# Reduction of Lupus Nephritis in MRL/*lpr* Mice by a Bacterial Superantigen Treatment

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## Summary

The effects of biweekly intravenous injections of Staphylococcus Enterotoxin B (SEB) into autoimmune MRL-*lpr*/*lpr* (MRL/*lpr*) mice were investigated. Rather than causing the expansion of V$\beta$8$^+$ T cells, SEB administration resulted in the reduction of V$\beta$8$^+$, CD4$^-$CD8$^-$ "double-negative" (DN) T cells. This was shown by FACS® analysis as this putative pathogenic population was diminished in both spleen and lymph node. The symptoms of systemic lupus erythematosus (SLE) in MRL/*lpr*, which include high titers of anti-DNA antibodies and circulating immune complexes and proteinuria, were reduced in SEB-treated mice in a dose-dependent manner. The clinical parameters of SLE in MRL/*lpr*, which include lymph node hyperplasia and necrotic vasculitis, were suppressed in 50-$\mu$g SEB-treated mice. T cells bearing V$\beta$6 T cell receptor, which does not interact with SEB, were not reduced with SEB administration. Thus, disease suppression was associated with a specific reduction in the number of V$\beta$8$^+$, DN T cells. These results implicate a possible therapeutic role of superantigen-based immunotherapy in V$\beta$-restricted, T cell–dominated clinical syndromes.

Superantigens (SuperAgs)[1] are molecules that in association with class II MHC activate T cells based solely on the V$\beta$ chain of the TCR (1, 2). These antigens are the most powerful polyclonal mitogens known, stimulating a large proportion of both murine and human T cells. The minor lymphocyte stimulating (Mls) antigen, a self-SuperAg, has recently been reported to be encoded by a retrovirus, but the Mls antigen has yet to be isolated (3–7). More is known about the bacterial SuperAgs, particularly the *Staphylococcus aureus* enterotoxin series (SEs), whose sequence and structure have been documented (1). SuperAgs are known to exert powerful effects on the developing TCR repertoire in both CD4$^+$ and CD8$^+$ T cells (8–11). Previous data from this laboratory (12, 13) and others (14) have demonstrated both clonal anergy and deletion of peripheral T cells bearing reactive V$\beta$8 TCR with in vivo administration of one of these SuperAgs, *Staphylococcus* Enterotoxin B (SEB), which engages V$\beta$7 and 8–bearing T cells (2, 8).

Recent work on different animal models of disease has demonstrated a V$\beta$ predominance of T cells involved in disease pathogenesis. Examples of this phenomenon include experimental allergic encephalomyelitis (EAE) in B10.PL mice (15) and lupus nephritis in MRL/*lpr* (16), both of which show a V$\beta$8 restriction in T cells. Specific immunotherapies aimed at intervention at the level of these pathogenic T cells have included anti-V$\beta$8 antibody (Ab) (17) and V$\beta$8 peptide therapy (18) in EAE, and anti-T cell (Thy-1.2 [19]), CD4 [20], B220 [21]) Ab treatment in MRL/*lpr* mice. These treatments have resulted in both the reduction in the number of "targeted" T cells and a corresponding improvement in the disease state. These findings suggested that the TCR-binding specificity of superantigens could be exploited for downregulation of T cell, and notably, pathogenic T cell subpopulations expressing TCRs of restricted heterogeneity. We have addressed this issue by SEB immunization of autoimmune MRL/*lpr* mice, in which CD4$^-$CD8$^-$ "double-negative" (DN) T cells expressing V$\beta$8.2 and V$\beta$8.3 TCRs are preferentially represented on the peripheral, pathogenic T cells (16). We demonstrate that SEB treatment in MRL/*lpr* mice results in a reduction in V$\beta$8$^+$, CD4$^-$CD8$^-$ peripheral T cells concomitant with disease suppression. Clinical and serological disease activity was reduced in SEB-treated mice in a dose-dependent manner.

[1] *Abbreviations used in this paper:* Ab, antibody; DN, double negative; EAE, experimental allergic encephalomyelitis; Mls, minor lymphocyte stimulating; SA-PE, streptavidin-phycoerythrin; SE, *Staphylococcus aureus* enterotoxin series; SEB, *Staphylococcus* Enterotoxin B; SuperAg, superantigen.

