

Liver Is a Possible Site for the Proliferation of Abnormal CD3⁺4⁻8⁻ Double-Negative Lymphocytes in Autoimmune MRL-*lpr/lpr* Mice

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Summary

MRL-*lpr/lpr* mice develop a severe autoimmune disease that resembles systemic lupus erythematosus in humans. The predominant immunological feature in these mice is the development of peripheral lymphadenopathy due to the expansion of an unusual T cell subset (TCR- α/β^{+5} CD3⁺4⁻8⁻B220⁺), which may be related to the onset of their autoimmunity. However, it is unknown whether such abnormal lymphocytes proliferate in the specific organs or not. We demonstrated in the present study that the number of liver nonparenchymal mononuclear cells (MNC) in the diseased MRL-*lpr/lpr* mice was 10 times greater than that of control MRL-*+/+* mice. Moreover, the freshly isolated liver MNC of MRL-*lpr/lpr* mice vigorously proliferated in vitro and consisted of abnormal CD3⁺4⁻8⁻ lymphocytes. Such in vitro proliferation was not observed in the MNC of other peripheral lymphoid organs. A potent natural cytotoxicity was also confined to the liver MNC in MRL-*lpr/lpr* mice. In vivo injection of [³H]TdR demonstrated that liver MNC incorporated [³H]TdR; such incorporation showed a peak on day 1, and the MNC-incorporated [³H]TdR appeared in the lymph nodes as late as day 5 after the injection. These results suggest that the liver is a possible site for the proliferation of abnormal lymphocytes, which may migrate thereafter into the peripheral organs in MRL-*lpr/lpr* mice.

MRL-*lpr/lpr* (MRL/*lpr*)¹ mice develop a severe autoimmune disease that resembles SLE and rheumatoid arthritis in humans (1, 2). The predominant immunological abnormality in these mice is the development of peripheral lymphadenopathy due to the expansion of an unusual T cell subset (TCR- α/β^{+} CD3⁺4⁻8⁻B220⁺). It is well known that MRL-*+/+* (MRL/*+*) mice also develop autoimmunity when they become old (>40 wk). If *lpr/lpr* genes are inserted into these mice (i.e., MRL/*lpr*), development of severe autoimmune disease in their youth (14 wk) is accelerated, accompanied by prominent lymphadenopathy. It is, therefore, postulated that the abnormal double-negative (DN; CD4⁻8⁻) lymphocytes constituting the lymphadenopathy may be responsible for the development of autoimmunity (e.g., helper function of autoantibody production for B lymphocytes and cytotoxicity against autoantigens) (3-7). Despite enormous accumulation of abnormal lymphocytes in the lymph nodes and spleen, the cells do not actively proliferate in these organs (8). As there is also no evidence of increased proliferation of such lymphocytes in the thymus and bone marrow, it raises the possibility that this lymphadenopathy is caused by mere accumulation of such cells in the periph-

eral organs (9, 10). However, we reveal here that such abnormal lymphocytes proliferate in the liver of MRL/*lpr* mice. Although it is well established that the liver is the major hematopoietic organ in the fetal stage (11), the present results show that the liver is still an important organ for the production of certain lymphocytes after birth.

Materials and Methods

Mice. Female MRL/*lpr* and MRL/*+* mice, 5-35 wk of age, were purchased from Charles River Japan, Inc., Japan, and fed under pathogen-free conditions.

Cell Preparation. Perfusion was performed to eliminate blood contained in the liver by gentle injection of 10 ml of PBS (0.01 M, pH 7.2) into the portal vein just before removal of the liver (12). To obtain mononuclear cells (MNC), the liver was removed, cut into small pieces by scissors, pressed through 100-gauge stainless steel mesh, and suspended in serum-free RPMI 1640 medium supplemented with antibiotics. This suspension was centrifuged at 250 g for 5 min at 4°C, and the supernatant was removed. The cell pellet was resuspended in an adequate amount (~20 ml/liver) of the medium, and nonparenchymal MNC were isolated by Ficoll-Isopaque density (1.090) gradient centrifugation. In the MNC preparation method applied here, the proportion of contaminating phagocytes (i.e., Kupffer cells) was negligible (<4%), as shown previously (12). MNC in other lymphoid organs, including the lung and the intestine, were collected by the Ficoll-Isopaque method,

