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Proliferation of CD3$^+$ B220$^-$ single-positive normal T cells was suppressed in B-cell-deficient *lpr* mice

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SUMMARY

It is known that *lpr* mice develop systemic lymphadenopathy and lupus erythematosus-like autoimmune disease that are associated with the accumulation of CD4$^-$ CD8$^-$ (double-negative; DN) CD3$^+$ B220$^+$ abnormal T cells as well as normal mature CD4$^+$ or CD8$^+$ single-positive (SP) CD3$^+$ T cells. In order to clarify the role of B cells in the lymphoproliferation and autoimmunity of *lpr* mice, we created B-cell-deficient C57BL/6 (B6) *lpr* mice (B6*lpr*/*lpr*μMT/μMT) by crossing B6*lpr*/*lpr* mice with B6μMT/μMT mice in which the B-cell development was arrested at pre-B stage owing to a targeted disruption of the immunoglobulin μ heavy-chain gene locus. In the B-cell-deficient B6-*lpr* mice, both lymphadenopathy and splenomegaly were markedly suppressed. Although the accumulation of both CD3$^+$ B220$^-$ SP normal T cells and CD3$^+$ B220$^+$ DN abnormal T cells was inhibited in the B-cell-deficient *lpr* mice, the decrease in numbers of CD3$^+$ B220$^-$ SP normal T cells occurred more strikingly than that of the CD3$^+$ B220$^+$ DN abnormal T cells. Glomerulonephritis did not develop in the B-cell-deficient *lpr* mice over 40 weeks. The present results indicate that the B cells thus play a crucial role in the extensive proliferation of normal CD3$^+$ B220$^-$ mature SP T cells rather than the accumulation of abnormal DN T cells.

INTRODUCTION

Mice homozygous for the *lpr* (lymphoproliferation) allele tend to develop extensive lymphadenopathy and systemic lupus erythematosus (SLE)-like autoimmune disease.[1–3] In the *lpr* mice, lymphadenopathy is associated with the accumulation of CD3$^+$ B220$^+$ CD4$^-$ CD8$^-$ (double-negative; DN) abnormal T lymphocytes as well as CD3$^+$ B220$^-$ and CD4$^+$ or CD8$^+$ single-positive (SP) mature T cells.[4,5] *lpr* mice have been shown to have a mutation in the *Fas* gene, where a retroviral transposon is inserted into the gene's second intron which interferes with the transcription of the *Fas* gene.[6] This mutation causes an impaired Fas-protein expression, thus resulting in the defect of Fas-mediated apoptosis.[7–9] Although the positive or negative selection of thymocytes is not affected in *lpr* mice,[10,11] the peripheral clonal deletion of autoreactive T cells and the elimination of activated T cells are impaired.[12] It was therefore suggested that the development of lymphoproliferation and autoimmune disease in *lpr* mice is due both to the impaired clonal deletion of autoreactive T cells in peripheral lymphoid organs and to the incomplete elimination of activated T cells which have responded to foreign antigens.[7]

The significant role of T cells in the pathogenesis of lymphadenopathy and autoimmune inflammation in *lpr* mice has previously been extensively documented. In MRL-*lpr* mice, lymphadenopathy and autoimmune inflammation were suppressed by thymectomy,[13,14] or by treatment with anti-Thy-1.[15] These data suggest that T cells play a central role in the pathogenesis of lymphoproliferation and in the autoimmunity of *lpr* mice. The development of autoimmunity was also blocked by crossing MRL-*lpr* mice with CD4 knock-out mice,[16] in major histocompatibility complex (MHC) class II knock-out mice,[17] where CD4$^+$ SP T cells are missing, or in β_2-microglobulin-deficient *lpr* mice lacking CD8$^+$ SP T cells.[18–20] Since β_2-microglobulin knock-out mouse is not only deficient in class I MHC but also in FcRn expression, the lack of autoimmunity in β_2-microglobulin knock-out mice might be due to lack of the FcRn receptor which regulates antibody levels *in vivo*. However, lymphadenopathy was not altered in these animals,[16–20] suggesting that lymphadenopathy

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Abbreviations: DN, double negative; lpr, lymphoproliferation; MHC, major histocompatibility complex; PCR, polymerase chain reaction; SP, single positive.

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disappeared in *lpr* mice missing whole T cells, but was still evident if either CD4 SP or CD8 SP were present.

In contrast, the role of B cells in the pathogenesis of the lymphoproliferation and autoimmunity of *lpr* mice was less clear. The treatment of MRL-*lpr* mice with anti-IgM antibodies from birth in order to eliminate B cells abolished the autoantibody production and reduced lymphadenopathy, but renal mononuclear cell infiltration with moderate lymphoproliferation was observed.[21] It was reported that in B-cell-deficient *lpr* F_2 mice with mixed genetic background of 129/Sv and C57BL/6, had no signs of autoimmunity, although the development of lymphadenopathy was less affected.[22] While these studies suggest a possible role of B cells in the autoimmunity of *lpr* mice, the role of B cells in the proliferation of lymphocytes in *lpr* mice remains unclear. It has been suggested that *lpr* mutation is not the single cause for the autoimmune disease in the MRL-*lpr* mice, since the inbred strain MRL +/+ mice develop late onset autoimmune syndrome. Also, homozygous *lpr* mutation in a B6-background resulted in a less severe autoimmune system than that seen with the MRL-background.[23] In addition, several studies have also suggested that autoimmunity and lymphoproliferation were affected by different genetic components in other autoimmune-prone strains.[24,25] Since B-cell-deficient *lpr* mice with the mixed background of 129/Sv, C57BL/6 and MRL mice were used in a previous study,[22] the results regarding the lymphoproliferation may be complicated by the influence of the autoimmune MRL genetic background. In the present study, to rule out the influence of the MRL genetic background in studying the role of B cells in the lymphoproliferation of *lpr* mice, we created B-cell-deficient *lpr* mice with a B6 genetic background after an eighth generation back-cross. In the B-cell-deficient *lpr* mice, lymphadenopathy was markedly suppressed. In addition, the accumulation of normal $CD3^+$ $B220^-$ SP mature T cells was remarkably suppressed compared to the decrease in the cell numbers of abnormal $B220^+$ DN T cells. These results demonstrate that B cells play a crucial role in the expansion of normal $CD3^+$ $B220^-$ SP mature T cells in *lpr* mice, whereas the proliferation of abnormal $CD3^+$ $B220^+$ DN T cells was less dependent upon the presence of B cells.