## Materials and Methods

*Animals.* MRL/Mp-*lpr*/*lpr* mice (4–6 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME).

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*Reagents and mAbs.* SEB was purchased from Sigma Chemical Co. (St. Louis, MO). Reagents used for direct staining were biotin-conjugated anti-: Vβ6 (22), Vβ8 (23), CD4-PE conjugate, and CD8-FITC conjugate from Becton Dickinson & Co. (Mountain View, CA). Secondary reagents, streptavidin-PE (SA-PE) and avidin-FITC, were also purchased from Becton Dickinson & Co.

*Treatment of MRL/lpr with SEB.* Treatment of MRL/*lpr* started at 6 wk of age before clinical onset of disease. Animals were injected with 50, 5, and 0.5 μg of SEB in 0.2 ml PBS or PBS alone intravenously through the tail vein every 2 wk.

*Fluorescence Staining (FACS®).* Spleen and axillary lymph node single cell suspensions from 6-mo-old MRL/*lpr* mice were treated with Tris-buffered 0.16 M ammonium chloride to lyse the RBC. $10^6$ cells were incubated with biotinylated anti-Vβ6 or anti-Vβ8 (purified from the respective B cell hybridomas by protein A–Sepharose [Pharmacia Fine Chemicals, Uppsala, Sweden]) for 30 min on ice. The cells were then washed and incubated with either SA-PE (when anti-CD8-FITC was used as the second antibody) or avidin-FITC (when anti-CD4-PE was used as the second antibody). After 30 min of incubation on ice, the cells were washed three times and incubated with anti-CD4-PE or anti-CD8-FITC (as mentioned above). Cells were washed and two-color analysis was performed by an Epics C fluorocytometer (Coulter Immunology, Hialeah, FL).

*ELISA for Anti-DNA Abs and Circulating Immune Complexes.* Polystyrene microtiter wells coated with double-stranded DNA (ds-DNA) or goat C1q were the kind gift of Dr. Marilyn Baltz (Sigma Chemical Co.). Blood from each mouse was pooled according to treatment group and collected before the biweekly injections. Sera was diluted in 0.05% Tween 20 in PBS at a 1:500 dilution and allowed to incubate on the plates for 60 min at room temperature. The plates were then washed three times with PBS-Tween, and 50 μl of a 1/1,000 dilution of anti-IgG and anti-IgM goat anti–mouse urease conjugate (Sigma Chemical Co.) was added to the plates. After incubation for 30 min, the plates were washed three times with PBS-Tween and twice with 0.15 M NaCl. The plates were then incubated with the urease substrate solution. The urease substrate solution was made by a protocol kindly provided by Sigma Chemical Co. In short, 8 mg of bromcresol purple was dissolved in 1.48 ml of 0.01 M NaOH and then diluted to 100 ml with water. 100 mg of urea and 3.7 mg of EDTA were dissolved and the pH was adjusted to 4.8 by the addition of 0.01 M NaOH. Colourimetric change was quantified by a Microplate Reader (MR 600; Dynatech, Chantilly, VA) at 590 nm OD.

*Proteinuria and Physical Symptoms.* Urine (from at least four mice per group) was pooled according to treatment group. Protein concentration and the presence of blood in urine was measured semiquantitatively by reagent strips for urine analysis (Labstix; Ames Corp., Etobicoke, Ontario). Physical symptoms were visually scored as: 0, no symptoms; 0.5, trace; 1–4, when visible symptoms are observed, with 4 being the most severe (physical symptoms include lymph node hyperplasia, immune complex vasculitis, and necrosis of the ears). Mortality was observed in the PBS and 0.5-μg SEB-treated groups at 4 mo of age. The scores representing physical

