The Journal of Immunology

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J Immunol 1998; 161:6427-6432; ;
http://www.jimmunol.org/content/161/11/6427

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Central T Cell Tolerance in Lupus-Prone Mice: Influence of Autoimmune Background and the *lpr* Mutation[1]

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Lupus-prone mice develop a systemic autoimmune disease that is dependent upon the B cell help provided by autoreactive $\alpha\beta$ $CD4^+$ T cells. Since autoreactive T cells with high affinity for self peptides are normally deleted in the thymus, their presence in these mice suggests the possibility that intrathymic negative selection may be defective. Here, we directly compared central T cell tolerance in response to a conventional peptide Ag in lupus-prone MRL/MpJ mice with a nonautoimmune strain using an MHC class II-restricted TCR transgene. Our results did not demonstrate any defects after Ag exposure in the induction of intrathymic deletion of immature $CD4^+CD8^+$ thymocytes, in TCR down-regulation, or in the number of apoptotic thymocytes in MRL/MpJ compared with nonautoimmune mice. Furthermore, we found that the *lpr* mutation had no influence upon the Ag-induced thymic deletion of immature thymocytes. These data support the notion that T cell autoreactivity in MRL/MpJ mice is caused by defects in peripheral control mechanisms. ***The Journal of Immunology*****, 1998, 161: 6427–6432.**

Systemic lupus erythematosus, the prototypical systemic autoimmune disease, is characterized by the production of high-titer autoantibodies to various cellular components, including chromatin and small nuclear ribonucleoproteins. It has become clear that autoreactive $\alpha\beta$ $CD4^+$ T cells are essential for the full induction of these autoantibodies (1–4). Such autoreactive T cells presumably escape normal tolerance mechanisms, with their presence in the periphery suggesting the possibility that negative selection in the thymus may be abnormal. However, work based on superantigen-induced T cell deletion has suggested that central tolerance is intact in lupus-prone mice (5–7), although others have suggested that central deletion of thymocytes in these animals is defective (8). Regardless, superantigen-induced central T cell deletion primarily affects $CD4^+$ thymocytes (9–11), whereas thymic deletion as a result of ligation of the TCR with a peptide Ag, which is the main pathway for elimination of autoreactive T cells, occurs at the level of premature $CD4^+CD8^+$ thymocytes (double-positive (DP)[4]) (12, 13). Thus, studies performed using superantigen-induced deletion may not apply to deletion of autoreactive thymocytes in response to peptide Ags. Previous studies have examined the tolerance of $CD4^+$ T cells and their precursors in response to peptide Ags in MRL/MpJ (MRL) mice with intact Fas expression (MRL/+Fas^{lpr}, (MRL/+)) as compared with Fas-deficient MRL mice (MRL/Fas^{lpr} (MRL/*lpr*)) (14–16), defining a role for Fas in peripheral tolerance (14). However, both of these strains develop systemic autoimmunity, and a comparison of central tolerance of lupus-prone to nonautoimmune mice has not been done to our knowledge. Such a study might define tolerance defects inherent to the autoimmune-prone background of such animals.

We compared the thymic deletion in MRL mice (both MRL/+ and MRL/*lpr*) with a nonautoimmune strain, B10.BR (H-2^k, like MRL), in response to a conventional Ag rather than a superantigen. Since developmental studies of single clones of T cells activated by peptide Ags are hampered by the low numbers of these cells in the repertoire, we used TCR transgenic mice that carry large numbers of T cells against a model Ag, pigeon cytochrome *c* (PCC) (17). We generated PCC-specific TCR transgenic mice that were crossed into both nonautoimmune (B10.BR) and autoimmune (MRL) backgrounds for more than six generations followed by an analysis of peptide-induced deletion of DP thymocytes. We found a comparable reduction in the number of DP thymocytes, down-regulation of the transgenic TCR, and an increase in the number of apoptotic thymocytes in MRL and B10.BR mice. Furthermore, contrary to a recent report (16), all of these changes were identical between MRL mice with an intact *fas* gene (MRL/+) and those homozygous for the *lpr* mutation (MRL/*lpr*). These experiments suggest that the central T cell tolerance in MRL mice is comparable with nonautoimmune mice of the same MHC background, and that the *lpr* mutation does not appear to affect negative selection in the thymus in response to a peptide Ag.

