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Autoimmune glomerulonephritis induced in congenic mouse strain carrying telomeric region of chromosome 1 derived from MRL/MpJ

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Summary. In lupus erythematosus-prone mice, including the BXSB, NZW and NZB strains, telomeric regions of chromosome 1 (Chr.1) contain major glomerulonephritis susceptibility loci such as *Bxs3*, *Sle1*, and *Nba2*. To assess whether strain MRL, a model for lupus erythematosus, had glomerulonephritis susceptibility loci on Chr.1, we created B6.MRLc1(82-100) congenic mice carrying MRL/MpJ Chr.1 (82-100cM) based on the C57BL/6 background and investigated renal pathology. From 6 months of age, B6.MRLc1 (82-100) showed the onset of diseases such as splenomegaly due to proliferation of CD3- or B220-positive cells, glomerular damage, and an increased serum anti-dsDNA antibody concentration, and these were earlier and severer in females. The score for glomerular damage was higher in B6.MRLc1(82-100) mice over 12 months old than in C57BL/6 or even in wild-type MRL/MpJ. Immune-complex depositions were demonstrated on glomerular basement membrane in B6.MRLc1(82-100) by immunohistochemistry and electron microscopy. For the percentage of IgG1-positive glomeruli, B6.MRLc1 (82-100) had significantly higher values than C57BL/6. In evaluations of clinical parameters, serum levels of blood urea nitrogen and the anti-dsDNA antibody in B6.MRLc1(82-100) were significantly higher than those in C57BL/6. In conclusion, B6.MRLc1(82-100) clearly developed autoimmune-mediated glomerulonephritis, and we demonstrated that MRL Chr.1 contained a novel glomerulonephritis susceptibility locus. We named this locus *Mag* (*MRL autoimmune glomerulonephritis*) and it provided new insights into the genetic basis and pathogenesis of lupus nephritis.

Key words: Autoimmunity, Chromosome 1, Glomerulonephritis, Kidney, MRL mice

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Introduction

Systemic lupus erythematosus (SLE) is a polycongenic autoimmune disease characterized by multiorgan inflammation and the production of pathogenic autoantibodies. These autoantibodies form an immune complex that can cause the glomerulonephritis (GN) called lupus nephritis. Lupus nephritis, a major cause of morbidity and mortality both in human SLE and animal lupus models, is the most serious complication of SLE (Klinman and Steinberg, 1995). As spontaneous animal models for lupus nephritis, (NZBxNZW) F1, BXSB, and MRL-Fas^{lpr} (MRL-*lpr*) mice have been extensively used to elucidate its complicated pathology (Santiago-Raber et al., 2004; Henry and Mohan, 2005). They develop fatal immune complex (IC) -mediated GN associated with the production of high titers of serum IgG antibodies (Abs) for nuclear antigens such as dsDNA (Santiago-Raber et al., 2004). On the other hand, experimental autoimmune GN, a model for human Goodpasture's disease, can be induced in Wister Kyoto (WKY) rats by immunization with the glomerular basement membrane (GBM) in an adjuvant (Reynolds et al., 2006). GN in these rats is characterized by anti-GBM Abs production, accompanied by focal necrotizing GN with crescent formation (Reynolds et al., 2006).

The MRL/MpJ mouse, generated from a series of crosses with strains C57BL/6J, C3H/HeDi, AKR/J, and LG/J, has some unique characteristics, such as

heightened wound healing and apoptosis of meiotic spermatocytes (Kon et al., 1999; Kon and Endoh, 2000; Leferovich et al., 2001; Masinde et al., 2001). In addition, the MRL-lpr mouse, a *Fas*-deficient strain derived from the MRL/MpJ, develops human SLE-like disease, characterized by skin lesions, lymphadenopathy, splenomegaly, arthritis, vasculitis and severe autoimmune GN (Andrews et al., 1978; Watanabe-Fukunaga et al., 1992). Interestingly, however, it has been suggested that the onset or severity of these symptoms is closely associated with not only the lpr allele, but also the genetic backgrounds of the mouse strains. For example, MRL-lpr mice develop GN leading to death at about 5 months of age (Cohen and Eisenberg, 1991), but renal pathology is negligible at 14 to 16 months in mice with C57BL/6 and C3H/HeJ backgrounds and homozygous for lpr (Kelley and Roths, 1985).

In recent studies, congenic and transgenic mouse strains have been mainly used to study the pathogenic mechanisms underlying autoimmune GN. Now, the efforts of researchers are focused on identification of candidate genes located within lupus susceptibility loci by investigating the renal histopathological features and the associated immunological disorders in transgenic and congenic mouse strains (Henry and Mohan, 2005). Many studies using these experimental models have suggested that the telomeric region of murine chromosome 1 (Chr.1) contains some autoimmune GN susceptibility loci such as *Bxs3* (71-99cM), *Sle1* (88cM), and *Nba2* (92-94cM) (Santiago-Raber et al., 2004).

Thus, the key to elucidate candidate genes of lupus nephritis is likely to be closely related to genetic background, especially the telomeric region of Chr.1 in murine models. In the MRL genetic background, lupus nephritis susceptibility loci were reported on chromosomes 4 and 5 (Miyazaki et al., 2005). However, although MRL strains are widely used for pathological analysis as human SLE models, analysis of the genetic background including lupus nephritis susceptibility loci in Chr.1 is not advanced. In the present study, we generated congenic mice (B6.MRLc1(82-100)) containing the telomeric region of MRL Chr.1 (82-100) and histopathologicaly demonstrated that B6.MRLc1(82-100) exhibited severe autoimune GN. This finding, with further studies of this lupus nephritis susceptibility locus (named *Mag* for *MRL autoimmune glomerulonephritis*), will provide important insights into the cause of this disease and new strategies for therapy.

Materials and methods

Creation of B6.MRLc1(82-100) congenic mice

Inbred strains, including C57BL/6 and MRL/MpJ, were purchased from an animal-breeding company (Japan SLC, Inc., Hamamatsu, Japan) and maintained in conventional conditions, feeding and drinking *ad libitum*. In the experimental animal care and handling, the investigators adhered to the "Guide for the Care and Use of Animals of the School of Veterinary Medicine of Hokkaido University." The F1 generation was produced by crossing female C57BL/6 to male MRL/MpJ mice. Female F1 mice were then backcrossed to male C57BL/6 to produce the N2 generation. N2 animals were typed for the microsatellite markers *D1Mit64, D1Mit123, D1Mit191, D1Mit200, D1Mit202, D1Mit107, D1Mit143, D1Mit15, D1Mit113, D1Mit403, and D1Mit361. D1Mit202, D1Mit107, D1Mit143, D1Mit15, D1Mit113* and *D1Mit403*, and were defined as *Mag* markers and localized on Chr.1 (81.6-100cM), which flanked murine lupus susceptibility loci reported in some previous reports (Santiago-Raber, 2004). The map positions of microsatellite loci were based on information from the Mouse Genome Database (MGD) of The Jackson Laboratory (www.informatics.jax.org/). Female mice heterozygous for *Mag* markers were bred to male C57BL/6 to produce the N3 generation. Subsequently, genotyping and backcrossing were consecutively repeated through the N16 generation, and N16 mice heterozygous for *Mag* were crossed with littermates. Finally, MRL/MpJ typed-mice homozygous for *Mag* were selected and maintained for from 5 to 10 generations by further inbreeding, and these animals were used in the present experiments.