¹ Abbreviations used in this paper: DN, double negative; MNC, mononuclear cells; MRL/*lpr*, MRL-*lpr/lpr*; MRL/*+*, MRL-*+/+*.

after which such organs were cut into small pieces with scissors and forced through 200-gauge stainless steel mesh (12). Bone marrow cells were obtained from the femur to flush out the marrow in conjunction with the Ficoll-Isopaque method (12). Peripheral blood was collected in a heparinized syringe, and MNC were purified by the Ficoll-Isopaque method.

DNA Synthesis Assay. For determination of spontaneous proliferation, 5×10^5 freshly isolated MNC in 200 μ l RPMI medium supplemented with 1% normal mouse serum and 5×10^{-5} M 2-ME were cultured with 0.5 μ Ci of [3 H]TdR (Amersham International, Amersham, England) for 12 h in a 96-well flat-bottomed microculture plate (Falcon Labware, Oxnard, CA) at 37°C in a 5% CO₂ incubator (12).

Immunofluorescence Test. Surface phenotype of cells was identified by using mAbs in conjunction with two-color immunofluorescence tests. The monoclonal reagents used here were fluorescein- or phycoerythrin-conjugated anti-mouse CD3, CD4 (L3T4), CD8 (Lyt-2), B220, and V β 8 of TCR (F23.1) antibodies (12). The fluorescence-positive cells were analyzed by FACScan (Becton Dickinson & Co., Mountain View, CA).

Cytotoxicity Assay. Cytotoxicity was examined by a specific 51 Cr-release assay of 4-h incubation analysis (12). Target cells used in this study included a murine lymphoid tumor cell line, YAC-1. The E/T ratios examined were 50:1, 25:1, and 12.5:1.

In Vivo Injection of [3 H]TdR. To investigate whether liver MNC proliferate in vivo in MRL/lpr mice, each mouse was intravenously injected with 740 KBq (0.1 ml of PBS) of [3 H]TdR, and the level of [3 H]TdR incorporation/ 5×10^6 MNC of various lymphoid organs was sequentially analyzed. The specific activity of [3 H]TdR used here was 185 GBq/mmol.

Results

Increase in Number and Spontaneous Proliferation of Liver MNC in MRL/lpr Mice. We previously established a method for isolating liver MNC after perfusion with PBS in conjunction with Ficoll-Isopaque gradient centrifugation (12). This method was applied to the isolation of liver MNC in MRL/lpr and MRL/+ mice (Table 1). In MRL/lpr mice, the onset of disease was at the age of \sim 14 wk. The MRL/lpr and

MRL/+ mice used here were 15 wk old. It was demonstrated that the mean number ($\times 10^6$) of liver MNC (8.3 ± 1.0) in MRL/lpr mice was 10 times greater than that (0.8 ± 0.1) in MRL/+ mice. In addition to such an abnormal increase in the cell number, the [3 H]TdR incorporation/ 5×10^5 cells ($18,314 \pm 8,527$) was 10 times greater than that ($2,666 \pm 1,088$) in MRL/+ mice. The activity of cell proliferation in liver MNC of MRL/lpr mice was estimated to be \sim 100 times greater than that of control MRL/+ mice. The cell proliferation assay was then analyzed in liver MNC of MRL/lpr mice of various ages to investigate whether the above result was related to the diseased state in these mice (Fig. 1). MNC of the lymph nodes were examined in parallel. The increased spontaneous proliferation of MNC in the liver, but not the lymph nodes, was clearly related to the age of the onset of autoimmunity (Fig. 1A). Prominent proliferation remained at a high level during the active stage of their disease, although it declined gradually as a function of age. Proliferation of MNC in the lymph nodes was not observed during the entire period. The cell proliferation assay was performed in various lymphoid organs of MRL/lpr and MRL/+ mice aged 15 wk (Fig. 1B). It was demonstrated that the increased proliferation of MNC was confined to the liver among the peripheral lymphoid organs in MRL/lpr mice. MNC of the bone marrow, that is the hematopoietic organ, greatly proliferated in both MRL/lpr and MRL/+ mice.