MATERIALS AND METHODS

Mice

C57BL/6-*lpr* (B6-*lpr*) mice were purchased from the Japan SLC Experimental Animals Inc., Shizuoka, Japan. The μMT mice with a mixed genetic background of 129/Sv and B6 strain[26] were back-crossed to B6 for eight generations. An F_8 heterozygous intercross (B6-μMT) should be more than 99% homozygous for the B6 genetic background. By crossing the B6-*lpr* mice and the B6-μMT mice, we first obtained heterozygous B6-*lpr*-μMT mice (B6-*lpr*/+ μMT/+) and then homozygous mice (B6-*lpr*/*lpr*μMT/μMT). The mice were screened for μMT and *lpr* alleles by a polymerase chain reaction (PCR). All mice were bred under specific pathogen-free conditions.

PCR-mediated detection of mutant alleles: genomic DNAs were isolated from the tail

To detect the μMT allele, PCR was performed with a primer set, comprising P-1988 (5′ TACAGCTCAG-CTGTCTGTGG 3′) and P-neor (5′ TCTATCGCCT-TCTTGACGAG 3′), which produced a 477-base pair (bp) DNA fragment. Another primer set, P-3542 (5′ CTGTCTTGCTTGCTCTGCTG 3′) and P-1988, was used to amplify the fragment from a wild-type allele. The PCR was performed at 95° for 60 seconds, 65° for 60 seconds and 72° for 90 seconds, for 40 cycles on a DNA thermal cycler. The PCR products were analysed by 2% agarose gel electrophoresis with ethidium bromide staining. To detect the *lpr* allele of the *Fas* gene, a primer set, P-13 (5′ CAGAGATG-CTAAGCAGCAG 3′) and LINS-2 (5′ CAGTCCGTTG-CTCCGATGT 3′) was used to produce a 580-bp fragment.[27] The PCR was performed at 94° for 45 seconds, 62° for 30 seconds and 72° for 60 seconds, for 40 cycles. Primer set, P-14 (5′ CAGAGATG-CTAAGCAGCAG) and P-13, produced a 330-bp fragment from the wild-type *Fas* gene allele,[27] after PCR was performed at 94° for 45 seconds, at 60° for 60 seconds and 72° for 90 seconds, for 40 cycles.

Flow cytometric analysis

The spleens, thymuses and the largest cervical lymph nodes (LN) from B6-*lpr*/*lpr*μMT/μMT, B6-*lpr*/*lpr*μMT/+, B6μMT/μMT and B6μMT/+ mice were removed at 15, 18, 21 and 24 weeks of age, and then the wet weights of these tissues were measured. Single-cell suspensions were prepared from these tissues and nucleated cells were counted. The cells were stained with various combinations of phycoerythrin (PE)-labelled anti-CD4 (Pharmingen, San Diego, CA), fluorescein isothiocyanate (FITC)-labelled anti-CD8α (Pharmingen), PE-labelled anti-B220 (Dainippon, Osaka, Japan), FITC-labelled anti-IgM (Cappel), FITC-labelled anti-B220 (Pharmingen), biotinylated anti-CD3ε (Pharmingen) and biotinylated anti-Thy-1.2 (Pharmingen) monoclonal antibodies. The cells were washed with phosphate-buffered saline (PBS) containing 2% fetal bovine serum and 0·1% NaN_3, followed by streptavidin–RED670 (Gibco, Grand Island, NY) staining. The cells were then fixed with 1% paraformaldehyde, and analysed by a flow cytometer.

Histological analysis

Blocks of kidney were obtained at autopsy and fixed with formalin, embedded in paraffin. Sections 4 μm thick were cut and were stained with either haematoxylin and eosin (H&E) staining or a periodic acid Shiff (PAS) reagent.

RESULTS

Generation of B-cell-deficient *lpr* mice with a B6 background

To generate *lpr* mice lacking B cells with a pure genetic background, we first back-crossed μMT mice to B6 mice. The μMT mice have a targeted insertion of a neomycin-resistant (neor) gene in the first membrane exon of the immunoglobulin heavy-chain μ constant region gene and homozygous (μMT/μMT) mice lack B cells due to an arrest of early B-cell development.[26] After eight generations of the back-cross, the resultant heterozygous mice (B6-μMT/+) were intercrossed with B6-*lpr* mice. The offspring of this intercrossing were then genotyped for μMT and *lpr* alleles by PCR as described in the Materials and Methods (Fig. 1a,c). The absence of B220-positive B cells in the peripheral blood of *lpr*/*lpr*μMT/μMT mice was confirmed by a flow cytometric analysis (Fig. 1b).