Materials and Methods

Mice and cell lines

All mice were housed in specific pathogen-free facilities at the Yale School of Medicine. B10.BR and MRL mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME); AND TCR transgenic mice in the B10.BR background (H-2^k) (17) that had been generated originally by S. Hedrick (University of California, San Diego, CA) were obtained from K. Bottomly (Yale University School of Medicine). AND transgenic animals were crossed with MRL mice for more than six generations, followed by appropriate intercrosses to generate AND transgenic MRL mice with or without the *lpr* mutation. AND transgenic mice were also crossed with mice carrying a disrupted TCR α gene to generate B10.BR and MRL mice

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Received for publication April 21, 1998. Accepted for publication July 28, 1998.

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[1] This work was supported in part by Grants 1K08AR01974 (to S.F.) and AR40072 and AR44076 (to J.C.) from the National Institutes of Health and by the Arthritis Foundation and the Lupus Foundation of America and their Connecticut chapters. S.L.P. was supported by the Medical Scientist Training Program, Yale University School of Medicine.

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[4] Abbreviations used in this paper: DP, double positive; MRL, MRL/MpJ; PCC, pigeon cytochrome *c*; TdT, terminal deoxynucleotidyl transferase; dUTP, deoxyuridine triphosphate; TUNEL, TdT-mediated UTP nick-end labeling; b-dUTP, biotin-21-deoxyUTP.

0022-1767/98/$02.00

(both +/+ and *lpr/lpr*) with only one population of $\alpha\beta$ T cells (4, 18). Screening for the *lpr* mutation, AND transgenic TCR α- and β-chains, and the H-2 haplotype was performed by PCR (19–21). CH27 lymphoblastoid cells were a gift of Charles Janeway (Yale School of Medicine) (22).

Flow cytometry

All conjugated mAbs with the exception of Texas Red-conjugated streptavidin (Life Technologies, Gaithersburg, MD) were purchased from Phar Mingen (San Diego, CA). Stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and CELLQuest software (Becton Dickinson).

In vitro deletion of $CD4^+CD8^+$ thymocytes

In these assays, intact thymic lobes or a mixture of thymocytes with CH27 lymphoblastoid cells were cultured at 37°C in presence of different concentrations of the PCC 88–104 peptide (KAERADLIAYLKQATAK, American Peptide Company, Sunnyvale, CA). At the conclusion of the in vitro culture period, cells were removed and counted. Control wells containing thymocytes alone, CH27 cells alone, and thymocytes plus CH27 cells without the addition of peptide were set up in a similar manner. The number of CH27 cells was subtracted from the total number of cells to calculate the number of thymocytes in the wells that contained a mixture of CH27 cells and thymocytes.

Terminal deoxynucleotidyl transferase (TdT)-mediated uridine triphosphate (UTP) nick-end labeling (TUNEL) assay

