

1 **Full-Text**

2 **Title:** Onset of autoimmune glomerulonephritis derived from the telomeric region of  
3 MRL-chromosome 1 is associated with the male sex hormone in mice

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17 **Running head:** Effects of gonadectomy on glomerulonephritis

## 1 **Summary**

2 Female B6.MRLc1(82–100) congenic mice develop severer autoimmune  
3 glomerulonephritis (AGN) than males. We assessed the effects of gonadectomy on the  
4 pathogenesis of AGN in these mice. One-month-old male and female mice were divided into  
5 sham-operated group (SG) and gonadectomized group (GG), and the pathological changes  
6 were investigated at 8 months. SG females exhibited higher spleen and thymus weights,  
7 serum total IgG and autoantibody levels, glomerular damage scores, and percent IgG- and  
8 CD3-positive glomeruli as compared to SG males. Gonadectomy exhibited more  
9 remarkable effects in males than in females. Spleen and thymus weights, urinary albumin  
10 excretion, glomerular damage scores, percent IgG- and CD3-positive glomeruli, and  
11 CD3-positive areas in the spleen were significantly higher in GG males than in SG males.  
12 CD3-positive cells were observed in both the thymic cortex and medulla in all animals  
13 except SG males. The expression ratio of active *Fc gamma receptor (Fcgr) 3* to inhibitory  
14 *Fcgr2b* in the kidneys, which we have previously demonstrated to have a great impact on  
15 pathogenesis in B6.MRLc1(82–100), was significantly higher in GG males than in SG males.  
16 These results suggested that the differences in the pathogenesis of AGN are primarily due  
17 to the inhibitory roles of the male sex hormones.

18 **Keywords:** autoimmune glomerulonephritis, sex differences, gonadectomy, MRL mouse,  
19 kidney

## 1 **Introduction**

2 The immune system is responsible for essential biological mechanisms such as  
3 immunological tolerance and the defense response to infection. Sex-related differences in  
4 these mechanisms have been reported. In humans, the mechanisms of cellular immunity,  
5 humoral immunity, and resistance to microbial infections have been shown to differ between  
6 the sexes, in which females exhibit stronger immune responses than males.<sup>1</sup> Interestingly,  
7 these immunological differences between sexes also have a great impact on the  
8 pathogenesis of autoimmune diseases. The female-to-male incidence ratios for systemic  
9 lupus erythematosus (SLE), Sjögren's syndrome, Hashimoto's disease, and rheumatoid  
10 arthritis, which are major autoimmune diseases, are reported to be 9:1, 9:1, 25:1, and 2:1,  
11 respectively.<sup>2-4</sup> In addition, the disease deteriorates in female SLE patients, leading to  
12 fluctuations in sex hormones, such as during pregnancy or menopause, or due to the  
13 dosage of anti-neoplastic drugs.<sup>1, 5-7</sup> The appearance of severe symptoms such as typical  
14 skin lesions, serositis, and renal disease is more common in male SLE patients than in  
15 female ones.<sup>6, 7</sup> Therefore, sex hormones or chromosomes are considered to be directly  
16 responsible for the development of sex differences concerning the immune system and the  
17 pathogenesis of autoimmune diseases.

18 Sex-related differences in the pathogenesis of autoimmune glomerulonephritis (AGN)  
19 have been investigated by using (NZB × NZW)F1 (NZB/WF1) mice and BXSB mice, which  
20 are commonly used for models of autoimmune diseases. In brief, the female NZB/WF1 mice  
21 had a shorter life span, higher serum autoantibody concentrations, and severer  
22 glomerulonephritis than the males.<sup>8-11</sup> On the other hand, among the BXSB mice, since the  
23 males developed severer glomerulonephritis than the females; it was suggested that the Y  
24 chromosome autoimmune acceleration (*Yaa*) gene played a role in this difference between

1 the sexes.<sup>12</sup> In a recent study, it was confirmed that the *Yaa* gene was in fact Toll-like  
2 receptor 7 (*Tlr7*), which is localized on the X chromosome encoding an RNA receptor, and  
3 that this gene is duplicated on the Y chromosome in BXSB mice.<sup>13</sup> Studies on MRL-Fas<sup>lpr</sup>  
4 (MRL-lpr) mice have reported varying tendencies of sex differences in pathological  
5 diseases: some have suggested that the female mice exhibit higher autoantibody levels and  
6 more severe glomerulonephritis than the males,<sup>14, 15</sup> while others have reported no sex  
7 differences between the mice in this regard.<sup>16–18</sup> Therefore, it has been considered that  
8 sex-related differences in the pathogenesis of autoimmune diseases are strongly affected  
9 not only by gene mutations in the individuals but also by their genetic backgrounds.