Genome polymerase chain reaction (PCR) for microsatellite markers

Genomic DNA was prepared from the tail of each animal in inbred strains and the congenic population. These samples were incubated in lysis buffer containing 50 mM Tris-HCl, 100 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, and 100 mg/ml proteinase K, overnight at 56°C, and then treated with two-phenol extraction. Finally, genomic DNA was purified by ethanol precipitation and resolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). A total of 11 markers were used to detect polymorphisms between MRL/MpJ and C57BL/6. PCR was carried out on a Promega PCR thermal cycler (iCycler, Madison, WI) with the cycling sequence of 94°C for 5 min (one cycle), followed by 35 cycles consisting of denaturation at 94°C for 40 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. The PCR mixture and enzymes were purchased from Bioline (London, UK). The amplified samples were electrophoresed with 2–6% Nusieve 3:1 agarose gel (FMC, Rockland, IL), stained with ethidium bromide, and finally photographed under an ultraviolet lamp.

Histopathological and immunohistopathological studies of kidneys and spleens

Mouse kidneys were fixed in 10% formalin for at least 72 h at room temperature. Paraffin sections (2 μm) were stained with PAS and PAM stains. To assess the severity of glomerular damage, 100 glomeruli per kidney

were examined by using PAS stained sections and scored according to the following criteria: grade 0, no recognizable lesion in glomeruli; grade 1, a little PAS-positive deposition, mild cell proliferation, mild membranous hypertrophy, and/or partial podocyte adhesion to the parietal layer of the renal corpuscle; grade 2, segmental or global PAS-positive deposition, cell proliferation, membranous hypertrophy, and/or glomerular hypertrophy; grade 3, the same as grade 2 with PAS-positive deposition in 50% of regions of glomeruli and/or severe podocyte adhesion to the parietal layer of the renal corpuscle; grade 4, disappearance of capillary and capsular lumina, global deposition of PAS-positive material, and/or periglomerular infiltration of inflammatory cells and fibrosis. PAM-stained specimens were used to investigate the membranous lesions in detail.

For electron microscopy, kidneys from 9-month-old MRL/MpJ and B6.MRLc1(82-100) mice were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde, postfixed in osmium tetroxide, dehydrated in a graded alcohol series, and embedded in Quetol 812. Ultrathin sections (40 nm) were double stained with uranyl acetate and lead citrate.

The streptavidin-biotin (SAB) method was used for detection of immunecomplex (IC) deposits in glomerulus and splenic CD3- or B220- (as a marker for T or B cell, respectively) positive cells. Immunohistochemistry was performed using biotin-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b Abs (Caltag Laboratories, South San Francisco, CA, USA) diluted 1:100, non-labeled rabbit anti-mouse CD3 Abs (Dako Japan, Kyoto, Japan) diluted 1: 200, and non-labeled rat anti-mouse B220 Abs (Southern Biotechnology Associates, Birmingham, AL, USA) diluted 1:1600. Incubation with primary Abs was performed overnight at 4°C. After rinsing with PBS, the sections for CD3 or B220 were incubated with non diluted biotin-conjugated goat anti-rabbit IgG Abs (Nichirei, Tokyo, Japan) or biotin-conjugated goat anti-rat IgG Abs (Caltag laboratory, Burlingame, CA, USA) diluted 1:100, respectively. After incubation with SAB, immunohistochemical reactivity was detected using 3-3'-diaminobenzidine-0.003% (V/V) H_2O_2 solution. Immunoreactions were stopped in distilled water, and then sections were counterstained with Mayer's hematoxylin. For antigen retrieval, pretreatments were performed after deparaffinization. Briefly, the sections for IgG1-, IgG2a-, and IgG2b were microwaved in 10mM citrate buffer (pH 6.0), and those for CD3 and B220 were incubated in 0.4% pepsin/0.2M HCl. The numbers of IgG1-, IgG2a-, and IgG2b-positive glomeruli were counted using PAS-stained sections and immunostained sections. The total number of IgGs-positive glomeruli with the immunostained sections (A) and total number of glomeruli with PAS-stained sections seriated to each immunostaining section (B) were counted, and Ax100/B was calculated as the numerical ratio of IgGs-positive glomeruli.

Serologic analysis and urinalysis

BUN and creatinine levels in serum were determined using the BUN-test-Wako and Creatinie-test-Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively. Proteinuria of mice was detected using Albusticks (Bayer Medical Corporation, Tokyo, Japan). The level of proteinuria was transformed into a score: no or only a trace of proteinuria was given score 0, 30mg/dl of proteinuria received score +1, 100mg/dl scored +2, 300mg/dl scored +3, and 1,000 mg/dl scored +4. Plasma glucose concentrations were measured using Medisafe-Mini (Terumo, Tokyo, Japan). The serum level of anti-dsDNA Abs was measured using a commercially available ELISA kit (ALPHA DIAGNOSTIC INTERNATIONAL, San Antonio, USA), according to the manufacturer's directions.

Statistical analysis

Results were expressed as the mean ± S.E. and analyzed statistically by using nonparametric methods. The Mann-Whitney U test was used for comparing two populations ($p<0.05$). The Kruskal-Wallis test was used for comparing over three populations, and multiple comparisons were performed using Scheffé's method when a significant difference was observed ($p<0.05$).

Results

Creation of B6.MRLc1(82-100) congenic mice

Figure 1a shows the result of genotyping on Chr.1 of C57BL/6, MRL/MpJ, and B6.MRLc1(82-100) genomic DNAs by using microsatellite markers. B6.MRLc1(82-100), *D1Mit64-200* (5.0-73.0 cM) and *D1Mit361* (101.5 cM) were C57BL/6-type homozygous, and *D1Mit202-403* (81.6-100.0 cM) were MRL/MpJ type homozygous. Genomic PCR for microsatellite markers on chromosomes 2-19 and X were also performed, and B6.MRLc1(82-100) were C57BL/6 type homozygous in these chromosomes (data not shown). Figure 1b shows a diagram of B6.MRLc1(82-100) Chr.1, and presents the gene names and genetic distances based on the Mouse Genome Database (MGD). MRL/MpJ type congenic intervals included some representative immune- or cell proliferation-associated genes such as *Fas ligand (Fasl), selectin (Sel), Fc gamma receptor (Fcgr), interferon activated gene (Ifi),* and *exonuclease1 (Exo1).*

GN in B6.MRLc1(82-100) congenic mice

Figure 2 shows the results of light microscopic examination of renal cortices from C57BL/6, MRL/MpJ, and B6.MRLc1(82-100). In B6.MRLc1(82-100), glomerular damage, including mild cell proliferation and membranous hypertrophy, was clearly observed from approximately 6 months of age and deteriorated with age, whereas no significant change was observed in the

