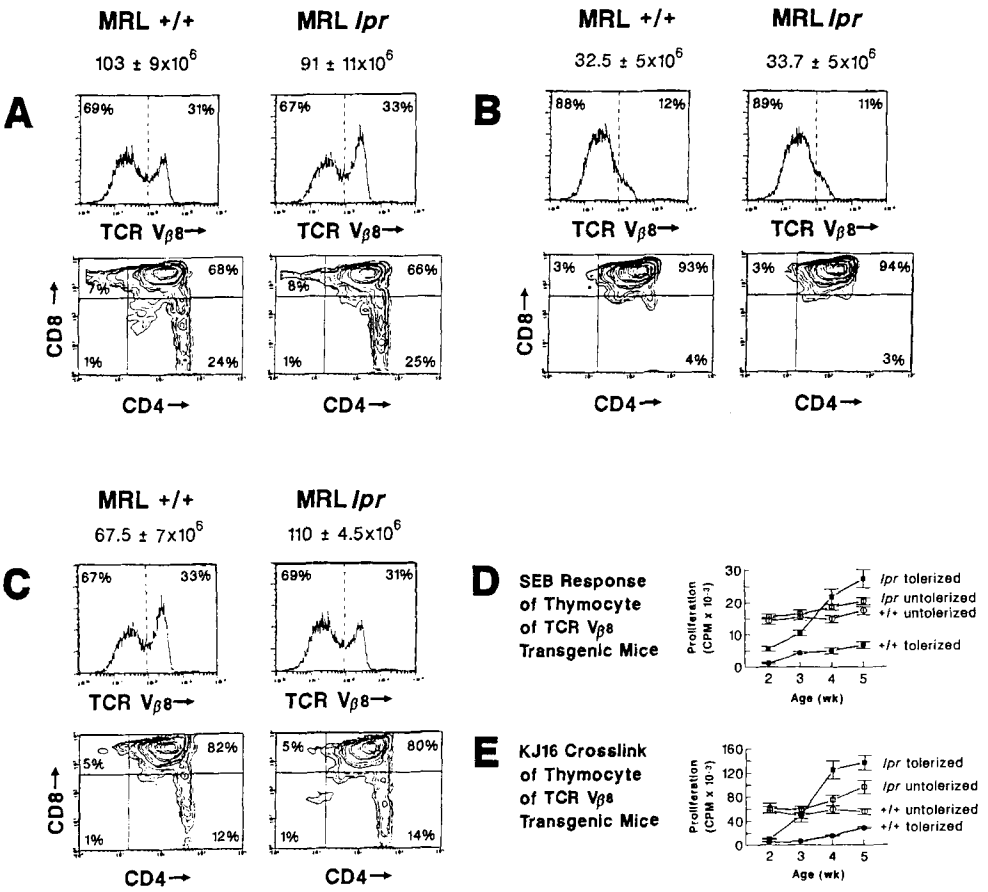

Figure 5. Analysis of thymocytes from Vβ8 transgenic mice during and after SEB tolerization. Thymocytes from 2-wk-old untolerized (*A*) and SEB-tolerized (*B*) mice, and 4-wk-old SEB-tolerized mice (*C*), were analyzed for the expression of TCR Vβ8, CD4, and CD8 as in Fig. 3. The total number of thymocytes (mean ± SEM) are shown above the panels. The time course of the proliferative response of thymocytes to stimulation with SEB (10 μg/ml) (*D*) and to crosslinking by plate-bound KJ16 mAbs (*E*) was estimated by [^{3}H]thymidine incorporation as in Figs. 1 and 2. Results were derived from three experiments with five mice per group.

Vβ8 TCR transgenic MRL-*lpr/lpr* mice exhibited a 15 times greater proliferative response compared with T cells from equivalently treated Vβ8 TCR transgenic MRL- + / + mice (slope = 15.3). In contrast, there was no difference in responsiveness comparing thymectomized MRL-*lpr/lpr* to MRL- + / + mice (slope = 1.03). These results indicate that failure to maintain peripheral T cell tolerance in MRL-*lpr/lpr* mice is due to an export of new untolerized T cells from the thymus to the periphery.