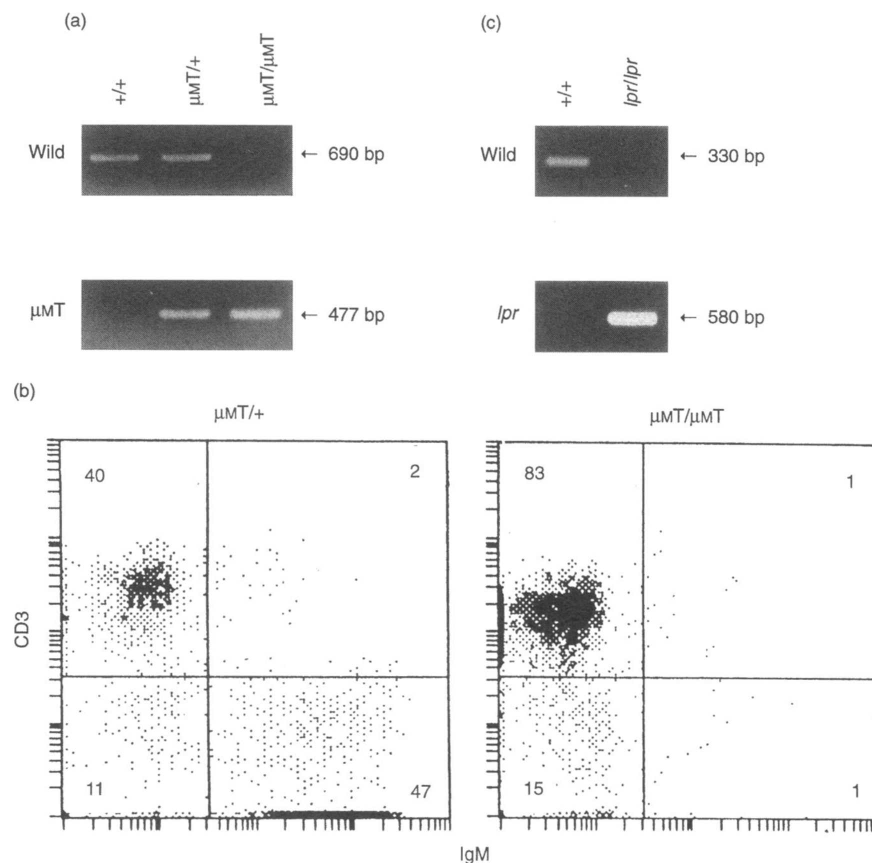

Figure 1. Genotyping of *lpr/lpr*μMT/μMT mice. (a) Genotyping of μMT-allele by PCR. Genomic DNAs were isolated from the tail. To detect the μMT allele, PCR was performed with a primer set P-1988 (5′ TACAGCTCAGCTGTCTGTGG 3′) and P-neor (5′ TCTATCGCCTTCTTGACGAG 3′), which produced a 477-bp DNA fragment. Another primer set, P-3542 (CTGTCTTGCTTGCTCTGCTG 3′) and P-1988, was used to amplify the fragment from a wild-type allele. The PCR was performed at 95° for 60 seconds, 65° for 60 seconds and 72° for 90 seconds, for 40 cycles on a DNA thermal cycler. The PCR products were analysed by 2% agarose gel electrophoresis with ethidium bromide staining. (b) B-cell-deficient phenotype of B6-*lpr/lpr*μMT/μMT mice. Peripheral lymphocytes were stained with FITC-anti-IgM and biotinylated-anti-CD3, and then analysed by flow cytometry. Mice with μMT/μMT did not possess lymphocytes with positive staining for IgM. (c) Genotyping of *lpr* alleles by PCR. To detect the *lpr* allele of the *Fas* gene, a primer set, P-13 (5′ CAGAGATGCTAAGCAGCAG 3′) and LINS-2 (5′ CAGTCCGTTGCTCCGATGT 3′) was used to produce a 580-bp fragment. The PCR was performed at 94° for 45 seconds, 62° for 30 seconds, and 72° for 60 seconds, for 40 cycles. Primer set P-14 (5′ CAGAGATGCTAAGCAGCAG) and P-13, produced a 330-bp fragment from the wild-type *Fas* gene allele, after PCR was performed at 94° for 45 seconds, at 60° for 60 seconds and 72° for 90 seconds, for 40 cycles.

Suppression of lymphadenopathy and splenomegaly in B-cell-deficient *lpr* mice

At 12–15 weeks of age, the B6-*lpr/lpr*μMT/+ mice began to develop lymphadenopathy which thereafter progressed. At 21 weeks of age, severe lymphadenopathy was observed in B6-*lpr/lpr*μMT/+ mice (Fig. 2). By contrast, in B6-*lpr*μMT/μMT mice, the development of lymphadenopathy was markedly suppressed (Fig. 2). The weight of the largest cervical LN of B6-*lpr/lpr*μMT/+ mice at 16–18 weeks of age was 104·5 ± 12·0 mg, while that of the B6-*lpr/lpr*μMT/μMT mice was 16·5 ± 6·5 mg (Table 1). Similarly, the weight of the spleens of the B6-*lpr/lpr*μMT/μMT mice (59·3 ± 7·4 mg) was markedly lower than that of the B6-*lpr/lpr*μMT/+ mice (209·8 ± 36·7 mg) (Table 2), although that of the B6-*lpr/lpr*μMT/μMT mice (59·3 ± 7·4 mg) was still a little higher than that of the B6μMT/+ mice without *lpr* mutation (33·0 ± 6·6 mg). Enlargement of spleens was also significantly suppressed in the B-cell-deficient *lpr* mice (Table 2). The cell-contents of the LN and spleens of the B-cell-deficient *lpr* mice were consistently markedly fewer than those of the *lpr* μMT/+ mice irrespective of the age studied (Tables 1 and 2). The accumulation of $CD3^+$ $B220^+$ $CD4^-$ $CD8^-$ DN abnormal T cells was observed in both LN (Fig. 3) and spleens (Fig. 4) of B6-*lpr/lpr*μMT/+ mice. Although the number of such DN abnormal T cells was reduced in both the LN (Table 1) and the spleens (Table 2) in B-cell-deficient *lpr* mice, the proportions of this abnormal T-cell population remained high (Figs 3 and 4). Normal SP T cells also accumulated in the LN and spleens of the B6-*lpr/lpr*μMT/+ mice (Tables 1 and 2). Surprisingly, the number of normal SP T cells drastically decreased in B6-*lpr*μMT/μMT mice.

Cell surface phenotyes of peripheral lymphoid cells

To investigate which population of T cells, either normal SP T cells or abnormal T cells, was preferentially affected in B-cell-