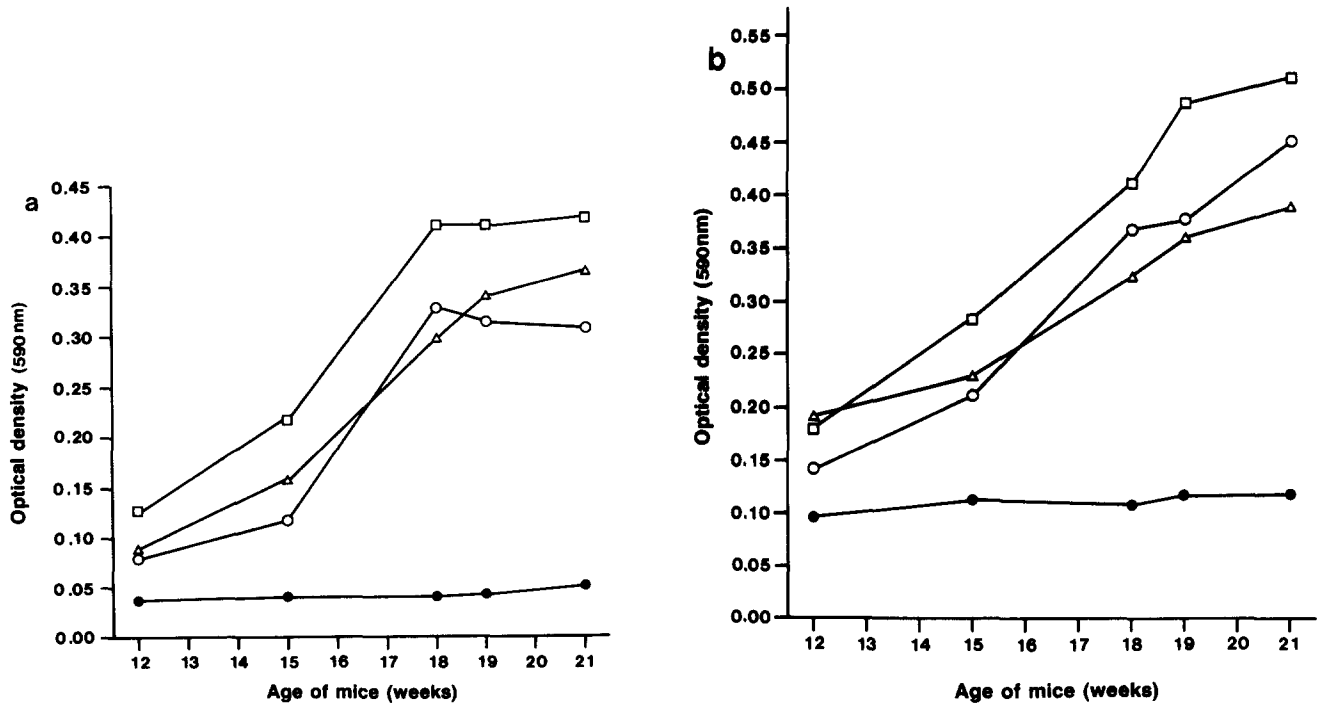


**Figure 1.** Circulating immune complex and anti-DNA titers as measured by ELISA. MRL-*lpr*/*lpr* mice were given biweekly intravenous injections (by tail vein) of 50, 5, and 0.5 μg of SEB in 0.2 ml PBS or PBS alone beginning at 6 wk of age. At weekly intervals, ~200 μl of blood was collected from each mouse and serum was pooled according to treatment group and stored at −20°C. (*a*) The results from C1q-coated plates; (*b*) DNA-coated wells. The data are representative of three separate experiments. Nonautoimmune C3H/HeJ and pre-autoimmune MRL/*lpr* (4 wk of age) registered OD readings <0.05 at 590 nm for both assays. 50 μg SEB (●), 5 μg SEB (○), 0.5 μg SEB (△), PBS (□).

symptoms were calculated by determining the total score for each group and then dividing by the number of animals alive in that group when the measurement was taken.

## Results

*Systemic Administration of SEB Reduces Both Immune Complex and dsDNA-specific Ab Titer.* Serologically, we followed both circulating immune complex (Fig. 1 *a*) and ds-DNA-specific antibody (Fig. 1 *b*) titers as measured by ELISA. The results show that while the PBS-injected controls manifested the aggressive increase in these indices correlated with disease onset at 3 mo of age, animals treated with 50 $\mu$g SEB showed a pre-autoimmune level, similar to that measured in nonautoimmune mice. The intermediate doses of SEB (5 and 0.5 $\mu$g) decreased both serological levels 1–1.5-fold, but did not have as great an effect as the 50-$\mu$g SEB treatment, which was associated with a fourfold difference in DNA-specific OD, and an eightfold difference in immune complex OD (from this point on, "SEB treatment" will refer to the 50-$\mu$g-treated group exclusively). We have also previously observed a decrease in ds-DNA-specific Ab titer using 20 $\mu$g of SEB (unpublished observations, C. Kim). Thus, SEB treatment induces a dose-dependent decrease in two serological parameters of SLE in MRL/*lpr* mice.