Loss of Tolerance In Vivo. To determine if the loss of neonatal-induced tolerance to SEB occurs in vivo as well as in vitro, SEB neonatally tolerized Vβ8 TCR transgenic + / + and *lpr/lpr* mice were injected with SEB (100 μg, i.p.) at 8 wk of age. There was a marked weight loss and a 60% mortality in *lpr/lpr* mice, but no effect in + / + mice (Fig. 7). In contrast, untolerized Vβ8 TCR transgenic + / + and *lpr/lpr* mice had equivalent weight loss and no mortality after injection with SEB (100 μg, i.p.) at 8 wk of age, comparable to previously reported SEB-induced weight loss (9, 10).

Discussion

T cell tolerance induction after SEB administration has previously been shown to involve both anergy induction and clonal deletion (11, 12). The effects of T cell activation by SEB are seen within 1 wk of SEB administration and consist of a limited expansion followed by clonal deletion of 50% of T cells (11). The remaining Vβ8$^+$ T cells do not respond to SEB. Biweekly administration of SEB into MRL-*lpr/lpr* mice also resulted in decreased CD4$^-$CD8$^-$B220$^+$ T cells and renal disease (13). The present experiments indicate that induction of T cell anergy and clonal deletion does occur in autoimmunme *lpr/lpr* mice, but is slightly less efficient than that observed in + / + mice. This result is consistent with previous reports that in the presence of the self-antigen, clonal deletion of self-reactive (Mls, MHC) T cells does occur in *lpr/lpr* mice (14–16). We also previously demonstrated that in D^b/HY TCR transgenic C57BL/6-*lpr/lpr* male mice, there was a small but detectable difference in intrathymic clonal deletion of self-reactive T cells (6).

Anergy induction with SEB is associated with the development of TCR^{dull}CD4^{dull}CD8^{dull} T cells in lymph node of Vβ8 TCR transgenic MRL- + / + and MRL-*lpr/lpr* mice. Cell size analysis indicates these are small, resting T cells. In TCR Vβ8 transgenic mice, downregulation of Vβ8 may occur in a unique manner. There is normal expression of TCRαβ and CD3 despite low levels of Vβ8, suggesting that there is simultaneous expression of endogenously rearranged TCR Vβ regions, as well as the TCR Vβ8 transgene (Fig. 3 *C*). The transgenic Vβ8 gene has been found to efficiently suppress