Phenotypic Characterization of the Lymphocytes. The surface phenotype of MNC was analyzed by two-color immunofluorescence tests in various lymphoid organs of MRL/lpr and MRL/+ mice (Fig. 2). Here, MNC was simultaneously stained with fluorescein-conjugated anti-CD3 antibody and phycoerythrin-conjugated anti-CD4 and anti-CD8 antibodies to identify the CD3 $^+$ 4 $^-$ 8 $^-$ cells and CD3 $^+$ 4 $^+$ (or 8 $^+$) cells. As shown previously (3, 4) and in this study, almost all of the abnormal CD3 $^+$ 4 $^-$ 8 $^-$ DN lymphocytes coexpressed the B220 antigens. Thus, if phycoerythrin-conjugated B220 antibody was added to the above staining schedule, all of the green single-color-positive cells disappeared (data not shown).

Table 1. Increase in Number and Spontaneous Proliferation of Liver MNC in Diseased MRL/lpr Mice

Exp.	No. of liver MNC/liver		3 H]TdR Incorporation	
	MRL/lpr	MRL/+	MRL/lpr	MRL/+
	$\times 10^6$		cpm \pm SD	
1	7.5	0.7	11,620 \pm 778	3,258 \pm 213
2	8.3	0.9	18,028 \pm 162	2,806 \pm 143
3	8.0	0.7	10,878 \pm 985	1,094 \pm 133
4	7.8	0.7	32,107 \pm 4,375	3,507 \pm 103
5	10.0	ND	18,930 \pm 1,422	ND
	8.3 \pm 1.0*	0.8 \pm 0.1*	18,314 \pm 8,527*	2,666 \pm 1,088*

MRL/lpr and MRL/+ mice, aged 15 wk, were used in this experiment. Data are expressed as the mean cpm and 1 SD of triplicate cultures. * Mean \pm SD.

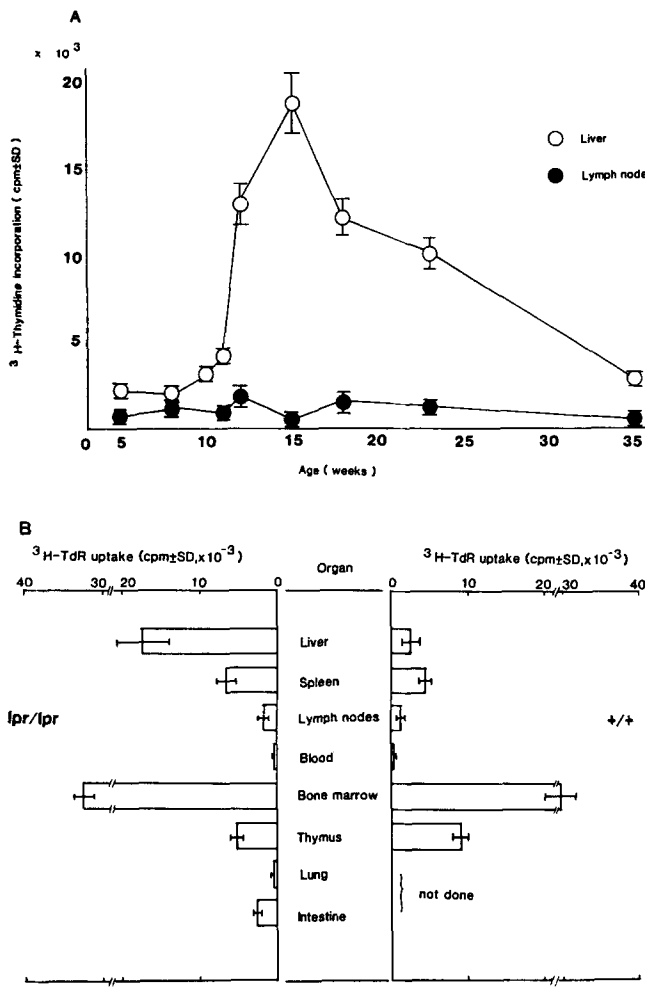


Figure 1. Spontaneous proliferation of MNC. (A) Age-dependent appearance of the spontaneous proliferation of liver MNC in MRL/lpr mice. MRL/lpr mice, aged 5–35 wk, were used in this experiment. (B) Analysis of the spontaneous proliferation of MNC from various lymphoid organs. Here, MRL/lpr and MRL/+ mice aged 15 wk were used. [³H]TdR incorporation in 5 × 10⁵ cells in a 12-h culture was analyzed.