*Specific Reduction of V$\beta$8$^+$, CD4$^-$CD8$^-$ T Cells in SEB-treated Spleens and Lymph Nodes.* To ascertain the effects of SEB immunization on the peripheral V$\beta$8 population in this autoimmune strain, the proportions of V$\beta$8$^+$ T cells in the splenic and lymph node CD4$^+$,CD8$^+$, and CD4$^-$CD8$^-$ T cell populations were compared between 6-mo-old controls and 50-$\mu$g SEB-treated mice. We were curious to determine if SEB could cause a decrease in peripheral V$\beta$8$^+$ T cells that were CD4$^-$ and CD8$^-$. The results of both splenic and lymph node analyses show that injections of SEB does produce a decrease in the V$\beta$8$^+$ DN T cell fraction, from 15% to 2% in spleen (Fig. 2 *a*) and 22% to 2% in LN (Fig. 2 *b*). Interestingly, there seemed to a compensatory increase in the V$\beta$6$^+$ CD4$^-$8$^-$ fraction with SEB treatment, from 3% to 8%. However, the V$\beta$6$^+$ DN T cell proportion did not show the enormous expansion normally observed in the V$\beta$8$^+$ DN T cell fraction. Also, SEB immunization led to minimal decreases in the proportions of V$\beta$8$^+$ CD4$^+$ and V$\beta$8$^+$ CD8$^+$ T cells.

The results imply that the abnormal DN T cells that are associated with both onset and acceleration of disease in the MRL/*lpr* strain are more susceptible to deletion than single-positive cells, and/or that SEB treatment has suppressed their time-dependent proliferation. Regardless, the results show that SEB treatment has decreased the level of these phenotypically aberrant cells and results in the suppression of disease.

*Mass of Lymphoid Organs from SEB-treated MRL/lpr Mice.* One of the phenotypic changes associated with disease onset is a massive lymphoid hyperplasia, which causes a profound increase in the size of lymphoid organs (24, 25). We therefore examined the splenic and lymph node sizes of mice from PBS controls and SEB-treated MRL/*lpr*.

As shown in Fig. 3, with SEB treatment, there is a decrease in both the spleen and the axillary lymph node. Specifically, spleens and axillary lymph nodes from PBS controls averaged 0.66 and 0.25 g, respectively. The SEB counterparts weighed 0.25 and 0.04 g. This decrease in splenic and axillary lymph node mass is due to a decrease in cell number in these lymphoid organs. The other lymph nodes demonstrated a similar decrease in cellularity and size with SEB treatment (data not shown).

*Reduction in Proteinuria and the Physical Symptoms of SLE.* Disease onset in these mice occurs at 3 mo of age with visible lymphoid hyperplasia, increasing titers of auto-Abs, and the development of nephritis with proteinuria. 50% mortality in the MRL/*lpr* strain, due to glomerulonephritis, occurs by 5 mo of age. To determine the effect of SEB treatment on