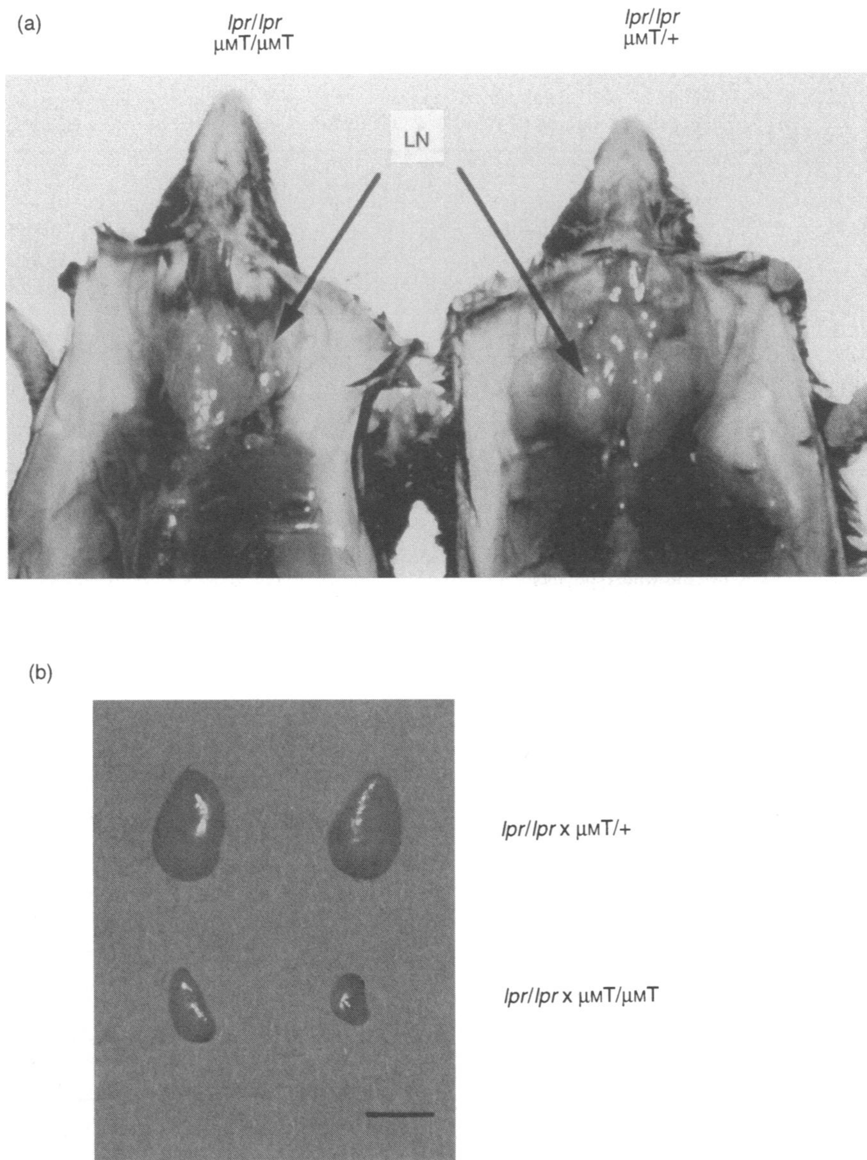

Figure 2. Suppression of lymphoproliferation in B-cell-deficient *lpr* mice. (a) At 21 weeks of age, severe lymphadenopathy was apparent in the B6-*lpr*/*lpr*μMT/+ mice (right). In contrast, lymphadenopathy was markedly suppressed in B-cell-deficient B6-*lpr*/*lpr*μMT/μMT mice (left). (b) Axillary LN of B6-*lpr*/*lpr*μMT/+ mice (top) and B6-*lpr*/*lpr*μMT/μMT mice at 21 weeks of age (bottom). In Fig. 2(b) the bar indicates 10 mm.

deficient *lpr* mice, we calculated the ratio of $CD3^+$ $B220^+$ abnormal T cells to $CD3^+$ $B220^-$ normal T cells in the LN. In the B6-*lpr*/*lpr* mice with B cells, $CD3^+$ $B220^+$/$CD3^+$ $B220^-$ ratio was $0{\cdot}60 \pm 0{\cdot}2$ at 16–18 weeks and $3{\cdot}9 \pm 1{\cdot}8$ at age of 21–24 weeks, whereas in B-cell-deficient *lpr*/*lpr* mice, this ratio was $2{\cdot}6 \pm 0{\cdot}6$ and $9{\cdot}1 \pm 3{\cdot}0$, respectively (Table 1), thus indicating that the expansion of normal T cells was more strikingly suppressed than that of abnormal T cells. A similar increase in the $CD3^+$ $B220^+$/$CD3^+$ $B220^-$ ratio was observed in the spleens of the B-cell-deficient mice (Table 2). It was thus collectively suggested that the proliferation of $CD3^+$ $B220^-$ normal SP T cells was more significantly suppressed than that of DN abnormal T cells in the B-cell-deficient *lpr* mice. Conversely, these results indicate that B cells are obligatory for the enhanced proliferation of normal SP rather than of abnormal DN T cells in *lpr* mice.

The ratio of $CD4^+$ $B220^-$/$CD8^+$ $B220^-$ cells in the LN of 24-week-old mice was also similar between the control *lpr* mice and B-cell-deficient *lpr* mice (1·31–1·36; Table 3), thus suggesting that the expansion of both $CD4^+$ and $CD8^+$ SP normal T cells was similarly suppressed by the absence of B cells.

Analysis of thymus

Since both the swelling of intrathoracic LN and the adhesion of thymuses with the LN were prominent, we carefully took the thymuses after removing the intrathoratic LN. After comparing the B6-*lpr*/*lpr*μMT/+ mice with the B6-*lpr*/*lpr*μMT/μMT mice, we could not observe any remarkable difference in either the weights or cell counts of the thymi between these animals (Table 4). We further analysed the

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Table 1. Analysis of lymph nodes

	Genotype*								
	lpr	μMT	*n*	Weight (mg)	Total cell counts (× 10^6)	CD3$^+$B220$^+$	CD3$^+$B220$^-$	B220$^+$IgM$^+$	CD3$^+$B220$^+$/ CD3$^+$B220$^-$
16–18-week old mice									
B6	+/+	μMT/+	3	2·8±1·0	1·3±0·1	0·0±0·0	1·0±0·2	0·3±0·1	0·0±0·0
B6	+/+	μMT/μMT	3	2·1±0·4	1·0±0·6	0·0±0·0	0·9±0·5	0·0±0·0	0·0±0·0
B6	lpr/lpr	μMT/+	4	104·5±12·0	133·9±36·1	36·8±14·6	61·1±12·8	30·7±13·6	0·6±0·2
B6	lpr/lpr	μMT/μMT	4	16·5±6·5	30·3±11·0	20·9±7·2	8·8±4·2	0·1±0·1	2·6±0·6
21–24-week old mice									
B6	+/+	μMT/+	3	2·6±1·3	1·4±0·4	0·1±0·1	1·0±0·3	0·3±0·1	0·1±0·1
B6	+/+	μMT/μMT	3	1·8±0·3	1·0±0·4	0·1±0·1	1·6±0·4	0·0±0·0	0·0±0·0
B6	lpr/lpr	μMT/+	5	128·5±28·7	143·2±13·5	59·3±8·5	20·7±4·9	57·5±9·8	3·9±1·8
B6	lpr/lpr	μMT/μMT	4	8·6±1·6	7·3±2·6	7·0±0·3	0·9±0·3	0·1±0·1	9·1±3·0

The cell numbers were calculated as total cell counts × (percentage of the cells with surface markers as indicated/100). All numbers were expressed as the mean ± SEM.

The ratio of CD3$^+$B220$^+$ to CD3$^+$B220$^-$ was expressed as the cell counts of CD3$^+$B220$^+$ cells/the cell counts of CD3$^+$B220$^-$ cells.

*Genotype was determined by PCR for the wild-type allele, *lpr* and μMT. The wild-type allele was expressed as +.