As shown in Fig. 2 A, abnormal CD3⁺4⁻8⁻B220⁺ DN lymphocytes were detected in all peripheral lymphoid organs, including the liver, of MRL/lpr mice. Such abnormal lymphocytes were extremely rare in the thymus and bone marrow. Such lymphocytes were not detectable at all in control mice. Some of the actual profiles are shown in Fig. 2 B. Liver MNC consisted of both CD3⁺4⁻8⁻ DN lymphocytes and CD3⁺4⁺ (or 8⁺) lymphocytes, whereas MNC of the lymph nodes consisted mainly of CD3⁺4⁻8⁻ DN lymphocytes. We then analyzed the phenotype of in vitro proliferating liver MNC in MRL/lpr mice (Fig. 2 C). As we observed that liver MNC depleted of CD3⁺ cells by complement-mediated cell lysis using anti-CD3 antibody still had the ability to proliferate in vitro, the phenotype of liver MNC depleted of CD3⁺ cells was examined before and after a 3-d culturing. The liver MNC depleted of CD3⁺ cells initially lacked the CD3, CD4, CD8, and TCR- α/β antigens (<4%). After the cul-

ture, the prominently increased expression of CD3 and TCR- α/β antigens (up to 14%), but not of CD4 and CD8 antigens (see the bottom of Fig. 2 C), was demonstrated. Here, the actual increase of the cell number was ~1.5-fold for the 3-d culture.

Time Kinetics of the Appearance of MNC-incorporated [³H]-TdR in Various Lymphoid Organs. To confirm that liver MNC actually proliferated in vivo in MRL/lpr mice, each mouse was intravenously injected with [³H]TdR, and the level of [³H]TdR incorporation/5 × 10⁶ MNC of various lymphoid organs was sequentially analyzed (Fig. 3). The liver MNC were demonstrated to incorporate [³H]TdR at a level almost comparable with that of bone marrow cells, showing a peak on day 1. MNC in the spleen were shown to have a considerable level of [³H]TdR incorporation as well, whereas that of the thymus was at the basal level during the entire period tested. Of particular interest was that MNC in the lymph nodes showed a peak of radioactivity on day 5. As significant radioactivity was not detected in the serum on days 4 and 5 (Fig. 3, dotted line), such MNC-incorporated [³H]TdR in the lymph nodes most probably originates in other lymphoid organs where cell proliferation occurs. Cells that incorporated [³H]TdR were demonstrated to be CD3⁺4⁻8⁻ cells, since the complement-mediated cell lysis using both anti-CD4 and -CD8 antibodies did not reduce the radioactivity (data not shown). On the other hand, such prominent in vivo [³H]TdR incorporation was not seen in MNC from any peripheral lymphoid tissues of MRL/+ control mice. Concerning the unique results of liver MNC of MRL/lpr mice in in vitro and in vivo studies, it is conceivable that MNC-incorporated [³H]TdR in the liver might migrate to the lymph nodes.

Cytotoxicity of Liver MNC. It has been previously demonstrated that liver MNC in MRL/lpr mice have potent cytotoxic activity against tumor target cells (13). We further examined whether such high cytotoxic activity was confined to liver MNC. The cytotoxic function against YAC-1 tumor cells was examined by a specific ⁵¹Cr-release assay of a 4-h analysis. The increased cytotoxicity was restricted to the liver MNC in MRL/lpr mice (Table 2). The complement-mediated cytotoxicity by using anti-asialo GM₁ and anti-CD3 antibodies revealed that not only asialo GM₁⁺ NK cells but also CD3⁺4⁻8⁻ cells mediated cytotoxicity against the NK-resistant target (data not shown).