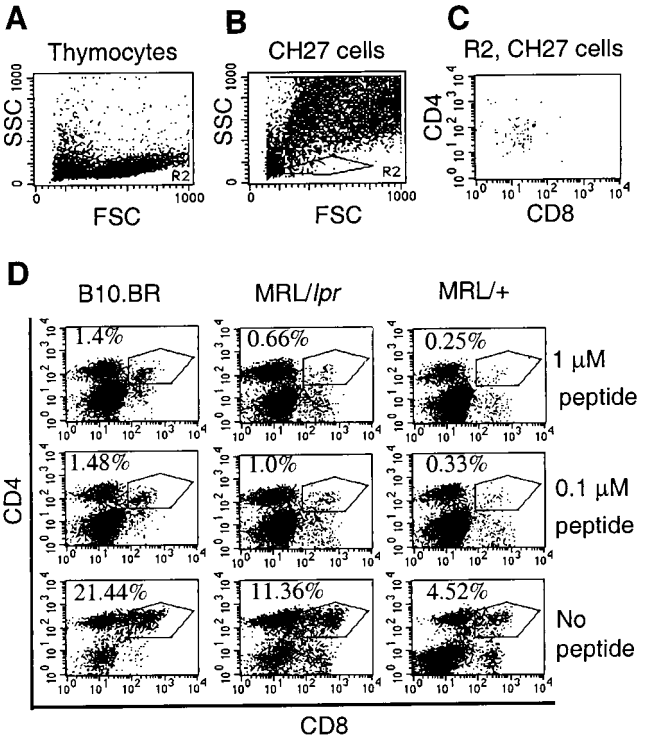
The TUNEL assay for the detection of apoptotic cells was performed by following a published procedure with minor modifications (23). Briefly, cells were fixed after surface staining, washed once in HBSS and once in TdT buffer (100 mM sodium cacodylate (pH 7.2), 0.2 mM 2-ME, and 2 mM $CoCl_2$), and incubated with TdT (United States Biochemical, Cleveland, OH) at a concentration of 0.1 U/μl in presence of 5 μM of biotin-21-deoxyUTP (b-dUTP) (Clontech, Palo Alto, CA) at 37°C for 20 min. The cells were washed, stained with Texas Red-conjugated streptavidin, and analyzed by flow cytometry.

Results and Discussion

Deletion of DP cells is equivalent in the thymi of MRL and B10.BR mice

Our preliminary studies of AND transgenic mice confirmed previous reports that the number and percentage of DP and single-positive cells can vary significantly between different transgenic mice (see Figs. 1*D* (row labeled No peptide) and 2 (row labeled Thymus without peptide)); this variation would make it difficult to use an in vivo system for the induction of central deletion when comparing different mice. As a result, a previously described in vitro method for assessing the deletion of DP thymocytes was used that allows for tight control and for comparison among different mice (24). We also found that, as reported previously (25), the initiation of effective DP deletion required presence of APCs (data not shown). We used CH27 lymphoblastoid cells, which express high levels of $I\text{-}E^k$ and costimulatory molecules, as APCs (data not shown). These cells can easily be distinguished from thymocytes by virtue of different forward and side scatter characteristics. Here and in all subsequent experiments we used this region (indicated as R2 in Fig. 1, *A* and *B*), which includes only thymocytes. We showed that 99.5% of all CH27 cells were gated out; the few cells that were included in this region did not express CD4 or CD8 molecules and did not interfere with the determination of the percentage of DP cells (Fig. 1*C*). Thus, the percentage of DP thymocytes was nearly identical in wells containing thymocytes alone and wells with thymocytes and CH27 cells without the addition of the PCC peptide (see Fig. 4, *middle* and *right panels*, respectively).

In these experiments, 1×10^6 thymocytes were initially cultured with 5×10^5 mitomycin C-treated CH27 cells for 24 h. The number of viable thymocytes was calculated by subtracting the number of CH27 cells from the total number of cells. Using this method, we showed that there was a marked reduction in the percentage (Fig. 1*D*) and actual number (Fig. 1*E*) of DP cells in response to



E

	1 μM peptide (% decrease)	0.1 μM peptide (% decrease)
B10.BR	93.5	93
MRL/lpr	94.2	91.2
MRL/+	94.5	92.5

FIGURE 1. Decrease in $CD4^+CD8^+$ (DP) thymocytes in response to APCs plus PCC peptide. Thymocytes from B10.BR, MRL/+, and MRL/*lpr* animals were cultured in duplicate wells with mitomycin C-treated CH27 cells and with two different concentrations of the PCC peptide. Thymocytes (*A*) and CH27 cells (*B*) were also cultured alone or together in the absence of the PCC peptide. After 24 h, cells were removed, counted, stained with conjugated Abs, and analyzed on a FACScan. *A* and *B*, Thymocytes in the R2 region were distinguished from CH27 cells by their forward and side scatter characteristics. *C*, CH27 cells were double-stained with anti-CD4 and anti-CD8 Abs. Few cells included in the R2 region do not express either CD4 or CD8 molecules as expected. *D*, Thymocytes were stained with anti-Vβ3, anti-CD4, and anti-CD8 Abs and analyzed by flow cytometry; the numbers in each FACS plot indicate the percentage of R2-gated thymocytes that stained with both CD4 and CD8 Abs (DP cells). *E*, The actual decrease in the number of DP cells in response to two different doses of the PCC peptide was calculated. Data are the average of duplicate wells from one mouse in each group; three mice in each group were analyzed.