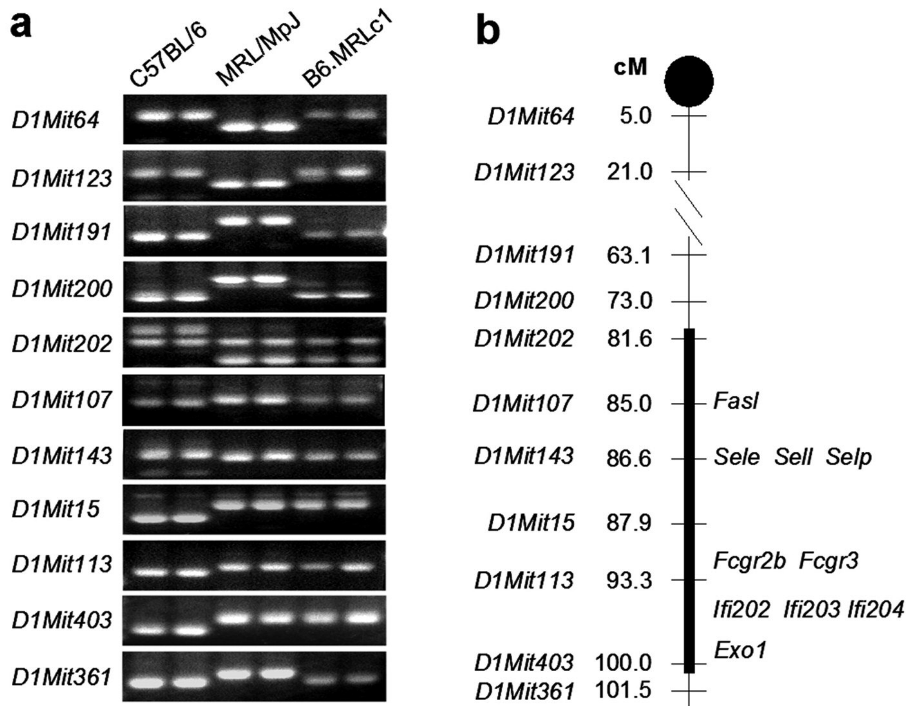

Fig. 1. Chromosome 1 of B6.MRLc1(82-100) congenic mouse. **a.** Genotyping in genomic DNA by using microsatellite markers. **b.** Schematic representation. The polymorphism between strains is detected as a size difference in the PCR products (panel a). In B6.MRLc1(82-100), *D1Mit64-200* (5.0-73.0 cM) and *D1Mit361* (101.5 cM) were C57BL/6 types, and *D1Mit202-403* (81.6-100.0 cM), defined as *Mag* markers, were MRL/MpJ types. The *Mag* markers used for genotyping are shown on the left-hand side (scale in cM, panel b). The MRL/MpJ type congenic interval is indicated by the bold line. Some immune- or cell proliferation-associated genes associated with congenic intervals, such as *Fas ligand* (*Fasl*), *selectin endotherial* (*Sele*), *selectin lymphocyte* (*Sell*), *selectin platelet* (*Selp*), *Fc gamma receptor* (*Fcgr*), *interferon activated gene* (*Ifi*), and *exonuclease1* (*Exo1*), are indicated on the right-hand side.

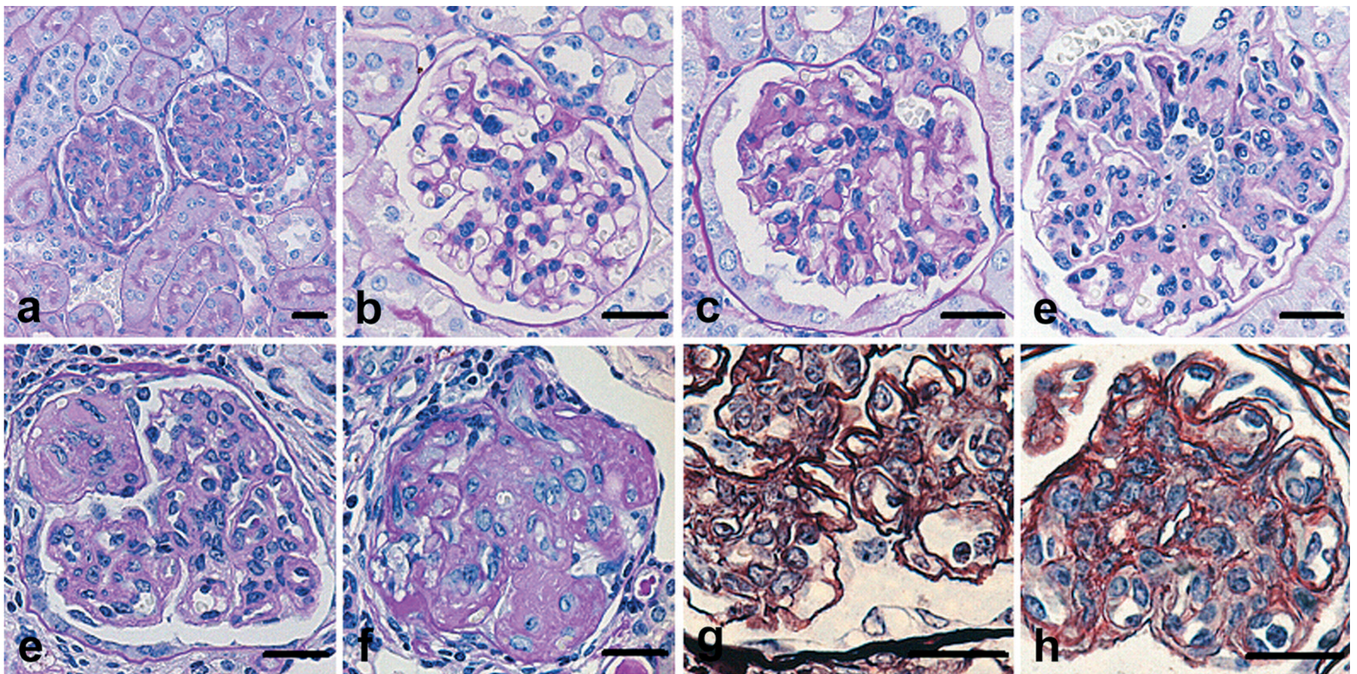

Fig. 2. Light micrographs of renal cortices from 6-month-old and over 12-month-old mice. **a.** 6-month-old female B6.MRLc1(82-100). **b.** 12-month-old male C57BL/6. **c.** 17-month-old male MRL/MpJ. **d.** 12-month-old male B6.MRLc1(82-100). **e-h.** 12-month-old female B6.MRLc1(82-100). In B6.MRLc1(82-100), mild cell proliferation and membranous hypertrophy are clearly observed in glomeruli from a 6-month-old but no significant changes are observed in the tubulointerstitial region (panel a). In **a** 12-month-old C57BL/6, no remarkable membranous or proliferative lesion is found (panel **b**). In glomeruli of MRL/MpJ and B6.MRLc1(82-100) older than 12 months, glomerular damage with thickening of glomerular basement membrane, proliferation of endothelial and mesangial cells, and expansion of the mesangial matrix, are observed (panels **c and d**). In a 12-month-old female B6.MRLc1(82-100), severe glomerular damage with the disappearance of capillary and capsular lumina, global deposition of PAS-positive material, and periglomerular infiltration of inflammatory cells and fibrosis, are observed. PAS- (panels **e and f**) and PAM-stain (panels g and h). Scale bars: 20 μm.