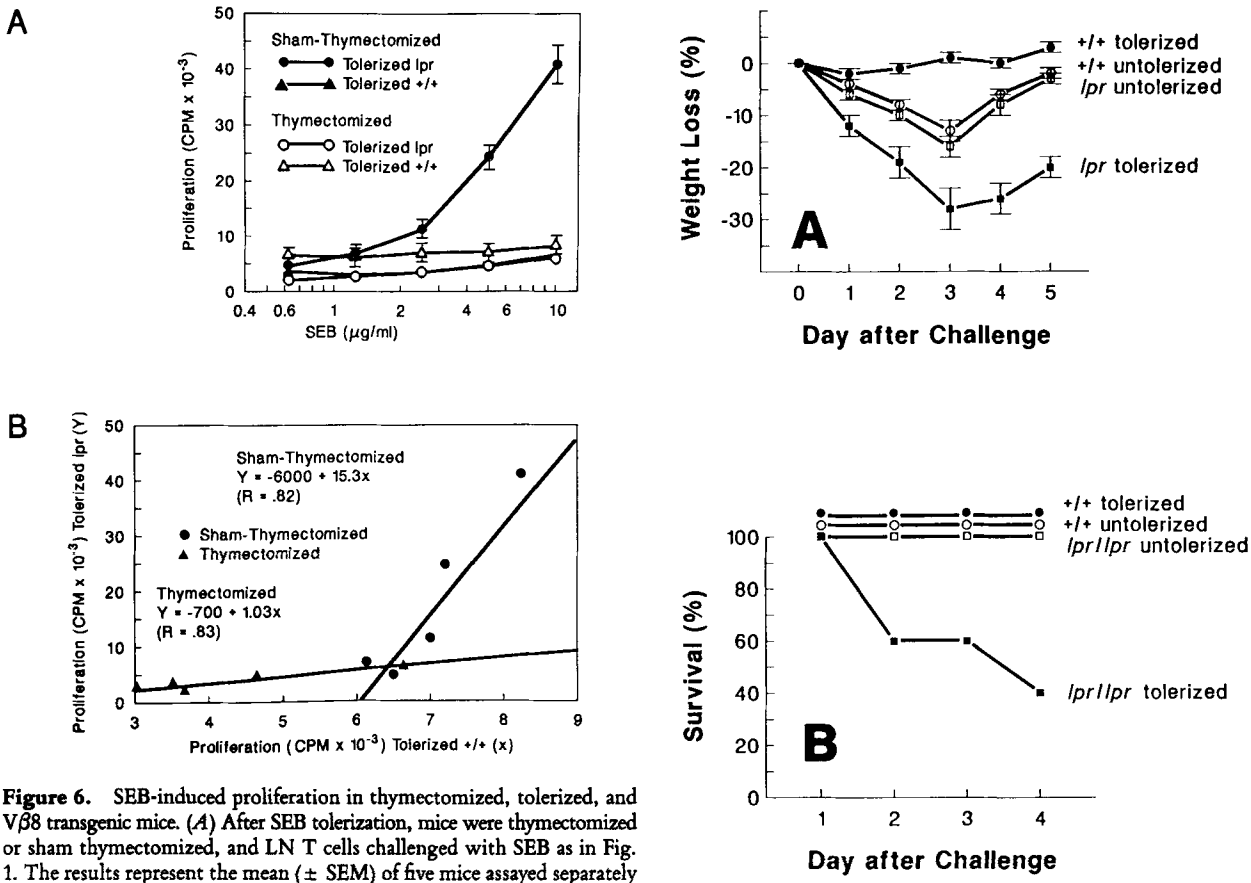

Figure 6. SEB-induced proliferation in thymectomized, tolerized, and Vβ8 transgenic mice. (*A*) After SEB tolerization, mice were thymectomized or sham thymectomized, and LN T cells challenged with SEB as in Fig. 1. The results represent the mean (± SEM) of five mice assayed separately in triplicate. (*B*) The proliferation of T cells from MRL-*lpr/lpr* mice was plotted against the proliferation of T cells from MRL-+/+ mice at the same concentration of SEB, and the least squares fit calculated, for sham-thymectomized (slope = 15.3) and thymectomized (slope = 1.03) mice.

Figure 7. SEB-induced weight loss (*A*) and survival (*B*) in SEB neonatally tolerized Vβ8 TCR transgenic MRL-*lpr/lpr* and MRL-+/+ mice. Neonatally SEB-tolerized, Vβ8 transgenic MRL-*lpr/lpr* and MRL-+/+ mice were challenged with SEB in vivo (100 μg, i.p.) at 8 wk of age. The mean (± SEM) values of five mice are presented.

endogenous Vβ rearrangement (8). SEB tolerization results in extensive clonal deletion of thymocytes exhibiting high expression of Vβ8 TCR resulting in increased visibility of the few thymocytes that express low levels of endogenously rearranged TCR Vβ regions and low expression of the Vβ8 transgene. Other investigators have previously observed the development $CD4^-CD8^-CD5^+IL\text{-}2R^+$ T cells in nontransgenic mice after treatment with SEB (17). In addition, we have previously observed downregulation of CD8 on T cells from the spleen of D^b/HY-reactive TCR transgenic D^b male +/+ and *lpr* mice (6). SEB-induced $TCR^{dull}CD4^{dull}CD8^{dull}$ T cells are seen during anergy induction in both transgenic and nontransgenic (data not shown) MRL-+/+ and MRL-*lpr/lpr* mice. Therefore, this T cell phenotype is a feature of neonatal anergy induction in normal mice, and is not related to the *lpr* gene or TCR transgene.