incubation with either 1 μM or 0.1 μM of PCC peptide. This decrease was similar between MRL mice with or without a functional Fas receptor (MRL/+ and MRL/*lpr*, respectively) and B10.BR animals. A similar degree of DP thymocyte deletion was noted when a 5-μM concentration of the peptide was used (data not shown). The most likely reason for the almost identical results observed at these three concentrations is that all three are above the threshold concentration of peptide ligand required for effective DP cell deletion (26, 27).

To demonstrate that these results were not biased due to a high level of expression of MHC class II and costimulatory molecules on CH27 cells, we repeated the experiments using intact thymic

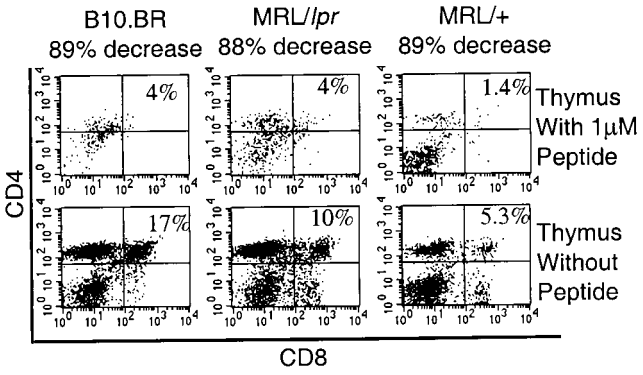


FIGURE 2. $CD4^+CD8^+$ (DP) cell reduction in intact thymic lobes. Intact thymic lobes from B10.BR, MRL/+, and MRL/*lpr* mice were isolated and cultured with 1 μM of PCC peptide at 37°C for 24 h; thymocytes were then removed, counted, surface stained, and analyzed as described in the legend to Fig. 1. The numbers at the top of each column illustrate the actual decrease in the number of DP cells in the wells with added peptide compared with wells in which no peptide was added; the numbers in each FACS plot indicate the percentage of R2-gated DP thymocytes. Data are representative of two separate experiments (two mice in each group).

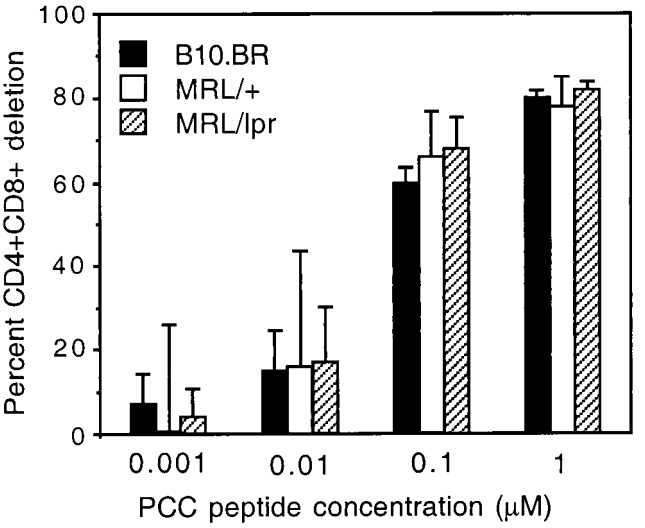


FIGURE 3. Submaximal stimulation of thymocytes does not reveal a defect in $CD4^+CD8^+$ (DP) thymocyte deletion in MRL mice. In these experiments, 1×10^6 thymocytes were cultured with 5×10^5 mitomycin C-treated CH27 cells for 20 h using four different concentrations of the PCC peptide in duplicate wells. Surface staining was performed as described in the legend to Fig. 1. The average decreases in the numbers of DP cells from three mice in each group are shown with error bars (indicating 1 SEM). Data were combined for TCR $\alpha^{-/-}$ and TCR $\alpha^{+/+}$, because there were no notable differences between the two groups.

lobes. Here, we cultured intact thymic lobes from 4-wk-old mice with 1 μM of PCC peptide for 24 h followed by surface staining (Fig. 2). Although the percentage of DP cells did not decrease as much as when thymocytes were added to CH27 cells, the actual number of DP cells declined to the same degree due to a marked reduction in the total number of viable cells.