Discussion

In the present study, we demonstrated that the number of liver MNC in the diseased MRL/lpr mice was 10 times greater than that of control MRL/+ mice. Moreover, the isolated liver MNC of MRL/lpr mice vigorously proliferate in vitro and consisted of abnormal CD3⁺4⁻8⁻ DN lymphocytes. In a morphological study, we also observed that >70% of MNC in the hepatic sinusoid were lymphoblastic in MRL/lpr mice, whereas almost all MNC (>95%) in the lymph nodes were small resting lymphocytes (Masuda et al., manuscript in preparation). It is, therefore, presumed that liver MNC, including abnormal DN lymphocytes, are in an

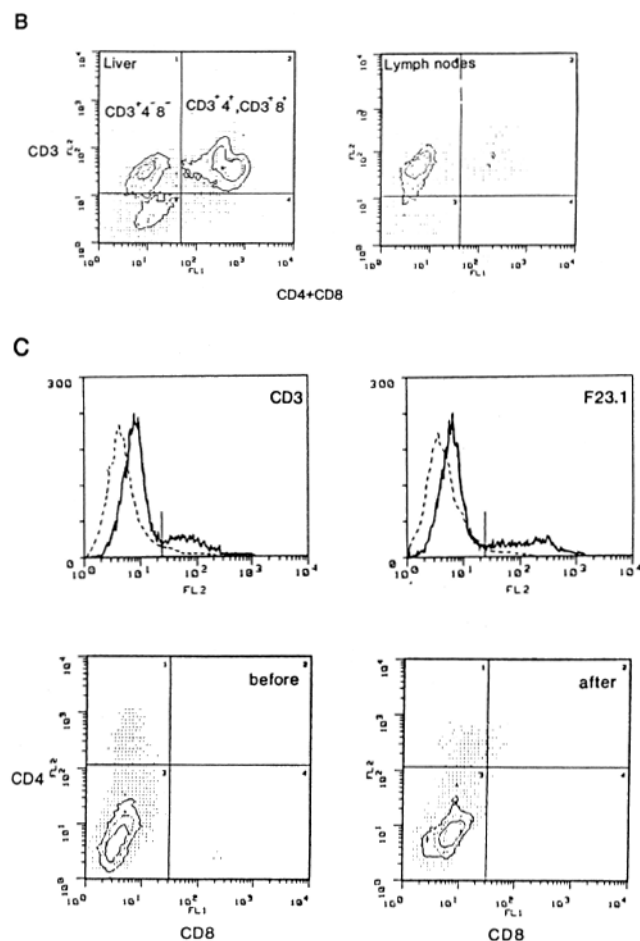
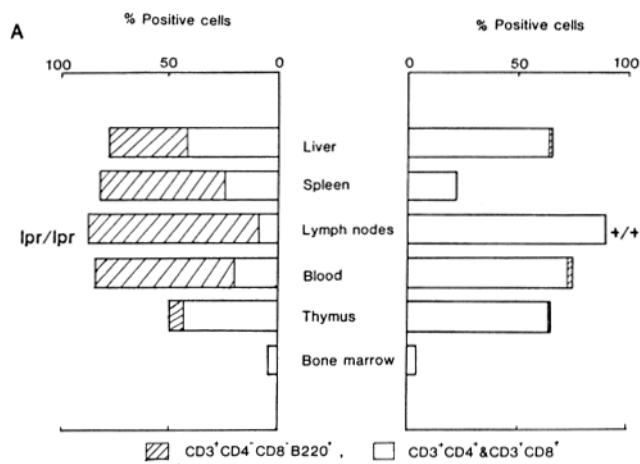


Figure 2. Analysis of surface phenotype of MNC. (A) Comparison of the distribution of $CD3^+CD4^+CD8^+B220^+$ DN lymphocytes in various lymphoid organs between MRL/*lpr* and MRL/+ mice. (B) Two-color profiles of MNC in the liver and lymph nodes from MRL/*lpr* mice. (C) Expansion of $CD3^+$ and $TCR_{\alpha\beta}^+$ cells from liver MNC in MRL/*lpr* mice; before culture (----) and after culture (—). As abnormal $CD3^+CD4^-CD8^-$ DN lymphocytes were always accompanied by the B220 antigens in MRL/*lpr* mice, we represented them as $CD3^+CD4^-CD8^-B220^+$ cells in this figure. In two-color profiles, the or-