Table 2. Analysis of spleens

	Genotype*								
	lpr	μMT	*n*	Weight (mg)	Total cell counts (× 10^6)	CD3$^+$B220$^+$	CD3$^+$B220$^-$	B220$^+$IgM$^+$	CD3$^+$B220$^+$/ CD3$^+$B220$^-$
16–18-week old mice									
B6	+/+	μMT/+	3	82·6±2·7	81·9±10·3	0·2±0·1	38·6±3·4	31·9±11·2	0·0±0·0
B6	+/+	μMT/μMT	3	33·0±6·6	19·1±3·7	0·1±0·1	16·9±3·4	0·2±0·1	0·0±0·0
B6	lpr/lpr	μMT/+	4	209·8±36·7	169·1±42·0	35·4±11·8	45·1±12·6	80·4±18·9	0·8±0·1
B6	lpr/lpr	μMT/μMT	4	59·3±7·4	52·4±9·9	24·7±7·0	23·2±2·7	0·1±0·0	1·1±0·2
21–24-week old mice									
B6	+/+	μMT/+	3	58·1±2·5	65·0±4·9	0·7±0·1	20·4±2·7	38·8±3·3	0·0±0·0
B6	+/+	μMT/μMT	3	44·5±3·4	25·0±3·8	0·4±0·1	21·7±3·6	0·3±0·2	0·0±0·0
B6	lpr/lpr	μMT/+	5	147·2±5·1	295·4±30·2	24·1±3·9	106·9±13·6	136·8±13·7	0·2±0·0
B6	lpr/lpr	μMT/μMT	4	54·6±4·5	59·1±7·3	26·0±4·5	26·8±4·1	0·4±0·2	1·0±0·2

The cell numbers were calculated as total cell counts × (percentage of the cells with surface markers as indicated/100). All numbers were expressed as the mean ± SEM.

The ratio of CD3$^+$B220$^+$ to CD3$^+$B220$^-$ was expressed as the cell counts of CD3$^+$B220$^+$ cells/the cell counts of CD3$^+$B220$^-$ cells.

*Genotype was determined by PCR for the wild-type allele, *lpr* and μMT. The wild-type allele was expressed as +.

single cell suspensions from the thymi by a flow cytometric analysis. The percentages and number of Thy-1$^+$ CD4$^+$ CD8$^+$ DP T cells, and Thy-1$^+$ CD4$^+$ CD8$^-$ or Thy-1$^+$ CD4$^-$ CD8$^+$ SP T cells, were also not markedly different between the control mice (B6 μMT/+) and the *lpr* mice (B6-*lpr*/*lpr*μMT/+). Thy-1$^+$ CD4$^-$ CD8$^-$ DN T cells were similarly observed in both μMT/+ *lpr* mice and B-cell-deficient *lpr* mice, representing 1–3% of the total number of thymocytes (Fig. 5). These results indicate that the number of abnormal DN T cells in the thymi of B6-*lpr* mice was very small, if any, and that the absence of B cells did not significantly alter the proportions of the T-cell subsets in the thymus.

Histological examination of the kidney

In H&E staining, mild to moderate glomerulonephritis was observed in the B6-*lpr*/*lpr*μMT/+ mice at the age of 36 weeks. In addition, significant accumulation of lymphocytes around vessels was observed (Fig. 6a) in a control *lpr* mouse, while no lymphocytic infiltration was present (Fig. 6b) in a B-cell-deficient *lpr* mouse. In a control *lpr* mouse, basal membrane thickening and mesangial hypercellularity were evident (Fig. 6c) as revealed by periodic acid Schiff staining, whereas no lesions were detectable in a B-cell-deficient *lpr* mouse (Fig. 6d). These observations thus indicate that B cells are necessary for the development of glomerulonephritis in *lpr* mice.

DISCUSSION

In the present study, to define the role of B cells in the lymphoproliferation associated with lymphadenopathy in *lpr* mice, we generated and analysed *lpr*/*lpr*μMT/μMT mice with a B6 genetic background and B-cell deficiency. We found in the B-cell-deficient *lpr* mice that: (i) lymphadenopathy and splenomegaly were markedly suppressed; (ii) the expansion of