Although there is a small tolerance defect in *lpr/lpr* mice during anergy induction with SEB, the main defect occurs within 1 wk after stopping SEB, suggesting different mechanisms underlie induction and maintenance of tolerance. The *lpr* defect has been found to be due to a mutation of the Fas antigen, which mediates apoptosis in the thymus (2–4). The observation of nearly normal intrathymic clonal deletion indicates that negative selection can occur in the thymus of *lpr/lpr* mice despite the mutated Fas antigen. The rapid return of SEB-reactive thymocytes after stopping SEB suggests that a Fas-dependent thymic apoptosis pathway is required for maintenance of T cell tolerance to SEB. Tolerance loss in the thymus of *lpr/lpr* mice is not simply due to increased production of thymocytes, because by 4 wk of age, there is a two- and threefold increase in thymocytes in +/+ and *lpr/lpr* mice, respectively, but there is a much greater increase in thymocyte proliferation after stimulation with SEB and KJ16 crosslinking by thymocytes from *lpr/lpr* compared with +/+ mice (Fig. 5). One possibility is that the Fas-dependent pathway is primarily used to eliminate "neglected" thymocytes. During SEB tolerization, SEB-reactive TCR $V\beta8^+$ T cells would be highly reactive due to bridging of the Vβ8 TCR and thymic MHC antigens by the SEB superantigen. Consequently, there would be few neglected $V\beta8^+$ T cells, and extensive clonal deletion of most $V\beta8^+$ thymocytes would occur. However, after SEB tolerization is stopped, neglected TCR $V\beta8^+$ T cells would be produced in high

numbers in nontransgenic as well as Vβ8 TCR transgenic mice. In MRL-+/+ mice, we propose that neglected Vβ8$^+$ T cells would undergo apoptosis primarily due to signaling through the Fas antigen. However, in MRL-*lpr/lpr* mice, such neglected thymocytes would not undergo apoptosis due to the Fas defect, nor would they undergo negative selection, as they would be neglected by the thymus. These Vβ8$^+$ T cells would persist in the thymus and escape to the periphery and remain reactive with SEB. The importance of new thymocytes in tolerance loss is demonstrated by the observation that MRL-*lpr/lpr* mice do not have a significant defect in maintenance of peripheral tolerance in the absence of the thymus, as thymectomy of SEB-tolerized mice at 2 wk of age completely eliminated the loss of tolerance to SEB at 4 wk of age.

The proposal that Fas mediates apoptosis of neglected thymocytes is consistent with our previous observation that a rearranged TCR gene inhibits lymphoproliferative disease by inhibiting production of CD4$^+$CD8$^+$TCR^{dull} T cells (5). In nontransgenic mice expressing endogenously rearranged TCR genes, a large percentage of CD4$^+$CD8$^+$TCR^{dull} T cells are thymocytes that fail to properly rearrange or express the TCR, resulting in production of neglected thymocytes that undergo apoptosis in the thymus (18). In *lpr* mice, the Fas defect in the apoptosis of neglected thymocytes might lead to escape of increased numbers of these unselected T cells to the periphery, resulting in lymphadenopathy and autoimmunity. We propose that a rearranged TCR transgene eliminates lymphoproliferation by decreasing production of neglected cells due to abnormal TCR rearrangement.