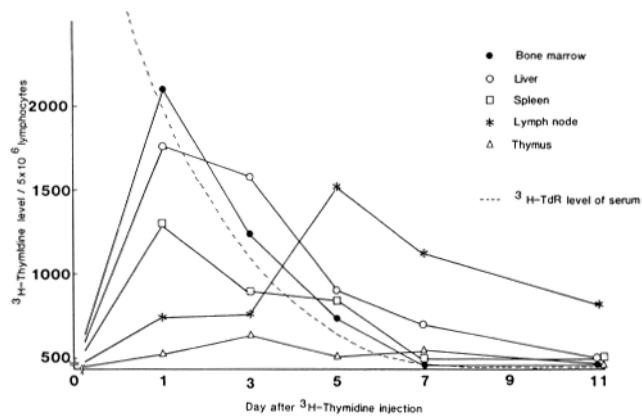


Figure 3. Time kinetics of the appearance of MNC-incorporated $[^3H]TdR$ in various lymphoid organs of MRL/*lpr* mice. 740 KBq of $[^3H]TdR$ was intravenously injected to each MRL/*lpr* mouse, aged 15 wks, and the level of $[^3H]TdR$ incorporation into 5×10^6 MNC of various lymphoid organs was sequentially analyzed up to 11 d after the injection. Each plot was produced by the mean value of MNC collected from two or three mice. The $[^3H]TdR$ levels of 2.5 μ l of serum were represented as a dotted line.

active state in terms of both cell proliferation and cytotoxic function. This raises the possibility that such cells may be responsible for the destruction of autologous cells in autoimmune disease when they migrate to the periphery. MNC in the spleen seem to have an intermediate feature between MNC in the liver and in the lymph nodes, with respect to their proliferation and cytotoxic activity. The results of *in vivo* $[^3H]TdR$ injection experiments suggest that hepatic DN lymphocytes may be a source of abnormal lymphocytes constituting the lymphadenopathy of lymph nodes.

We previously established that MNC could be isolated from liver of mice after perfusion with PBS from the portal vein, in conjunction with Ficoll-Isopaque gradient centrifugation (12). The isolated MNC mainly consisted of T lymphocytes and NK cells, but only a small proportion of Kupffer cells (<4%). When we estimated the number of hepatic MNC in a small aliquot of the mixed cell suspension (i.e., the mixture of hepatocytes and hepatic MNC) before the application of gradient centrifugation, the number of hepatic MNC was approximately five times greater than that of hepatic MNC after application of gradient centrifugation. Such a cell yield was not variable between MRL/*lpr* and MRL/+ mice. As the cell yield of liver MNC isolated by the present method was $\sim 20\%$, the actual cell number of liver MNC in the diseased MRL/*lpr* mice was $\sim 4 \times 10^7$ cells/liver. This number

dinat is indicated by green (fluorescein) and the abscissa is indicated by red (phycoerythrin). Complement-mediated cell lysis was applied to eliminate $CD3^+$, $CD4^+$, or $CD8^+$ cells in conjunction with unconjugated aliquots of the anti- $CD3$, $CD4$ (L3T4), and $CD8$ (Lyt-2) antibodies (murine IgM or IgG2b), which are complement-fixing reagents. Here, the anti- $CD3$ antibody was murine IgM and was not mitogenic for T lymphocytes.

Table 2. A comparison of the Cytotoxicity of MNC in Various Lymphoid Organs between MRL/lpr and MRL/+ Mice

Mice	Organ	Percent cytotoxicity against YAC-1 cells		
		50:1*	25:1	12.5:1
MRL/lpr	Liver	46.5	42.1	34.5
	Spleen	3.5	2.7	1.5
	Lymph nodes	10.1	8.8	7.3
	Blood	8.3	7.9	4.8
	Bone marrow	0.6	1.7	3.2
	Thymus	0.1	0.5	1.5
MRL/+	Liver	1.4	2.0	1.2
	Spleen	0.6	0.1	0.5
	Lymph nodes	0.1	0.1	0.4
	Blood	1.6	1.4	0.5
	Bone marrow	1.1	0.2	0.3
	Thymus	2.7	0.6	0.1

MRL/lpr and MRL/+ mice, aged 15 wk, were used in this experiment. Data are expressed as the mean percent cytotoxicity of triplicate assays.