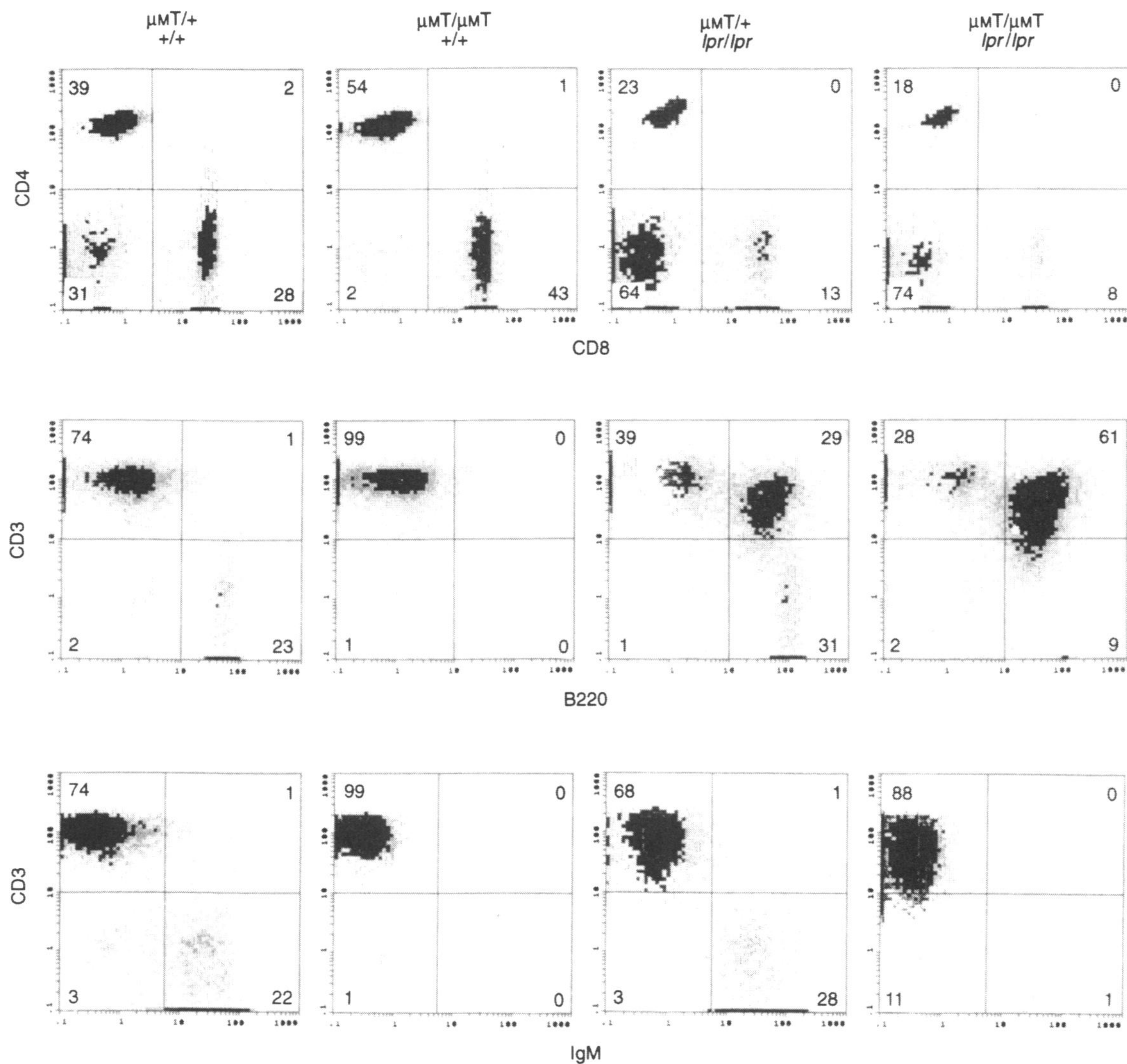

Figure 3. Flow cytometric analysis of LN. Single cell suspensions of axillary LN from 18-week-old mice of the indicated genotypes were stained with PE-labelled anti-CD4 (pharmingen), FITC-labelled anti-CD8α (Pharmingen), PE-labelled anti-B220 (Dainippon Laboratories), FITC-labelled anti-IgM (Cappel), and biotinylated anti-CD3ε (Pharmingen) monoclonal antibodies, and analysed by flow cytometry. The dot plot profiles of the cells are shown falling in a lymphocyte-gated population defined by both a forward- and side-scatter analysis. The percentages of the gated population in quadrants are noted. Note that the $CD3^+$ $B220^+$ DN T cells were present in the LN of both the control B6-*lpr*/*lpr*μMT/+ mice and B-cell-deficient B6-*lpr*/*lpr*μMT/μMT mice.

both $CD3^+$ $B220^-$ SP normal T cells and $CD3^+$ $B220^+$ abnormal DN T cells was suppressed; (iii) however, the expansion of $B220^-$ normal SP T cells was suppressed more strikingly than in $B220^+$ abnormal DN T cells; (iv) the expansion of both $CD4^+$ and $CD8^+$ normal SP T cells was equally suppressed in the lymph nodes; and (v) the development of glomerulonephritis was prevented.

Although it has been suggested that T cells play an important role in the development of autoimmunity in *lpr* mice, the role of B cells in the lymphoproliferation remains unclear.[21,22] The treatment of *lpr* mice from birth with anti-IgM antibodies to eliminate B cells inhibited the development of glomerulonephritis but did not significantly prevent the development of systemic vasculitis and lymphadenopathy.[21] Shlomchik *et al.* made B-cell-deficient *lpr* mice with a mixed background of 129/SV, C57BL/6 and MRL mice and reported that the development of lymphadenopathy was not inhibited in 4·5-month-old mice but was suppressed in 5·5–6-month-old mice.[22] The study could not rule out the possible contribution of an MRL autoimmune genetic backgound in the lymphoproliferation phenotype. In the present study, we generated *lpr* mice with a B6 genetic background to rule out the possible contribution of the autoimmune MRL genetic background. In contrast to the previous report, the development of lymphadenopathy was prevented in B-cell-deficient *lpr* mice regardless of their age, suggesting that B cells are crucial for the development of lymphoproliferation in *lpr* mice. In the previous study, Schlomchik *et al.* was unable to clarify the mechanism of suppression of autoimmunity in B-cell-deficient *lpr* mice. Surprisingly, as shown by this study, the accumulation of normal SP T cells was suppressed more strongly than that of abnormal DN T cells, which led to the complete suppression of lymphadenopathy and autoimmunity in *lpr* mice. The finding contrasted with the previous rationale in which