Submaximal stimulation of thymocytes by the PCC peptide does not reveal any defects in thymic deletion of DP thymocytes in MRL mice compared with B10.BR animals

Although the experiments discussed above did not reveal any differences in DP thymocyte deletion between MRL and B10.BR mice, they were conducted under conditions that induced nearly maximal deletion. To rule out the possibility that lower doses of Ag would induce deletion in nonautoimmune mice but not in autoimmune animals, we performed the same assay using a range of PCC concentrations. In addition, we shortened the incubation time from 24 to 20 h to further decrease the strength of the deleting signal delivered to DP thymocytes (23). Using these measures, we were able to induce submaximal deletion of DP thymocytes; however, we did not detect any difference between MRL and B10.BR animals, even with the induction of less than maximal deletion (Fig. 3). It is important to note that doses as low as 0.01 μM of peptide did not reveal any obvious defects in thymic deletion in MRL mice with or without the *lpr* mutation, suggesting a lack of any significant role for Fas in the TCR-mediated deletion of DP thymocytes under these conditions.

Role of endogenous TCR α rearrangement and Fas *deficiency in deletion of PCC-reactive thymocytes*

The presence of transgenic, rearranged TCR α and β genes results in an inhibition of the rearrangement of endogenous TCR α and β genes such that the majority of $\alpha\beta$ T cells carry the transgenic TCR only. Nonetheless, the inhibition of endogenous TCR rearrangement is less stringent at the TCR α locus than at the TCR β locus (28). As a result, T cells can be generated that carry the transgenic β-chain associated with the endogenous α-chain; such T cells may express two TCRs, one with an endogenous α-chain and another with the transgenic Vα11 (28–30). It is not clear whether such T cells undergo thymic deletion in response to the cognate peptide as do the transgenic T cells. To address this issue, we generated AND transgenic MRL mice with a genetically disrupted endogenous TCR α gene (TCR $\alpha^{-/-}$; Refs. 4 and 18). All $\alpha\beta$ T cells in these animals, which were backcrossed to the MRL background for six or more generations, carried the transgenic Vα11 Vβ3 TCR chains (data not shown). Using the same techniques described above, we demonstrated that the central deletion of thymocytes in TCR $\alpha^{-/-}$ AND transgenic mice was similar to that seen in mice which have an intact endogenous TCR α gene (compare Figs. 1*E* and 4). These studies suggest that in T cells that express the endogenous TCR α gene product, the transgenic TCR α gene is also functional and subject to negative selection. Due to the relatively small number of cells carrying the endogenous α gene, it is possible that the deletion of these cells is not due to TCR ligation and is instead a result of cell death and the release of toxic materials in the culture. However, this possibility seems unlikely, because the number of DP cells was similar between TCR $\alpha^{-/-}$ and TCR α intact AND transgenic MRL/*lpr* animals even when DP thymocyte death was submaximal (Figs. 3 and 5).

Since programmed cell death or apoptosis is defective in MRL/*lpr* mice, it is reasonable to question whether thymic deletion in these animals is also defective. Nonetheless, most investigators have found no significant defect in the process of thymic deletion in Fas deficiency (14, 15, 31); however, a recent report indicated an early role for Fas in the modulation of the negative selection of thymocytes (16). We addressed this question by comparing the negative selection of thymocytes in TCR $\alpha^{-/-}$ animals, which could allow us to detect small differences between the mice without the potential interference caused by expression of the endogenous TCR α genes. We found that after 24 h of in vitro culture with the PCC peptide, the degree of DP thymocyte deletion was equivalent between MRL/+ and MRL/*lpr* mice (Fig. 4); this result was also found in mice with intact endogenous TCR α genes (Fig. 1*D*).