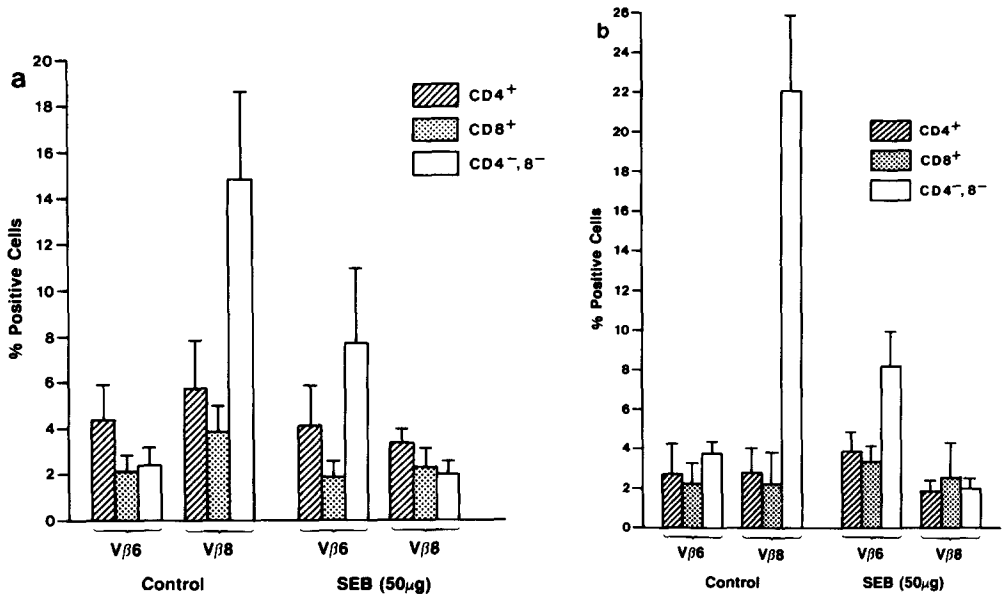


**Figure 2.** Flow cytometric analysis of MRL/*lpr* spleen and lymph node cells. MRL/*lpr* mice were treated with 50 $\mu$g SEB or PBS alone and were killed at 6 mo of age, and the spleens (*a*) and axillary lymph nodes (*b*) were removed. Single cell suspensions were analyzed by two-color FACS® analysis with biotinylated anti-V$\beta$6 and -V$\beta$8 Abs, and CD4-PE and CD8-FITC Abs. The results are presented as the mean and SD of three separate experiments.

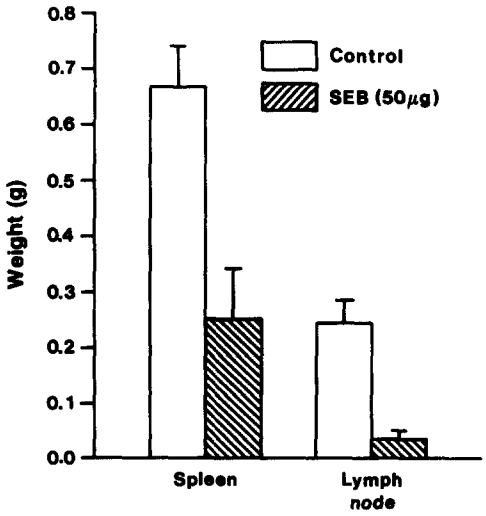


**Figure 3.** Mass of lymphoid organs from MLR/*lpr* mice. Spleen and axillary lymph nodes were removed from 6-mo-old MRL/*lpr* mice treated with 50 μg SEB or PBS and weighed. The data are presented as the mean mass per spleen or axillary lymph node and SD of four different experiments.

a clinical level, we measured both renal function by assaying proteinuria and followed the physical symptoms of disease.

In Table 1, it is clearly shown that SEB treatment has inhibited renal degradation (0.3 g/liter at 5 mo of age), while the PBS controls demonstrate a gross defect in renal function (3.0 g/liter). The 0.5-μg SEB-treated group shows an apparent acceleration of renal degradation, whereas the 5-μg SEB-treated group reaches abnormal levels at 5 mo of age. In fact, by 21 wk of age, all the treatment groups, except 50-μg SEB, show high levels of proteinuria as well as the presence of blood in the urine, a further indicator of glomerulonephritis.

The visible physical symptoms that signal lupus in this strain include lymph node hyperplasia, necrosis of the ears, and immune complex vasculitis of the skin (24, 25). Table 2 shows that SEB treatment has been effective in suppressing the physical signs of disease and parallels the results of SEB treatment on the other parameters of SLE in the MRL/*lpr* strain.

## Discussion

T cell–dominated clinical syndromes in which T cells preferentially express TCRs of restricted heterogeneity provide an unique opportunity to study the effects of T cell–directed therapies. The use of mAbs against specific T cell markers have been used in the treatment of both EAE (anti-Vβ8 Abs) and lupus in MRL/*lpr* (anti-Thy-1.2, CD4, B220). These therapies have resulted in the reduction of the "targeted" T cells as well as a decrease in disease activity. However, the effects of passive Ab therapy are generally short-lived and require frequent injections of large amount to be effective.