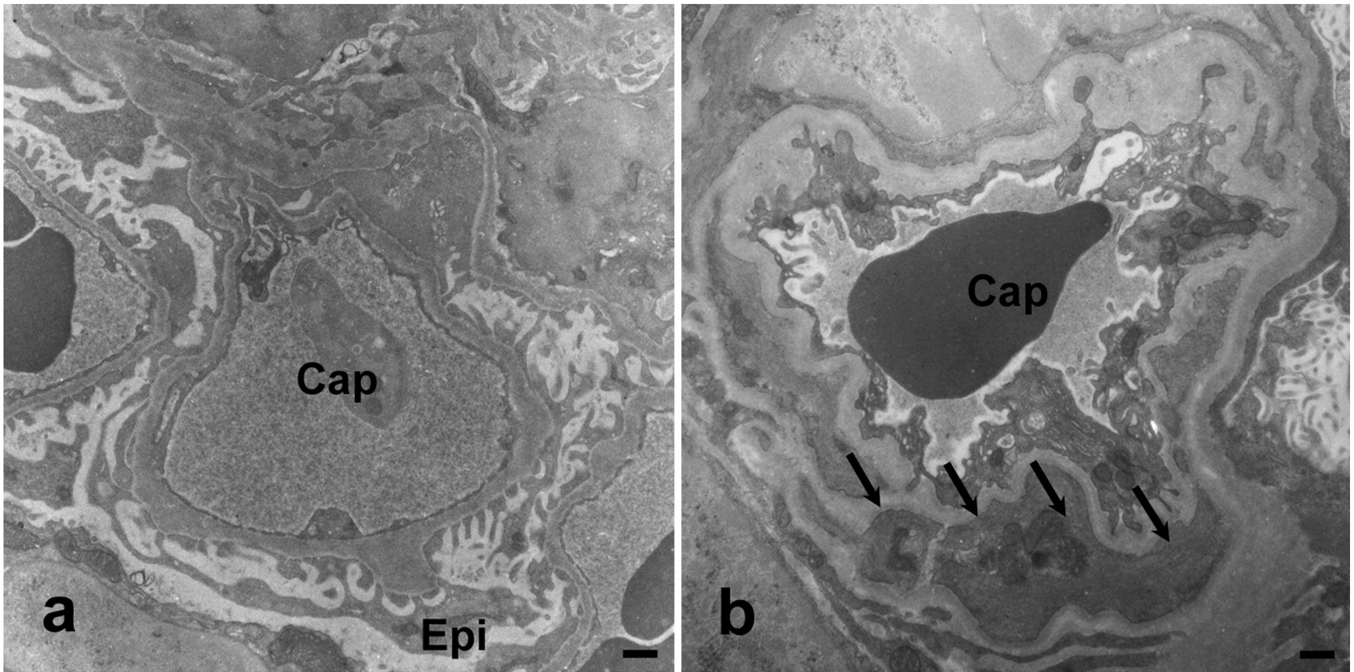

Fig. 3. Transmission electron micrographs of glomeruli in 9-month-old female mice. **a.** MRL/MpJ. **b.** B6.MRLc1(82-100). In MRL/MpJ, minor glomerular abnormalities such as epithelial foot-process fusion were observed (panel **a**). In B6.MRLc1(82-100), wrinkling of GBM and electron-dense deposits (arrows) between double contoured GBM were clearly observed (panel **b**). Cap: capillary lumen. Epi: epithelial cell. Scale bars: 5 μm.

tubulointerstitial region (Fig. 2a). In the 12-month-old C57BL/6, although a slight age-related change was observed, few membranous or proliferative lesions were found (Fig. 2b). In striking contrast, the glomeruli of B6.MRLc1(82-100) showed thickening of the GBM, proliferation of endothelial and mesangial cells, and expansion of the mesangial matrix (Fig. 2d-h). Additionally, some glomeruli of female B6.MRLc1(82-100), but not males, showed quite severe damage, including the global deposition of PAS-positive material and the disappearance of capillary and capsular lumina (Fig. 2f). Staining of B6.MRLc1(82-100) glomeruli with PAM clearly showed diffuse thickening of the GBM, with spike-like alternations or a double contour of GBM (Fig. 2g,h). In electron microscopic examination, minor glomerular abnormalities, such as epithelial foot-process fusion, were observed in glomeruli of 9-month-old MRL/MpJ (Fig. 3a). In contrast, electron-dense deposits were clearly observed between double contoured GBM of age-matched B6.MRLc1(82-100) (Fig. 3b). Figure 4 shows the results of glomerular damage scores in B6.MRLc1(82-100). Glomerular damage scores of B6.MRLc1(82-100) increased with age, and females had a significantly higher value than males at 12 months of age. In the comparison among animals older than 12 months, B6.MRLc1(82-100) had a higher score than wild-type C57BL6 and MRL/MpJ, and significant differences were observed between B6.MRLc1(82-100)

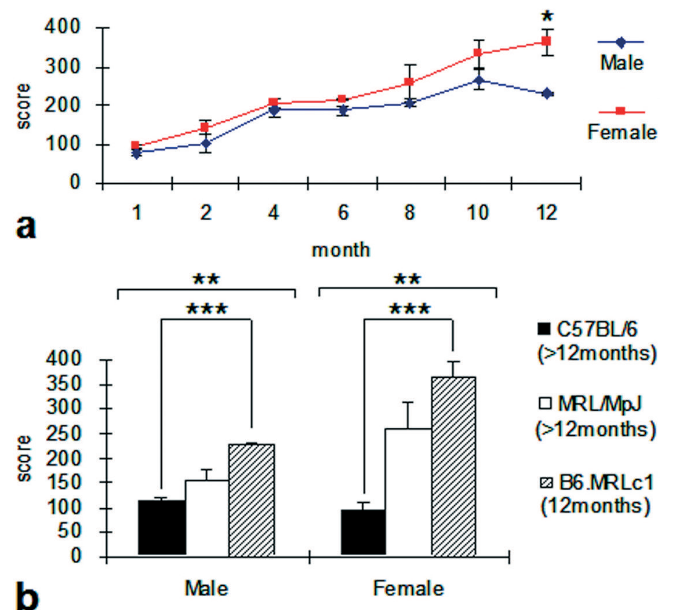

Fig. 4. Glomerular damage score. **a.** Time course in B6.MRLc1(82-100). **b.** Comparison of C57BL/6, MRL/MpJ, and B6.MRLc1(82-100) over 12 months old. Each value represents the mean ± S.E. *: Significantly different from males of the same strain (Mann-Whitney U test, $p<0.05$). **: Significant strain difference in each sex group (Kruskal-Wallis test, $p<0.05$). ***: Significant differences in multiple comparisons following the Kruskal-Wallis test (Scheffé method, $p<0.05$). n≥ 4 (a) or 5 (b). B6.MRLc1: B6.MRLc1(82-100).

and C57BL/6 for both sexes.

IgG deposition in the glomeruli of B6.MRLc1(82-100) congenic mice

C57BL/6, MRL/MpJ, and B6.MRLc1(82-100) older than 12 months were used to assess the depositions of IC in glomeruli. Figure 5 shows light micrographs of the glomeruli immunostained with IgG1, IgG2a and IgG2b. IgG1- and IgG2b-positive reactions were observed in the GBM of all groups. However, there were very few IgG-positive glomeruli in C57BL/6. IgG2a-positive reactions were detected in the glomeruli of MRL/MpJ but not in those of C57BL/6 and B6.MRLc1(82-100). As for the ratios of IgG1-positive glomeruli, the values of B6.MRLc1(82-100) increased at 10 months of age (Fig. 6a), and MRL/MpJ showed the highest values for both sexes in the comparison among strains (Fig. 7a). IgG2a-positive glomeruli were not observed in B6.MRLc1(82-100) at any examined age (data not shown). In contrast, MRL/MpJ showed the highest values for the ratio of IgG2a-positive glomeruli; the values of females were significantly greater than those of males (Fig. 7b). The ratios of IgG2b-positive glomeruli of B6.MRLc1(82-