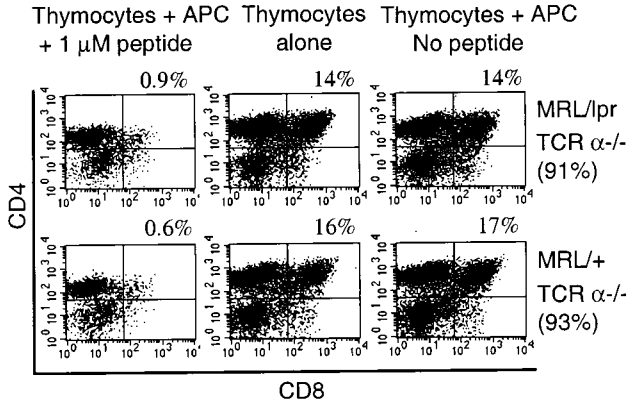


FIGURE 4. Effect of endogenous TCR α gene expression and the *lpr* mutation on DP thymocyte deletion. In these experiments, thymocytes from TCR $\alpha^{-/-}$ AND transgenic MRL/+ and MRL/*lpr* mice were removed and treated as described in the legend to Fig. 1. The numbers above each panel illustrate the percentage of DP cells; the numbers to right of the panels show the actual decrease in the number of DP cells when the PCC peptide was added. The degree of DP cell deletion was comparable with that of mice with an intact endogenous TCR α gene (see Fig. 1). Data are also shown for wells in which APCs and thymocytes were cultured together without the addition of the PCC peptide to illustrate that the presence of APCs does not influence the percentage of DP cells measured (compare *middle panels* with *right panels*). Averages of duplicate wells are shown and are representative of two separate experiments (three mice in each group).

*Down-regulation of the TCR and apoptosis of thymocytes in response to TCR stimulation in MRL/*lpr *mice is comparable with normal mice*

DP thymocytes that are destined to die as a result of exposure to a high concentration of cognate peptide down-regulate their TCR (32). To assess TCR expression, we compared MRL/*lpr* and B10.BR mice, since our earlier results showed no significant difference in DP thymocyte deletion between MRL/+, MRL/*lpr*, and B10.BR animals. In these experiments, we first incubated CH27 cells with the PCC peptide for 3 h at 37°C. Cells were then washed, fixed with 1% paraformaldehyde, and used as APCs in the presence of 1 μM of PCC peptide. After 18 h of incubation, the reduction in mean fluorescence intensity of the transgenic Vβ3$^+$ DP thymocytes in response to 1 μM of PCC peptide was as efficient in MRL/*lpr* mice as in B10.BR animals (Fig. 5*B*, 46% vs 41%, respectively). The degree of DP deletion was also similar between AND transgenic MRL/*lpr* and B10.BR animals, although in both groups of animals this deletion was less pronounced than that observed after 24 h of culture (compare Figs. 1*E* and 5*A*).

There is a slight difference in the baseline TCR expression level between MRL and B10.BR animals in this experiment. However, our experience with large numbers of these mice has not demonstrated any consistent differences in TCR expression between mice of different strains (data not shown). Moreover, the differences in expression noted here could not have affected our results, because the response to peptide exposure is measured in thymocytes from the same animals with and without addition of the peptide.

We subsequently assessed the number of apoptotic thymocytes after peptide exposure using the TUNEL assay. This system uses the ability of the enzyme TdT to incorporate biotinylated dUTP at DNA strand break points in apoptotic cells (33, 34), a method which is more sensitive than vital dyes for the detection of dying cells (23). Here we induced apoptosis in B10.BR and MRL/*lpr* thymocytes to determine whether small defects in cell death may