The results presented in this report indicate a reduction of disease in SEB-treated MRL/*lpr* mice past 5 mo of age, the time when the PBS and 0.5-μg SEB groups show 50% mortality (data not shown; and 24, 25). However, SEB treatment does not seem to suppress the disease completely. At 7 mo of age, some mice treated with 50 μg of SEB developed lymph node swelling. This, however, was not due to an expansion of $V\beta8^+$ or $V\beta6^+$ DN T cells as measured by

**Table 1.** *Proteinuria*

| | Treatment | | | |
|---|---|---|---|---|
| Age | 50 μg SEB | 5 μg SEB | 0.5 μg SEB | PBS |
| *wk* | | *g/liter* | | |
| 12 | <0.3 | <0.3 | 1.0 | 1.0 |
| 13 | <0.3 | <0.3 | 1.0 | 1.0–3.0 |
| 14 | <0.3 | <0.3 | 1.0 | 1.0–3.0 |
| 15 | <0.3 | 0.3–1.0 | 1.0* | 1.0–3.0 |
| 16 | <0.3 | 0.3 | 1.0 | 1.0–3.0 |
| 17 | <0.3 | 1.0 | 1.0–3.0 | 1.0–3.0 |
| 18 | 0.3–1.0 | 0.3–1.0 | 3.0 | 3.0‡ |
| 19 | 0.3–1.0 | 0.3–1.0 | 3.0–20‡ | 3.0 |
| 20 | 0.3 | 1.0–3.0 | 3.0–20‡ | 3.0 |
| 21 | 0.3–1.0 | 3.0–20‡ | 3.0–20§ | 3.0–20§ |

Measured from 3 mo of age (by Labstix).
* Trace, nonhemolyzed blood.
‡ Hemolyzed blood.
§ Large blood.

**Table 2.** *Physical Symptoms (Lymphoid Hyperplasia, Necrosis of Ear, Hair Loss)*

| | Treatment | | | |
|---|---|---|---|---|
| Age | 50 μg SEB | 5 μg SEB | 0.5 μg SEB | PBS |
| *wk* | | | | |
| 12 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0.3 | 0.3 |
| 14 | 0 | 0 | 0.3 | 0.3 |
| 15 | 0 | 0 | 0.35 | 0.4 |
| 16 | 0 | 0 | 0.45 | 0.61 |
| 17 | 0 | 0.1 | 0.85 | 0.67 |
| 18 | 0 | 0.1 | 1.1 | 0.72 |
| 19 | 0 | 0.31 | 0.85 | 0.61 |
| 20 | 0 | 0.31 | 0.85 | 1.0 |
| 21 | 0.1 | 0.44 | 0.95 | 1.06 |

No symptoms, 0; trace, 0.5. Symptoms scored 1–4, (4 most severe). Total score for each group is divided by total number of mice.

FACS® analysis (data not shown). The possibility that clonal expansion of another Vβ DN T cell population causes disease in SEB-treated MRL/*lpr* mice remains to be investigated. By 9 mo, the first indications of immune complex vasculitis are observed as well as mortality in this group. Thus, SEB treatment has a dramatic effect on delaying disease onset, but cannot completely abrogate the disease in this strain.

Work from this laboratory (12, 13) has shown anergy and reduction of $V\beta8^+$, $CD4^+$ peripheral T cells 21 d after a single injection of 50 μg of SEB in BALB/c mice. It is thus possible that reductions of disease in MRL/*lpr* mice may require only two or three SEB injections, and this is currently being investigated. However, the active processes involved in the development of disease in this strain may not permit duration of the effects of SEB injection comparable to that observed in BALB/c mice. The proliferation of DN T cells that is genetically programmed in MRL/*lpr* mice may overcome an abbreviated treatment regimen. We have observed that after termination of treatment at 5 mo of age, MRL/*lpr* mice treated with 50 μg of SEB develop high levels of anti-DNA Abs by 10 mo of age (unpublished observations, A. De Hoyos and A. Ochi).