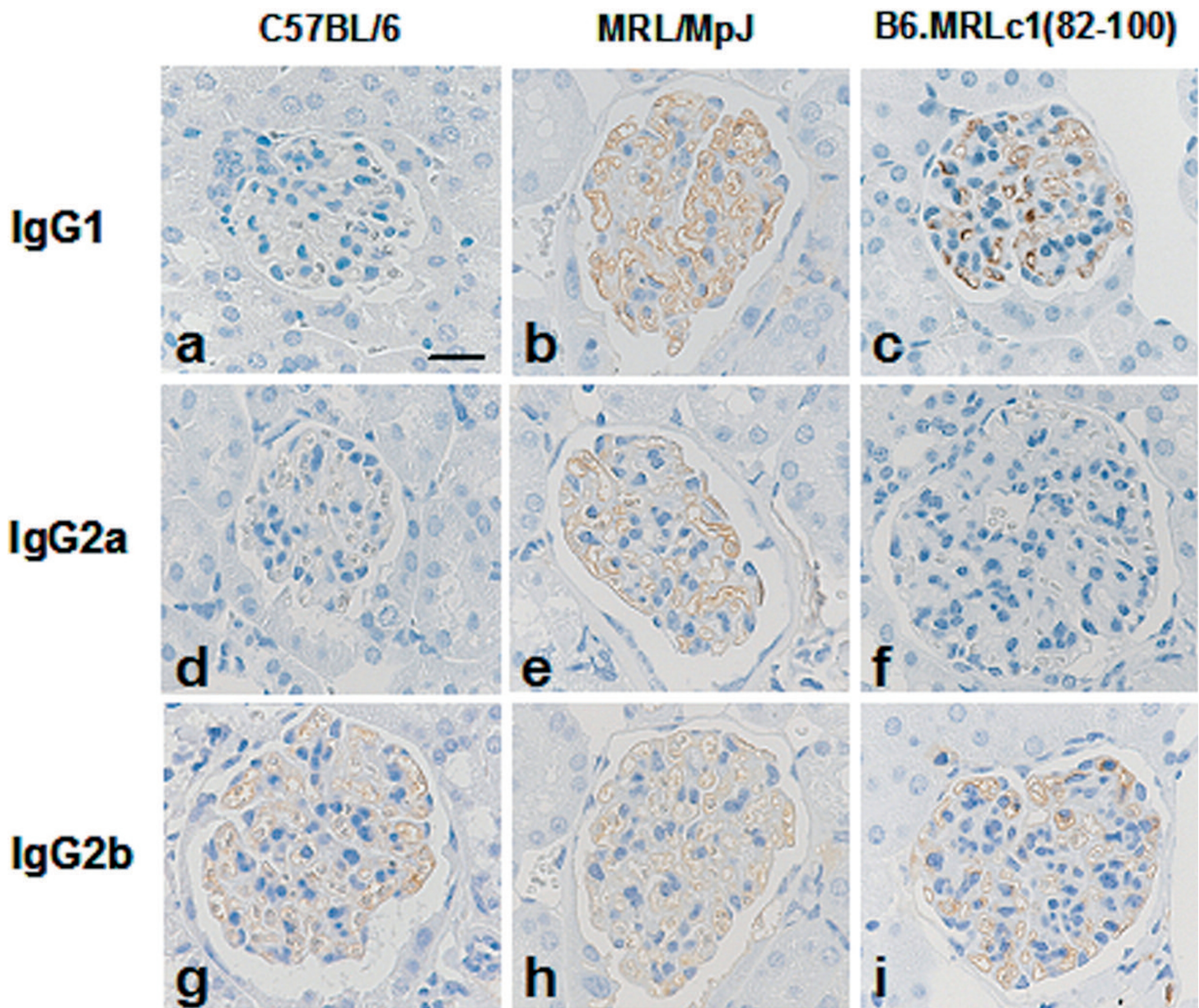

Fig. 5. Immunohistochemical detection of IgG subtypes in mice over 12 months old. **a-c.** IgG1. **d-f.** IgG2a. **g-h.** IgG2b. Clear immunoreactions of IgG1 are observed in glomerular basement membranes of MRL/MpJ (panel **b**) and B6.MRLc1(82-100) (panel **c**) but these reactions are very few in C57BL/6 (panel **a**). Similar reactions of IgG2a are observed in the glomeruli of MRL/MpJ (panel **e**) but not in those of C57BL/6 (panel **d**) and B6.MRLc1(82-100) (panel **f**). IgG2b-positive reactions are observed in the glomeruli of all strains (panel **g-i**). Scale bar: 20 μm.

100) increased at 6 and 10 months of age in females and males, respectively (Fig. 6b). In the comparison of this parameter among strains, no significant differences were observed but a tendency for B6.MRLc1(82-100) to have higher values than C57BL/6 was observed in female groups (Fig. 7c).

Pathophysiology in B6.MRLc1(82-100) congenic mice

A summary of the clinical features of C57BL/6, MRL/MpJ, and B6.MRLc1(82-100) at 6 months of age is presented in Table 1. Most female B6.MRLc1(82-100) showed splenomegaly in macroscopy, and the spleen weight/body weight of B6.MRLc1(82-100) was significantly higher than those of MRL/MpJ for both sexes and those of C57BL/6 in female groups. In immunohistochemistory, CD3 or B220 single-positive cells were mainly observed in splenic lymph follicles of all animals (Fig. 8a-f). Especially, B6.MRLc1(82-100) showed follicular hyperplasia, and CD3- or B220-positive areas in B6.MRLc1(82-100) were more widely observed than those in other strains (Fig. 8c,f).

The total kidney weight/body weight was the highest

Table 1. Summary of clinical parameters in C57BL/6, MRL/MpJ, and B6.MRLc1(82-100) at 6 months of age.

	C57BL/6		MRL/MpJ		B6.MRLc1(82-100)	
	Male	Female	Male	Female	Male	Female
Body Weight (g)	28.3	29.5	47.3[B]	43.7[B,Co]	32.0	27.4
Total kidney weight/ Body weight (%)	1.4	1.2	1.7[Co]	1.4	1.2	1.1
Spleen weight/ Body weight (%)	0.3	0.4	0.2	0.3	0.5[M]	1.0[*,B,M]
Blood Glucose (mg/dl)	238.8	247.4	240.5	186.5	233.9	194.5
Blood Urea Nitrogen (mg/dl)	24.9[*]	17.0	22.0	31.0[*,B]	36.0[B,M]	35.1[B]
Creatinine (mg/dl)	0.5	0.4	0.2	0.2	0.4	0.5[M]
Urine protein score (+)	1.5[*]	0.7	2.1	1.3	1.6	1.3
Anti-dsDNA antibody (μg/ml)	65.0	61.9	127.1[B]	316.9[B]	114.9[B]	248.2[B]

Values: mean. n≥ 4; *: Sex difference (Mann-Whitney U test, p<0.05); B: Strain difference vs. C57BL/6; M: Strain difference vs. MRL/MpJ; Co: Strain difference vs. B6.MRLc1(82-100) (multiple comparisons following the Kruskal-Wallis test, Scheffé method, p<0.05).

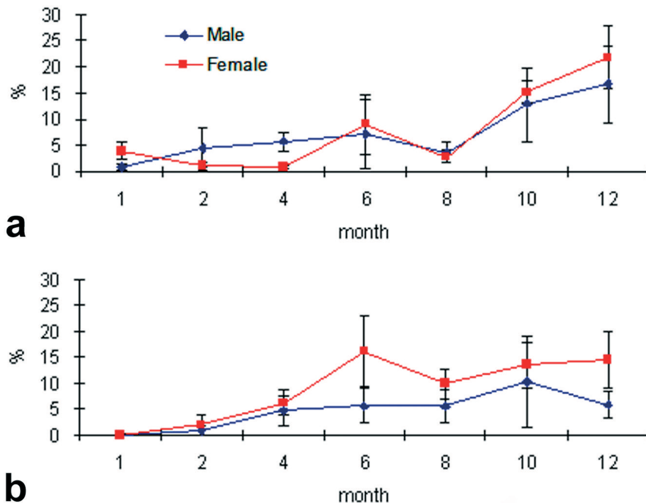

Fig. 6. Time course of the ratios of IgG-positive glomeruli in B6.MRLc1(82-100). **a.** IgG1. **b.** IgG2b. Each value represents the mean ± S.E. *: Significantly different from males of the same strain (Mann-Whitney U test, $p<0.05$). n≥ 4.