* E/T ratio.

is almost comparable with that of the spleen in normal young adult mice feeding under pathogen-free conditions. On the other hand, the number of liver MNC ($\sim 4 \times 10^6$ cells/liver) in MRL/+ mice was comparable with those of other strains of mice, as shown in our previous study (12).

In the in vitro culture system, we observed that liver MNC depleted of CD3⁺ cells still had the ability to proliferate, and that the prominent increased expression of CD3 and TCR- α/β antigens, but not of CD4 and CD8 antigens, was demonstrated. TCR- α/β ⁺CD3⁺4⁻8⁻ cells, therefore, might expand from either null cell precursors or a minor remaining population of TCR- α/β ⁺CD3⁺4⁻8⁻ cells. On the other hand, we could not induce in vitro such TCR- α/β ⁺CD3⁺4⁻8⁻ cells from bone marrow cells in MRL/lpr mice. It is conceivable that, even if the earliest precursors of abnormal lymphocytes themselves come from the bone marrow (14), such lymphocytes may have to encounter hepatic microenvironments for maximum expansion.

There are several reports that neonatal thymectomy significantly reduces the lymphadenopathy and autoimmunity in MRL/lpr mice (15–17). However, there is no evidence that abnormal lymphocytes proliferate in the thymus based on the

results of the present in vitro and in vivo studies. Therefore, two possibilities can be proposed to explain this phenomenon. The first possibility is that the precursor cells of abnormal lymphocytes in the bone marrow of MRL/lpr mice pass through the thymus and proliferate in the liver. Even in this case, the thymus may not be a major site for the expansion of these cells. The second possibility is that abnormal lymphocytes in the liver may directly come from the bone marrow and be generated by entirely extrathymic proliferation. Thus, the liver MNC depleted of CD3⁺ cells in MRL/lpr mice are still able to proliferate in vitro and produce CD3⁺4⁻8⁻ DN lymphocytes. In this case, as abnormal TCR- α/β ⁺ DN lymphocytes are generated in the liver, it is easy to consider that such cells may be autoreactive due to the escape from thymic education. In recent studies, the possibility of extrathymic maturation of TCR- α/β ⁺ and TCR- γ/δ ⁺ cells has been shown (18–20). There is also an earlier report that the transplantation of the thymus from a normal mouse to a thymectomized MRL/lpr mouse did not prevent lymphadenopathy and autoimmunity (16). This experimental result also seems to support the second possibility. In this case, we prefer to consider the possibility that normal CD4⁺ (and/or CD8⁺) cells, which come from the thymus and are abundant in the liver of MRL/lpr mice (Fig. 2, A and B), may support the growth of abnormal lymphocytes in the liver, since the percentages of normal CD4⁺ and CD8⁺ cells existing in the liver, spleen, and lymph nodes correlate well with the proliferation activity of CD3⁺4⁻8⁻ cells in these organs. This conception is supported by several reports that anti-CD4 antibody treatment in vivo reduced the onset of autoimmunity in MRL/lpr mice (21, 22).

We have recently demonstrated that liver MNC, in not only aged MRL/+ mice (>40 wk of age), but also in other autoimmune mice such as BXSB and NZB/W F₁ strains, are characterized by increased cell proliferation activity accompanying the increased number of liver MNC (Ohteki et al., unpublished observation). This evidence suggests that the liver may be the most important site for proliferation of abnormal autoreactive lymphocytes in mice with a wide range of autoimmune diseases. However, the above autoimmune mice do not show prominent lymphadenopathy in the peripheral lymphoid organs. It is, therefore, conceivable that MRL/lpr mice have an additional feature, such as the accumulation of abnormal lymphocytes in the peripheral lymphoid organs, probably due to the homing abnormality of such lymphocytes, that may be responsible for the acceleration of their autoimmunity (10). As increased TCR- α/β ⁺ DN cells were also demonstrated in the patients with SLE (7), whether such lymphocytes also proliferate in the liver of patients with autoimmune diseases is under investigation.

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