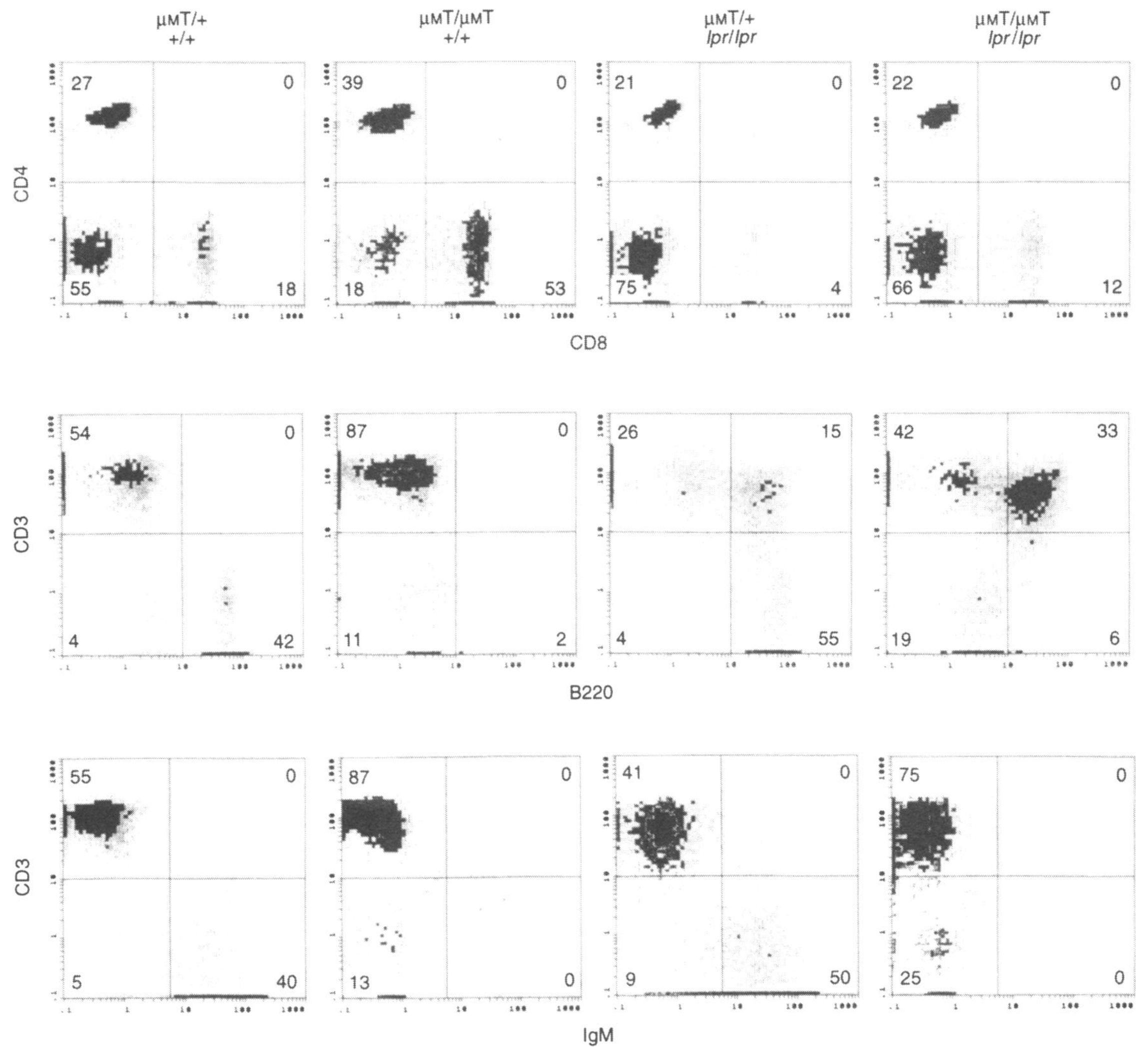

Figure 4. Flow cytometric analysis of spleen cells. CD3$^+$ B220$^+$ DN T cells were present in the spleen of both the control B6-*lpr/lpr*μMT/+ mice and B-cell-deficient B6-*lpr/lpr*μMT/μMT mice. Single cell suspensions of spleen cells from 18-week-old mice of the indicated genotypes were stained with PE-labelled anti-CD4 (pharmingen), FITC-labelled anti-CD8α (Pharmingen), PE-labelled anti-B220 (Dainippon Laboratories), FITC-labelled anti-IgM (Cappel), and biotinylated anti-CD3ε (Pharmingen) monoclonal antibodies, and then were analysed and displayed.

Table 3. CD4/CD8 ratio in lmyph nodes

	Genotype*			24-week-old mice				
	lpr	μMT	*n*	CD$^+$B200$^+$ (%)	CD8$^-$B220$^+$ (%)	CD4$^+$B220$^-$ (%)	CD8$^+$B220$^-$ (%)	CD4$^+$B220$^-$/CD8$^+$B220$^-$
B6	+/+	μMT/+	2	0·9	0·5	40·7	32·7	1·30
B6	+/+	μMT/μMT	2	0·2	0·1	51·5	45·0	1·17
B6	lpr/lpr	μMT/+	2	3·0	0·6	29·9	23·6	1·36
B6	lpr/lpr	μMT/μMT	2	3·6	0·2	16·8	16·4	1·31

All numbers represent the percentages of isolated lymphocytes as determined by a flow cytometric analysis. All data were expressed as the mean of two mice.

The ratio of CD4$^+$B220$^+$ to CD8$^+$B220$^-$ was expressed as the percentage of CD3$^+$B220$^+$ cells/the percentage of CD3$^+$B220$^-$ cells.

*Genotype was determined by PCR for the wild-type allele, *lpr* and μMT. The wild-type allele was expressed as +.

accumulation of DN T cells had been believed to play a central role in the pathogenesis of *lpr* mice.[1–3] In addition, the study revealed that the number of SP T cells in B-cell-deficient *lpr* mice was at a comparable level to that of normal B6 mice, thereby indicating that B cells are critical for the expansion of normal SP T cells in *lpr* mice. Indeed, Liu *et al.* reported that B cells are critically required for the proliferation of CD4$^+$ T cells.[28] It was reported that when mature CD4$^+$ T cells from

Table 4. Analysis of thymi

	Genotype*		16–18-week-old mice								
	lpr	μMT	*n*	Weight (mg)	Total cell counts (× 10^6)	Thy-1$^+$B220$^+$	Thy-1$^+$B220$^-$	CD4$^+$CD8$^+$	CD4$^+$CD8$^-$	CD4$^-$CD8$^+$	CD4$^-$CD8$^-$
B6	+/+	μMT/+	3	58·9±18·1	93·0±21·7	0·3±0·2	90·9±21·9	81·4±21·9	8·5±3·7	1·8±1·7	1·7±0·7
B6	+/+	μMT/μMT	3	51·7±6·9	120·0±21·5	0·2±0·1	118·2±21·9	103·4±11·6	12·1±7·4	1·8±1·1	2·0±0·6
B6	lpr/lpr	μMT/+	3	79·3±21·7	121·0±29·3	0·5±0·5	117·6±28·1	104·1±20·7	11·1±7·6	2·8±0·6	3·4±1·7
B6	lpr/lpr	μMT/μMT	3	67·0±5·8	123·1±9·0	0·2±0·1	118·8±9·1	105·6±8·8	11·1±2·3	1·4±1·4	3·3±0·8

The cell numbers were calculated as total cell counts × (percentage of the cells with surface markers as indicated/100). All numbers were expressed as the mean ± SEM.
*Genotype was determined by PCR for the wild-type allele, *lpr* and μMT. The wild-type allele was expressed as +.

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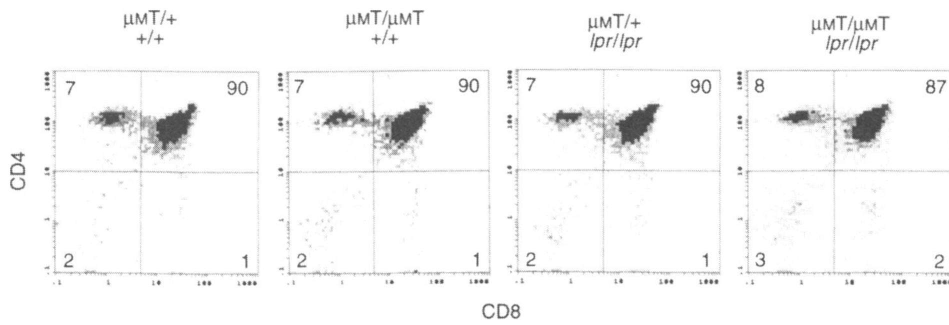

Figure 5. Flow cytometric analysis of thymocytes. Single cell suspensions of thymocytes from 18-week-old mice of the indicated genotypes were stained with PE-labelled anti-CD4 (Pharmingen), and FITC-labelled anti-CD8α (Pharmingen) monoclonal antibodies, and then analysed and displayed. No DN T cells were detected in the thymi of either control B6-*lpr*/*lpr*μMT/+ mice or B-cell-deficient B6-*lpr*/*lpr*μMT/μMT mice.