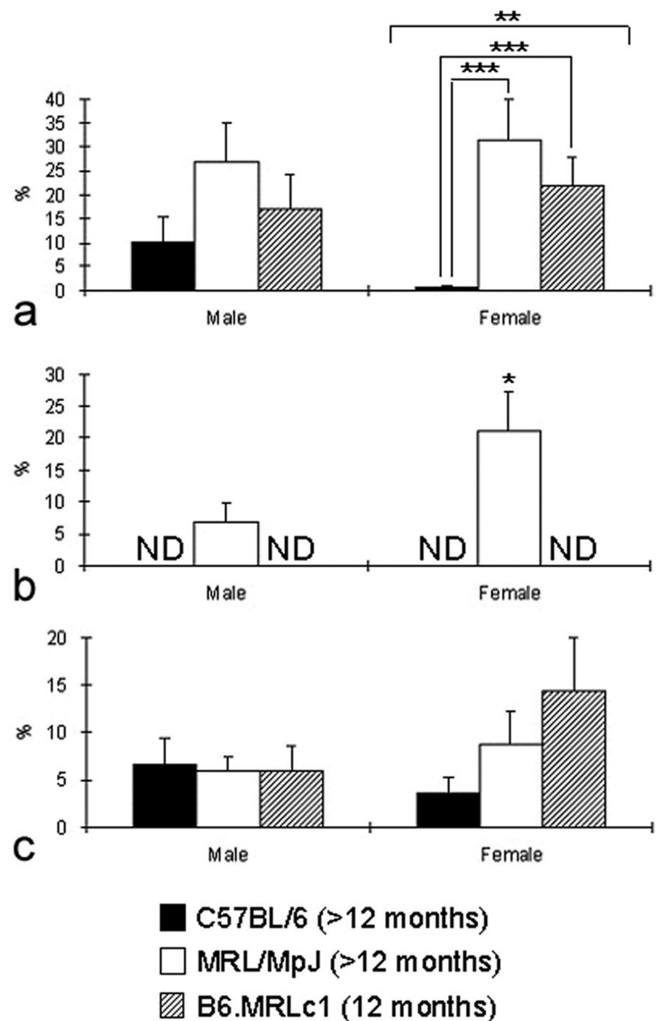

Fig. 7. Comparison of the ratios of IgG-positive glomeruli in over 12-month-old mice. **a.** IgG1. **b.** IgG2a. **c.** IgG2b. Each value represents the mean ± S.E. *: Significantly different from males of the same strain (Mann-Whitney U test, $p<0.05$). **: Significant strain difference in each sex group (Kruskal-Wallis test, $p<0.05$). ***: Significant differences in multiple comparisons following the Kruskal-Wallis test (Scheffé method, $p<0.05$). ND: not detected. n≥5. B6.MRLc1: B6.MRLc1(82-100).

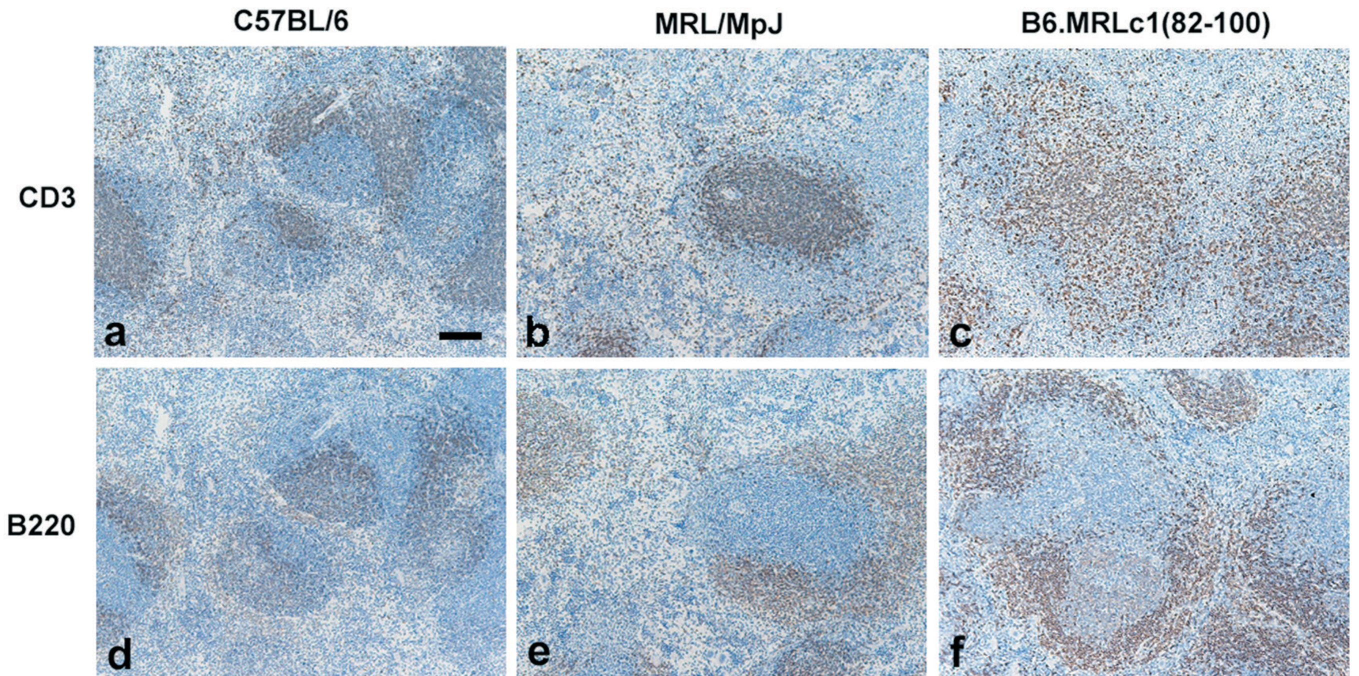

Fig. 8. Immunohistochemical detection of CD3- or B220-positive cells in 6-month-old mice spleen. **a-c.** CD3. **d-f.** B220. In all animals, CD3- or B220-positive cells were mainly observed in splenic lymph follicle. Notably, CD3- or B220-positive areas in B6.MRLc1(82-100) (panels **c and f**) were more widely observed than those in both C57BL/6 (panels a and d) and MRL/MpJ (panels **b and e**). Scale bar: 200 μm.