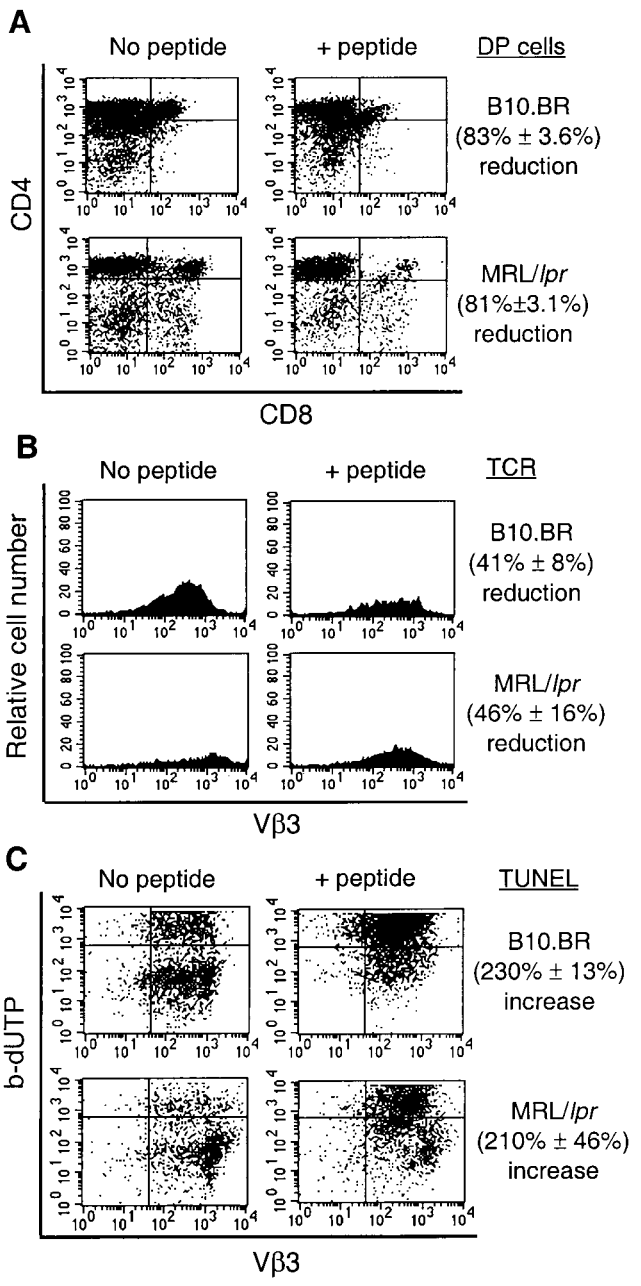


FIGURE 5. TUNEL assay to assess apoptosis in thymocytes of B10.BR and MRL/*lpr* mice. An in vitro deletion assay was set up similar to Fig. 1, except that thymocytes were removed and stained after 18 h. Following surface staining with anti-CD4, anti-CD8, and anti-Vβ3 Abs, apoptotic cells were identified using b-dUTP incorporation (TUNEL assay; see Results for details). The same region as shown in Fig. 1 was used to include the thymocytes alone. *A*, The reduction in the number of DP cells for B10.BR and MRL/*lpr* mice is indicated to the right of each panel (mean ± SE from three mice). *B*, The down-regulation of Vβ3 surface expression on DP thymocytes in response to the addition of the PCC peptide is illustrated. Numbers represent the percent decrease in mean fluorescence intensity ± SE of DP-gated cells in wells in which peptide was added compared with wells without the addition of peptide. *C*, Apoptotic, transgenic DP thymocytes characterized by high-medium surface staining with Vβ3 and incorporation of b-dUTP were increased to the same degree in the B10.BR and MRL/*lpr* mice after an 18-h culture with PCC peptide. Note that as shown in Fig. 1*B*, the TCR expression level decreases (shifts to the left) on TUNEL high thymocytes. These experiments were repeated once and represent three mice in each group. Data were combined for TCR $\alpha^{-/-}$ and TCR $\alpha^{+/+}$, because there were no notable differences between the two groups.

exist in the latter mice as a result of either the autoimmune background or the *lpr* mutation. After an 18-h culture of thymocytes without PCC, ~30% of transgenic DP cells became TUNEL-positive in both B10.BR and MRL/*lpr* animals. This number increased by an average of 2.3-fold in B10.BR and 2.1-fold in MRL/*lpr* mice after exposure to the PCC peptide (Fig. 5*C*). Such similarities between nonautoimmune and MRL mice are comparable with our findings on the number of DP cells and the down-regulation of TCR after peptide exposure. Of note, the incubation time of 18 h induced submaximal deletion of DP thymocytes in response to the dose of PCC peptide used, thus increasing the sensitivity of our assay for detecting small differences between the mice.