We cannot yet fully explain how a TCR transgene might inhibit a defective Fas-dependent pathway of thymocyte development, yet not eliminate autoimmune features of *lpr* mice (5, 6). Failure to eliminate autoantibody production could be due to the requirement that B cell development in *lpr/lpr* mice is also affected by the defected Fas-related apoptosis pathway, resulting in autoreactive B cells. This is consistent with the observation of an intrinsic B cell defect in *lpr/lpr* mice (19). A B cell abnormality, by itself, has been noted to result in autoantibodies, but not severe autoimmune disease (20). In addition to B cell abnormalities, we find an increase in autoreactive T cells expressing the rearranged TCR transgene in the TCR transgenic *lpr/lpr* mice (6). It is possible that the Fas antigen plays a role in negative selection and clonal deletion of autoreactive T cells, as well as on neglected T cells. Alternatively, as described above, some of the T cells expressing the self-reactive TCR transgene might develop into neglected thymocytes requiring Fas-induced apoptosis. In both cases, TCR transgenic mice would produce abnormal, potentially autoreactive T cells, but at lower numbers compared with nontransgenic mice. This is supported by the data in Table 1, in which the production ratio for IL-2 and IFN-γ in tolerized relative to untolerized mice is reduced in the presence of the TCR Vβ8 transgene. A third possibility is that some aspects of organ disease in *lpr* mice might be related to failed apoptosis of abnormal cells unrelated to immune defects. Fas is expressed in the heart, liver, and ovaries (4), and defective apoptosis of cells at nonlymphoid sites might lead to tissue damage associated with mononuclear cell infiltrate. This is exemplified by the observation that synovial hyper-proliferation in MRL-*lpr/lpr* mice has been reported to precede T cell infiltration and might be related to an apoptosis defect of a synovial cell (21).

SEB has previously been shown to induce rapid weight loss after in vivo administration in normal mice, which was initially proposed to be due to production of IL-2 (9). The data presented in this paper indicate that after neonatal tolerance induction, SEB does not induce weight loss in TCR Vβ8 transgenic MRL-+/+ mice, but induces a greater than normal weight loss and 60% mortality in SEB neonatally tolerized TCR Vβ8 transgenic MRL-*lpr/lpr* mice. This SEB hyper-responsiveness in vivo is consistent with the in vitro tolerance studies in which after 5 wk of age, a greater than normal response of T cells from neonatally tolerized MRL-*lpr/lpr* mice to SEB was observed compared with untolerized mice. SEB has also been shown to induce TNF-α, IL-1, and IL-6 production by blood mononuclear cells (22). These cytokines have been found to be increased in *lpr/lpr* mice (23, 24), and release of cytokines in vivo could lead to an acute septic shock–like state, weight loss, and death.

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References

1. Cohen, P.L., and R.A. Eisenberg. 1991. *Lpr* and *gld*: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243.
2. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A.

Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature (Lond.).* 356:314.
3. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S.-I. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell.* 66:233.
4. Watanabe-Fukunaga, R., C.I. Brannan, N. Itoh, S. Yonehara, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. *J. Immunol.* 148:1274.
5. Mountz, J.D., T. Zhou, J. Eldridge, K. Berry, and H. Bluethmann. 1990. Transgenic rearranged T cell receptor gene inhibits lymphadenopathy and accumulation of $CD4^-CD8^-$ $6B2^+$ T cells in *lpr/lpr* mice. *J. Exp. Med.* 172:1805.
6. Zhou, T, H. Bluethmann, J. Eldridge, M. Brockhaus, K. Berry, and J.D. Mountz. 1991. Abnormal thymocyte development and production of autoreactive T cells in T cell receptor transgenic autoimmune mice. *J. Immunol.* 147:466.
7. White, J., A. Herman, A.M. Pullen, R. Kubo, J.W. Kappler, and P. Marrack. 1989. The Vβ-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell.* 56:27.
8. Uematsu, Y., S. Ryser, Z. Demblic, P. Borgulya, P. Krimpenfort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. In transgenic mice the introduced functional T cell receptor β chain prevents expression of endogenous β genes. *Cell.* 52:831.
9. Marrack, P., M. Blackman, E. Kushnir, and J. Kappler. 1990. The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells. *J. Exp. Med.* 171:445.
10. Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P.H. Krammer, and H. Wagner. 1992. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J. Exp. Med.* 175:91–98.
11. Rellahan, B.L., L.A. Jones, A.M. Kruisbeek, A.M. Fry, and L.A. Matis. 1990. In vivo induction of anergy in peripheral $V\beta8^+$ T cells by staphylococcal enterotoxin B. *J. Exp. Med.* 172:1091.
12. Kawabe, Y., and A. Ochi. 1991. Programmed cell death extrathymic reduction of $V\beta8^+$ $CD4^+$ T cells in mice tolerant to *Staphylococcus aureus* enterotoxin B. *Nature (Lond.).* 349:245.
13. Kim, C., K.A. Siminovitch, and A. Ochi. 1991. Reduction of lupus nephritis in MRL/*lpr* mice by a bacterial superantigen treatment. *J. Exp. Med.* 174:1431.
14. Kotzin, B.L., S.K. Babcock, and L.R. Herron. 1988. Deletion of potentially self-reactive T cell receptor specificities in $L3T4^-$, $Lyt\text{-}2^-$ T cells of *lpr* mice. *J. Exp. Med.* 168:2221.
15. Singer, P.A., R.S. Balderas, R.J. McEvily, M. Bobardt, and A.N. Theofilopoulos. 1989. Tolerance-related Vβ clonal deletions in normal $CD4^-8^-$, TCR-α/β^+ and abnormal *lpr* and *gld* cell populations. *J. Exp. Med.* 170:1869.
16. Mountz, J.D., T.M. Smith, and K.S. Toth. 1990. Altered expression of self-reactive TCR V_β regions in autoimmune mice. *J. Immunol.* 144:2159.
17. Patel, M., D.D. Taub, Y.S. Lin, and T.J. Rogers. 1990. Immunosuppressive activity of staphylococcal enterotoxin B. I. Characterization of staphylococcal enterotoxin-B-induced suppressor cells. *Cell. Immunol.* 131:159.
18. Shortman, K., D. Vremec, and M. Egerton. 1991. The kinetics of T cell antigen receptor expression by subgroups of $CD4^+8^+$ thymocytes: delineation of $CD4^+8^+3^{2+}$ thymocytes as post-selection intermediates leading to mature T cells. *J. Exp. Med.* 173:323.
19. Sobel, E.S., T. Katagiri, K. Katagiri, S.C. Morris, P.L. Cohen, and R.A. Eisenberg. 1991. An intrinsic B cell defect is required for the production of autoantibodies in the *lpr* model of murine systemic autoimmunity. *J. Exp. Med.* 173:1441.
20. Mountz, J.D., H.R. Smith, R.L. Wilder, J.P. Reeves, and A.D. Steinberg. 1987. CS-A therapy in MRL-*lpr/lpr* mice: amelioration of immunopathology despite autoantibody production. *J. Immunol.* 138:157.
21. Tanaka, A., F.X. O'Sullivan, W.J. Koopman, and S. Gay. 1988. Etiopathogenesis of rheumatoid arthritis-like disease in MRL/1 mice. II. Ultrastructural basis of joint destruction. *J. Rheumatol.* 15:1.
22. Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik. 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. *J. Exp. Med.* 169:333.
23. Murray, L.J., R. Lee, and C. Martens. 1990. *In vivo* cytokine gene expression in T cell subsets of the autoimmune MRL/Mp-*lpr/lpr* mouse. *Eur. J. Immunol.* 20:163.
24. Davidson, W.F., C. Calkins, H. Hugin, T. Giese, and K.L. Holmes. 1991. Cytokine secretion by C3H-*lpr* and -*gld* cells. Hypersecretion of IFN-γ and tumor necrosis factor-α by stimulated $CD4^+$ T cells. *J. Immunol.* 146:4138.