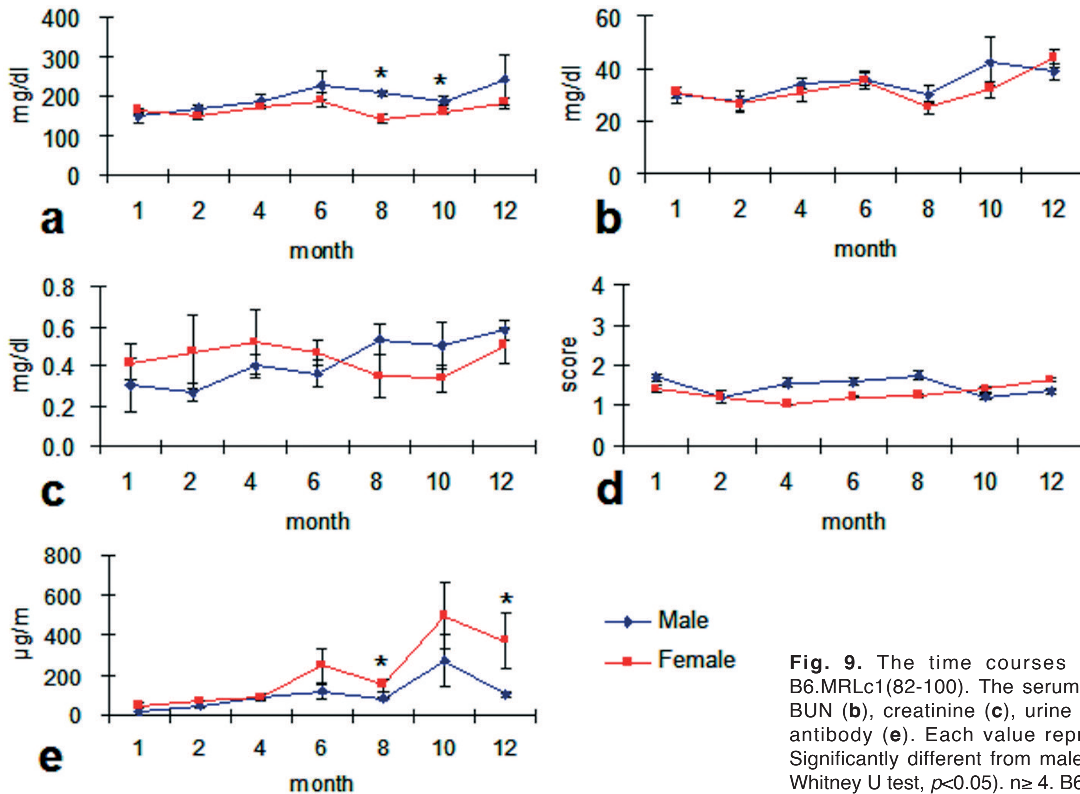

Fig. 9. The time courses of clinical parameters in B6.MRLc1(82-100). The serum levels of blood glucose (**a**), BUN (**b**), creatinine (**c**), urine protein (**d**), and anti-dsDNA antibody (**e**). Each value represents the mean ± S.E. *: Significantly different from males of the same strain (Mann-Whitney U test, p<0.05). n≥ 4. B6.MRLc1: B6.MRLc1(82-100).

in MRL/MpJ for both sexes, and significant differences were observed between MRL/MpJ and B6.MRLc1(82-100). For blood glucose, a hallmark of diabetic nephropathy, no significant difference was observed among the strains. In B6.MRLc1(82-100), the blood glucose levels of males were significantly higher than those of females at 8 and 10 months, but the values of both sexes changed within constant values (185.1±6.2 mg/dl) during the measurement (Fig. 9a). To assess the deterioration of renal functions, the serum levels of blood urea nitrogen (BUN) and creatinine, as well as urine protein were measured (Table1 and Fig. 9b-d). The serum BUN levels were the highest in B6.MRLc1(82-100) for both sexes, and those of B6.MRLc1(82-100) were significantly higher than those of C57BL/6 for both sexes and those of MRL/MpJ in male groups. For serum creatinine, B6.MRLc1(82-100) had significantly higher values than MRL/MpJ in female groups. No strain difference was observed in urine protein. In B6.MRLc1(82-100) of both sexes, the serum BUN increased at 10 months of age, but the serum creatinine concentration and urinary protein score were almost constant during the observations (Fig. 9b-d).

Autoantibody production in B6.MRLc1(82-100) congenic mice

In B6.MRLc1(82-100), the serum levels of anti-dsDNA Abs in females increased from 6 months of age, while those in males showed a mild increase with age (Fig. 9e). Significant sex differences were observed at 8 and 12 months, when females had higher values than males. At 6 months of age, the values of MRL/MpJ and B6.MRLc1(82-100) were significantly higher than those of C57BL/6 for both sexes (Table 1).

Discussion

Light microscopic examination of B6.MRLc1(82-100) kidneys showed severe glomerular damage, including proliferative and membranous lesions. Furthermore, histometric analysis of the glomerular damage morphometrically demonstrated that B6.MRLc1(82-100) had severer glomerular damage than aged C57BL/6 (older than 12 months). Therefore, it was clear that the glomerular damage of B6.MRLc1(82-100) was not due to age-related changes in the mouse strain. Electron microscopy demonstrated the presence of electron-dense deposits between double contoured GBM in B6.MRLc1(82-100). Some studies have reported that electron-dense deposits include the deposition of IC and play a major role in the pathogenesis of lupus nephritis (Ferluga et al., 2000; Hvala et al., 2000). Furthermore, IgG-positive reactions were observed in the GBM of B6.MRLc1(82-100), and the deposition of IC was immunohistochemically demonstrated. From these findings, it was clarified that B6.MRLc1(82-100) developed immune-mediated GN. This is the first report that MRL/MpJ has a lupus nephritis susceptibility locus on Chr. 1 (82-100), which we named *Mag*.

Interestingly, original phenotype for MRL strain such as arthritis, skin lesion, or vasculitis was not observed in B6.MRLc1(82-100) carrying MRL-type telomeric region of chromosome 1 (data was not shown). In many previous studies, it has been clarified that *Paam1* (*progression of autoimmune arthritis in MRL mice 1*), *Paam2*, *Lprm1* (*Lymphoproliferation modifier 1*; vasculitis susceptibility loci for MRL strain), and *Lprm2* were localized on chromosome 15, 19, 4, and 3, respectively (Kamogawa et al., 2002; Santiago-Raber et al., 2004). Therefore, B6.MRLc1(82-100) might not show these phenotypes because of a lack of autoimmune disease susceptibility loci derived from MRL/MpJ genetic background except for telomeric region of chromosome 1. Furthermore, the histopathological features of GN in B6.MRLc1(82-100) were also severer than those in MRL/MpJ. In a previous study, *Lprm3* (*Lymphoproliferation modifier 3* on chromosome 14) was reported as a lupus nephritis resistant locus in the MRL genetic background (Wang et al., 1997). Additionally, Rozzo et al. investigated the effect of the genetic background in the contribution of NZB loci to lupus nephritis, and reported that congenic mice with a C57BL/6 background showed severer GN than those with a BALB/c background (Rozzo et al., 1999). These findings suggested that B6.MRLc1(82-100) suffer from severe GN because of the lack of lupus nephritis resistant loci derived from the MRL/MpJ genome or the presence of an unknown GN accelerating factor derived from the C57BL/6 genome.

As for IgG subtypes, B6.MRLc1(82-100) had IgG1- and IgG2b-positive glomeruli and higher values than C57BL/6 for the ratio of IgG1-positive glomeruli. Interestingly, IgG2a depositions were observed in the glomeruli of MRL/MpJ but not in those of C57BL/6 and B6.MRLc1(82-100). Recent studies suggested that alterations of Th1/Th2 balance influenced the pathogenesis of autoimmune GN. Shimizu et al. demonstrated that Th1- and Th2-type autoimmune responses contributed to proliferative and membranous GN, respectively. IgG2 subtypes (Th1 isotypes) were mainly detected in proliferative GN, and glomeruli in membranous GN showed a predominance of IgG1 (Th2 isotypes) deposition in IL-27 receptor-deficient MRL/lpr mice (Shimizu et al., 2005). Furthermore, it was reported that IgG2a was the most abundant IgG subtype in the serum of the MRL/lpr mouse (Balomenos et al., 1998). From these findings, we consider that the *Mag* interval included a factor that could affect the deposition of IgG subtypes, especially IgG1 rather than IgG2, by influencing the Th1/Th2 balance directly or indirectly.