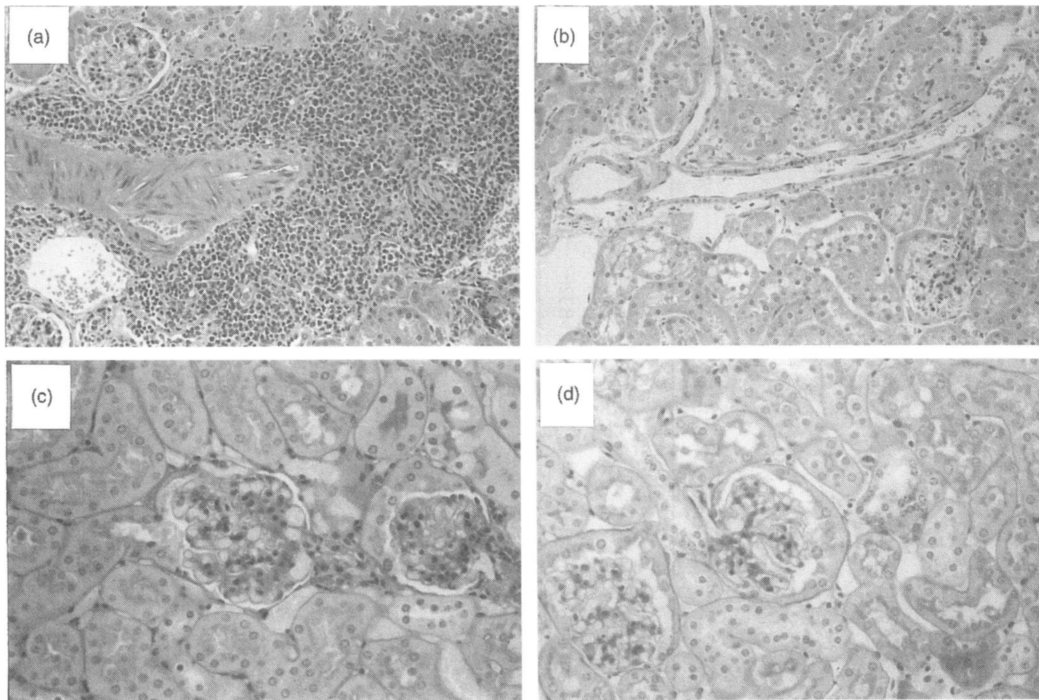

Figure 6. Representative histopathology of kidney sections in a *lpr* mouse and a B-cell-deficient *lpr* mouse at the stage of 36 weeks. (a) Renal vessel in a *lpr* (μMT/+*lpr*/*lpr*) mouse: H&E, × 400. (b) Renal vessel in a B-cell-deficinet *lpr* (μMT/μMT*lpr*/*lpr*) mouse: H&E, × 400. In a control *lpr* mouse, significant accumulation of lymphocytes around vessels was observed (a), while no lymphocytic infiltration was present (b) in the B-cell-deficient *lpr* mouse. (c) Glomeruli in a *lpr* mouse, periodic acid Schiff, ×400. (d) Glomeruli in a B-cell-deficient *lpr* mouse, periodic acid Schiff, ×400. In a control *lpr* mouse, basal membrane thickening and mesangial hypercellularity were evident, whereas no lesions were detectable in the B-cell-deficient *lpr* mouse.

lpr mice were transplanted into wild-type mice, these T cells became DN T cells, indicating that the $CD3^+$ $B220^+$ DN cells accumulating in *lpr* mice are derived from mature SP T cells.[29] The proliferation of $B220^+$ DN abnormal T cells in *lpr* mice therefore appears to be secondary to the proliferation of SP T cells which requires B cells. On the other hand, although the accumulation of abnormal T cells was also suppressed, a significant number of $CD3^+$ $B220^+$ DN cells was still observed in LN and spleen even in 21–24-week-old mice, suggesting that proliferation of $CD3^+$ $B220^+$ DN cells could be induced in the absence of B cells. Although B cells play an important role as antigen-presenting cells and in providing costimulatory signals to T cells,[30–35] it has been reported that T-cell priming normally occurs in B-cell-deficient mice and is possibly mediated by the other antigen-presenting cells, such as macrophages and dendritic cells which are intact in the B-cell-deficient mice,[36,37] indicating that T cells are well activated in the absence of B cells. In addition, peripheral T-cell tolerance also normally occurred in B-cell-deficient mice, suggesting that activation-induced cell death is operative even in the absence of B cells.[37,38] However, in *lpr* mice, the activation-induced cell death mediated by the FasL-Fas system is impaired due to the mutation of *Fas* gene, and such activated T cells may therefore be able to proliferate to some extent and accumulate in the spleens and LN in the accumulation of the SP activated T cells in the *lpr* mice may be strongly accelerated by the

presence of B cells. Since it was speculated that proliferating $CD3^+$ $B220^+$ DN T cells cells in *lpr* mice may be derived from antigen-primed T cells which escaped from activation-induced cell death,[29] the decrease in numbers of abnormal T cells in B-cell-deficient *lpr* mice may be mainly caused by the decrease of activated SP T cells. But the decrease of the abnormal T cells in B-negative *lpr* mice was not severe compared to that of normal SP T cells, indicating that proliferation of the abnormal $CD3^+$ $B220^+$ T cells is not dependent on the presence of B cells.

Although the mechanisms of the B-cell-dependent proliferation of normal SP T cells in *lpr* mice remain unclear, the B7-1-and B7-2-mediated costimulatory signals through CD28 expressed on T cells seem to play an important role in the proliferation of T cells.[34,35] In a separate study, we found that the progression of insulitis was significantly suppressed and the development of diabetes was prevented in B-cell-deficient non-obese diabetic mice (NOD),[39] thus suggesting that B cells are required for the proliferation of autoreactive T cells in NOD mice. Such B-cell-mediated costimulatory signals may be essential for the activation and/or proliferation of peripheral T cells in these autoimmune-prone mice.

Another explanation for the accumulation of T cells in *lpr* mice is that B cells might produce stimulatory antibodies and/or cytokines required for the proliferation of T cells. Autoreactive B cells may survive and continue to produce autoantibody in *lpr* mice due to the absence of Fas-mediated apoptosis after activation. Some of the autoantibodies may possibly stimulate the proliferation of T cells, as autoantibodies against thyroid stimulating hormone receptor activate thyroid cells to produce and release thyroid hormones in Basedow's disease.[40] In addition, there remains a possibility that unknown cytokines secreted by B cells and/or the ligand expressed on B cells may enhance the proliferation of T cells. Further studies are required to delineate the exact mechanisms by which B cells contribute to the expansion of normal SP T cells in *lpr* mice.

In conclusion, the present study clearly demonstrated that B cells play a significant role in the proliferation of $CD3^+$ $B220^-$ normal SP T cells which escaped from Fas-mediated apoptosis. The B-cell-deficient *lpr* mouse with a B6 genetic background is thus considered to be an excellent animal model for studying the role of B cells in the proliferation of T cells with a defect in the process of Fas-mediated apoptosis.

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