Our results regarding the role of Fas in the induction of apoptosis in immature thymocytes are in agreement with previous studies (14, 15, 31); however, Castro et al. recently described a significant role for Fas early on (1.5–2 h of culture) after the exposure of thymocytes to anti-TCR Abs (16). Similar results were reported by these authors when MHC class II-restricted TCR transgenic mice were exposed to cognate peptide in vivo for 4–12 h. These early effects could be blocked with Fas-Fc fusion protein. The explanation for the discrepancy between our results and those reported by these authors is likely the strength of TCR signal delivered to immature thymocytes: whereas these authors reported an ~20%-30% increase in apoptosis, we detected an ~200% increase in apoptotic cells after TCR stimulation. Thus, it seems that at an early timepoint when TCR ligation has not yet transduced a negative signal, Fas deficiency (or a blockade of Fas signaling with Fas-Fc) could result in a slow takeoff of apoptosis; however, over time, with sufficient activation of TCR signaling, the main pathways of apoptosis that are independent of Fas become dominant. Hence, the results noted by these authors are likely due to insufficient activation of the thymocytes through the TCR. To test this hypothesis, we assessed the degree of apoptosis in thymocytes stimulated with low concentrations of the PCC peptide using the TUNEL assay (see Fig. 3, legend). We found that although the increase in the number of apoptotic cells in response to exposure to 0.1 μM of peptide for 18 h was similar between the MRL/+ and B10.BR animals (an increase of 83% vs 71%, respectively), it was less for the MRL/*lpr* animals (a 26% increase, data not shown). These conclusions on the lack of a significant role for Fas in central deletion are supported by a recent report on the presence of an unidentified calcineurin- and phosphatidylinositol 3-kinase-independent pathway for TCR-CD28 induction of apoptosis in DP thymocytes (35).

Taken together, the studies presented here demonstrate that central T cell deletion in response to a conventional peptide in spontaneously autoimmune Fas-intact and Fas-deficient MRL mice is comparable with a nonautoimmune strain. Previous studies comparing central T cell tolerance in MRL and nonautoimmune mice were based on the responses of thymocytes to superantigens, a response that predominantly depends upon the interaction of the Vβ chain with MHC class II molecules outside of the Ag-binding region (36–38). In addition, superantigen exposure results in the elimination of thymocytes at the transitional stage from DP to $CD4^+$ or $CD8^+$ stage, whereas peptide Ags cause the deletion of immature DP thymocytes (9–13). These two fundamental differences in thymic deletion in response to peptide Ags vs superantigens prompted us to carry out our current studies. Using three different parameters to judge central T cell tolerance (namely, DP thymocyte deletion, TCR down-regulation, and induction of apoptosis), we found no differences between MRL and B10.BR mice in response to PCC Ag. We did find that an 18-h culture or the use of lower doses of the Ag resulted in a less efficient deletion of DP thymocytes (Figs. 3 and 5*A*), but these lower values were still equivalent between B10.BR and MRL mice. The lack of a defect in central T cell tolerance in the latter compared with a nonautoimmune strain suggests that the presumed presence and activation of autoreactive T cells in MRL mice with an intact *Fas* gene is due to defects in peripheral tolerance independent of the *Fas* defect. The basis for such defects in general are not clear but could involve a failure of one or more checkpoints (reviewed in Ref. 39).

In conclusion, our data support the concept that central T cell tolerance in spontaneously autoimmune MRL mice is intact. The autoreactive T cells that are presumed to be responsible for B cell help likely arise as a result of defects in peripheral mechanisms that normally control autoreactive T cells.

Acknowledgments

We thank Drs. Steven Hedrick (University of California, San Diego, CA) and Kim Bottomly (Yale University School of Medicine) for their gift of B10.BR and transgenic mice. We also thank Dr. Nick Crispe for his helpful remarks and careful review of the manuscript.

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