As indices of renal function, the serum levels of BUN and creatinine, along with urine protein, were measured. Although BUN of B6.MRLc1(82-100) increased with age and became higher than those of C57BL/6, creatinine and urine protein of B6.MRLc1(82-100) showed standard values during the examination, and no correlation between these parameters and

glomerular damage scores was observed. In recent reviews, it was suggested that hyperfiltrated proteins were reabsorbed in the proximal tubules and that these proteins increased tubular cell turnover as both apoptosis and proliferation (Exaire et al., 1972; Thomas et al., 1999). Recently, therefore, proteinuria has been recognized as a marker for inflammatory tubulointerstitial injury rather than glomerular injury (Thomas and Schreiner, 1993; Thomas et al., 1999), corresponding to the present findings that significant tubulointerstitial injuries were not observed in B6.MRLc1(82-100). Although B6.MRLc1(82-100) showed severe GN, the disorder of renal function, including proteinuria, might be kept mild by a compensational mechanism such as protein reabsorption in the proximal tubules.

The serum level of blood glucose, a hallmark of diabetic nephropathy, was maintained at the baseline during the examination in B6.MRLc1(82-100). In addition to histopathological findings, we confirmed that glomerular damage of B6.MRLc1(82-100) was not due to diabetic nephropathy. On the other hand, the serum level of the anti-dsDNA Abs showed an increase with age in B6.MRLc1(82-100), being significantly higher than in C57BL/6. These results showed that B6.MRLc1(82-100) clearly developed autoimmune disease. However, we could not detect a good correlation between the serum level of anti-dsDNA Abs and other parameters, such as glomerular damage, and the number of IgG-positive glomeruli. Several immunogenetic studies demonstrated that not all lupus-prone mice producing anti-dsDNA Abs developed nephritis (Yoshida et al., 1981; Izui et al., 1984) and that anti-dsDNA Abs were insufficient to cause nephritis (Lefkowith and Gilkeson, 1996). Recently, in addition to nuclear components such as dsDNA, ssDNA, and histones, it has been suggested that novel factors are involved in the formation of severe murine lupus nephritis, such as the interferon-inducible 202 proteins (Ifi202p) within the Ifi200 family, or heat shock protein 70 (Hsp70), which is a member of the HSP family (Hueber et al., 2004). Ifi202p, in particular, are encoded on the *Ifi202* gene cluster included by *Mag* and are important regulators of cell growth and differentiation (Johnstone and Trapani, 1999). Furthermore, Xin et al. reported that enhanced expression of Ifi202p in B6.Nba2 mice increased the susceptibility to lupus by inhibiting apoptosis (Xin et al., 2006). Therefore, not only anti-nuclear Abs but also other factors such as increased Ifi202p may contribute to the formation of severe GN in murine lupus.

In MRL/MpJ and B6.MRLc1(82-100), sex differences were observed in glomerular damage scores, the numbers of IgG-positive glomeruli, and the serum levels of anti-dsDNA Abs, as females had higher values than males for these parameters. In human lupus patients, similar sex differences were observed and the incidence of disease occurs with a female to male ratio of 9:1 (Soto et al., 2004; Grimaldi et al., 2005). In murine lupus models, NZB/NZW F1 and MRL/lpr develop lupus by 5 months of age, exhibit high titers of anti-nuclear Abs, and have GN, and females exhibit more severe disease than males (Bell et al., 1973; Pisetsky et al., 1980; Honda et al., 1999). A recent study suggests that sex hormones, especially estrogen, exacerbate murine lupus in an autoimmune-prone genetic background by affecting B cell maturation, selection, and activation (Grimaldi et al., 2005). Briefly, in the studies using estrogen-treated R4A-γ2b BALB/c mice, which spontaneously develop autoimmune disease, Grimaldi et al. suggested that estrogen increased the level of Bcl-2, which enhanced survival of autoreactive B cells, and those of CD22 and SHP-1, which raise the threshold for B cell receptor crosslinking required for the deletion of autoreactive B cells (Grimaldi et al., 2002). From these findings, it was strongly suggested that sex differences observed in MRL/MpJ and B6.MRLc1(82-100) were caused by the effect of estrogen. Therefore, further studies to assess the sex-based pathological differences in MRL-background GN, focusing specifically on B cell tolerance mediated by estrogen, are required.

In the telomeric region of Chr.1, *Bxs3* (71-99), *Sle1* (88), and *Nba2* (92-94) were reported as lupus nephritis susceptibility loci in BXSB, NZW, and NZB mice, respectively (Santiago-Raber et al., 2004). Recently, *Ifi202* was suggested by microarray analysis to be a major candidate susceptibility gene in *Bxs3* and *Nba2*, and it was suggested that polymorphism in the *Ifi202* promoter region correlated with the contribution of *Nba2* to lupus (Rozzo et al., 2001; Haywood et al., 2006). Interestingly, the MRL strain has a polymorphism similar to the NZB strain in the *Ifi202* promoter region (Rozzo et al., 2001). However, B6.NZB-Nba2 congenic mice, which have the NZB Chr.1 (79-109 cM) and C57BL/6 background, do not develop GN, but only splenomegaly and high serum levels of IgG antinuclear Abs (Rozzo et al., 2001). In the present study, both GN and splenomegaly were observed in B6.MRLc1(82-100) which has MRL/MpJ Chr.1 (82-100 cM) and C57BL/6 background. Therefore, not only *Ifi202*, but also other candidate genes may play important roles in the development of autoimmune GN in lupus-prone mice, including B6.MRLc1(82-100).

Interestingly, B6.MRLc1(82-100) clearly showed splenic follicular hyperplasia caused by proliferations of CD3- and B220-single positive cells which were indicating T cells and B cells respectively. Recently, the *Fc gamma receptor* family genes, which are widely expressed on immune cells and regulate immune responses positively (*Fcgr1* and *Fcgr3*) and negatively (*Fcgr2*), are recently attracting the attention of many researchers as lupus nephritis candidate genes on murine Chr.1. Briefly, it was reported that mutant BXSB mice deficient in *Fcgr1* and *Fcgr3* were resistant to induction of autoimmune GN (Lin et al., 2006). In addition, it was reported that the polymorphism of promoter region in *Fcgr2b* (Chr.1 92.3cM) was shared in lupus-prone mice,

including MRL strains, and that this polymorphism was linked to down-regulation of *Fcgr2b* expression levels on activated B cells, leading to up-regulation of IgG auto-Abs and autoimmune disease (Jiang et al., 2000; Lin et al., 2000). From these findings, we considered that MRL-type mutation of *Fcgr2b* might link to pathogenesis of GN by causing the hyperactivation of immune cells, such as splenic follicular hyperplasia.

In conclusion, we created B6.MRLc1(82-100) congenic mice, which have the MRL/MpJ type Chr.1 (82-100cM) and C57BL/6 genetic background, and demonstrated that B6.MRLc1(82-100) developed severe autoimmune GN with splenomegaly, evaluated serum levels of anti-dsDNA Abs, and the IgG deposition in glomeruli. Our findings could help to elucidate the complex genetic background that underlies the pathogenesis of lupus nephritis and determine therapeutic targets for this malignant disease.

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