The data presented in this report are consistent with a cause and effect relationship between a decrease in DN $V\beta8^+$ T cells and amelioration of disease. However, a role of $CD4^+$ T cells in disease pathogenesis in this strain cannot be ruled out. It has been previously reported that $CD4^+$ T cells can contribute to lymphoproliferation and auto-Ab production in MRL/*lpr* mice (20). As we have previously shown that the dose of SEB used to reduce disease in MRL/*lpr* mice is tolerogenic for $CD4^+$ T cells in normal mice, the possibility exists that these salutary effects result, in some measure, from anergy (but not deletion) of mature $CD4^+$, $V\beta8^+$ T cells. Alternately, $CD4^+$ T cells that are involved in disease propagation may have arisen, in part, from the reacquisition of the CD4 accessory molecule by the DN T cells in the periphery (26–28). Thus, it may be impossible to distinguish the relevance of T cell anergy vs. reduction in amelioration of disease in MRL/*lpr* mice. The advantage of SEB therapy, however, lies in the fact that SEB interacts with "reactive" T cells that are $CD4^+$,$CD8^+$, and $CD4^-$ $CD8^-$ (29). The factors that cause these reactive T cells to undergo deletion or anergy when exposed to SEB remain to be determined. Similarly, the factors that lead to activation (30) or suppression (12–14) of T cells consequent to in vivo SEB administration remain to be determined.

The application of SEB to the treatment of MRL/*lpr* mice provides a potential new technique in T cell–directed therapies. Although the data demonstrate that $V\beta8^+$ $CD4^-CD8^-$ expansion in lymphoid tissues is dramatically prevented in mice, the mechanisms whereby this therapy downregulates autoantibody and immune complex production are currently unclear. Previous data have demonstrated that SEB injection can cause suppression in the humoral response in normal mice (31), and thus it is possible that SEB can suppress anti-DNA antibody-producing B cells in MRL/*lpr* mice.

The exact role of DN T cells in disease initiation and/or pathogenesis remains to be elucidated. Previous reports have shown that these cells have undergone thymic selection and expression of surface CD4 and CD8 (32, 33). There are currently two main paradigms as to the origin of these DN T cells (34). They may encode TCRs with autoreactive specificities or they may arise consequent to defective positive selection and may not have specificity for self at all. Regardless of their origins DN T cells are clearly involved in disease pathogenesis, and their ablatement by SEB treatment suppresses the disease.

In an alternate approach, peptide therapy has recently been shown to be effective in both preventing and reversing EAE in Lewis rats (18). This strategy invokes a 21–amino acid peptide derived from the second complementarity determining region of the Vβ8 TCR and presumably induces an autoregulatory response against Vβ8-bearing T cells. This treatment has a surprisingly rapid therapeutic effect, and the lack of foreign antigenic determinants as well as its extended regulatory effects makes this approach very promising. So far the mechanism of therapeutic effects is unclear. As yet, peptide therapy has not been investigated in the MRL/*lpr* strain. It would be necessary and important to compare therapeutic benefits of SEB and peptide-mediated approaches.

One potential problem associated with SEB administration in vivo is its enterotoxic effect. SEs are the common cause of food poisoning in man. However, a recent report has suggested that different portions of the SEB molecule are responsible for its T cell mitogenic and enterotoxic effects (35). Our observations support this contention, as no detrimental effect was observed in SEB-treated mice except for a transient weight loss of 5–10% in 50-μg-treated mice (data not shown; and 36).

In summary, T cell–mediated pathogenesis of autoimmunity in MRL/*lpr* mice is implied by the clinical improvements induced by such interventions as neonatal thymectomy (37, 38) and administration of anti-Thy-1.2 (19), CD4 (20), and B-220 (21) mAbs. This strain then provides a unique model for studying the efficacy of T cell–directed therapies at improving and/or ablating disease. We have shown that a Vβ-specific SuperAg that functionally inactivates and deletes peripheral T cells markedly reduces disease activity in these autoimmune mice. Whether Vβ-dominant T cells play a causative role in disease initiation and progression or arise through some bystander effect remains to be elucidated. However, Vβ-specific therapy by SuperAgs may have potential for future clinical application and warrants further investigation.

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