10 In our previous study, we generated B6.MRLc1(82–100) congenic mice carrying the  
11 telomeric region of chromosome 1 of the MRL strain, establishing that these mice were  
12 useful as AGN models, including the candidate gene for this disease on chromosome 1  
13 (82–100 cM; *MRL autoimmune glomerulonephritis*, i.e., *Mag*).<sup>19, 20</sup> The disease was active  
14 in female B6.MRLc1(82–100) mice, which was reflected by glomerular damage, glomerular  
15 immune-complex deposition, and the serum levels of anti-double strand DNA (dsDNA).<sup>19</sup>  
16 Although we assumed sex hormones to be responsible for these sex differences, no  
17 evidence of this relationship was available between sex hormones and the pathogenesis of  
18 AGN in the B6.MRLc1(82–100) mice. As mentioned above, the mechanisms responsible for  
19 sex differences in the pathogenesis of AGN have been analysed using spontaneous  
20 disease models such as the NZB, BXSB, or MRL mice. However, the secondary effects of  
21 the numerous disease-susceptible genes present in these models have rendered it difficult  
22 to precisely assess the roles of sex hormones in the pathogenesis of AGN. In contrast, since  
23 B6.MRLc1(82–100) mice do not exhibit any disease-susceptible loci other than *Mag*, this

1 model would be able to reflect the effects of sex hormones in AGN more accurately than  
2 other models.

3 In the present study, male and female B6.MRLc1(82–100) mice were castrated to assess  
4 the roles of sex hormones in the pathogenesis of AGN. We show here that the male sex  
5 hormone has considerable inhibitory effects on the pathogenesis of AGN in  
6 B6.MRLc1(82–100) mice.

## 1 **Materials and Methods**

### 2 Gonadectomy and sham operation

3 B6.MRLc1(82–100) mice were generated using the method described in our previous  
4 report,<sup>19, 20</sup> and they were maintained under conventional conditions. With regard to the care  
5 and handling of experimental animals, the investigators adhered to the ‘Guide for the Care  
6 and Use of Animals’, issued by the School of Veterinary Medicine, Hokkaido University.  
7 One-month-old male and female mice were castrated or sham operated under anaesthesia  
8 induced by a mixture of ketamine (75 mg/kg, i.p.) and medetomidine (1 mg/kg, i.p.).  
9 Postoperative emergence was performed by atipamezole (2 mg/kg, i.p.). The mice were  
10 separated into female and male gonadectomized groups (GGs, n = 3 each) and  
11 sham-operated groups (SGs, n = 3 each), and they were weighed every 2 weeks.

### 12 13 Preparations for materials

14 Urine samples were collected from the animals at 8 months of age, and they were then  
15 sacrificed by exsanguination via the carotid arteries, under deep anaesthesia induced by a  
16 ketamine-medetomidine mixture. From each animal, serum samples were collected, and the  
17 kidneys, spleen, and thymus were quickly removed and weighed. A fraction of each tissue  
18 sample was fixed in 10% formalin for at least 72 h at room temperature and then embedded  
19 in paraffin for histopathological analysis. The remaining tissue was frozen in RNAlater  
20 (Ambion, TX, USA) and stored at –30°C until use for extraction of total RNA.

### 21 22 Serological analysis and urinalysis

23 The serum levels of both the sex hormones were measured using an enzyme  
24 immunoassay kit (Cayman Chemicals, MI, USA), according to the manufacturer’s directions.

1 The serum levels of blood urea nitrogen (BUN), total IgG, and anti-dsDNA antibodies  
2 (Abs) and the urinary albumin concentration were determined using the BUN-test-Wako kit  
3 (Wako Pure Chemical Industries, Osaka, Japan), the mouse IgG ELISA quantitation kit  
4 (Bethyl, TX, USA), the mouse anti-dsDNA Ab ELISA kit (Alpha Diagnostic International, TX,  
5 USA), and the Lebis Albumin assay kit (Shibayagi, Gunma, Japan), respectively, according  
6 to the suppliers' protocols.

#### 7 8 *Histopathological analysis of the kidneys*

9 The severity of glomerular damage was assessed as described previously.<sup>20</sup> In brief,  
10 paraffin-embedded tissue sections (2 µm) of both male and female GG and SG mice were  
11 stained with periodic acid-Schiff reagent (PAS). Digital images of 30 glomeruli per kidney  
12 were obtained, and the number of nuclei per glomerulus and the diameters of the renal  
13 corpuscles were measured using ImageJ ver. 1.32j (NIH, MD, USA).

#### 14 15 *Immunohistochemical analysis of the kidneys, spleen, and thymus*

16 IgG2b-positive glomeruli in the kidneys and CD3- and B220-positive cells in the kidneys,  
17 spleen, and thymus were detected using biotin-conjugated goat anti-IgG2b Abs (Caltag  
18 Laboratories, CA, USA), unlabeled rabbit anti-CD3 Abs (Dako, Kyoto, Japan), and rat  
19 anti-B220 Abs (Cedarlane Laboratories, Homby, Ontario, Canada), respectively, as  
20 described previously.<sup>19, 20</sup> Randomized histoplanimetric analysis was performed using the  
21 PAS-stained kidney sections and the immunostained sections. The IgG2b- or CD3-positive  
22 glomeruli were quantified according to a previously described procedure. In brief, the total  
23 number of IgG2b- or CD3-positive glomeruli in the immunostained sections and the total  
24 number of renal corpuscles in the PAS-stained sections (serial with immunostained

1 sections) were determined, and the index for the number of IgG2b- or CD3-positive  
2 glomeruli was expressed as the value per 100 renal corpuscles.

3 The CD3- and B220-positive areas in the spleen sections were measured as described  
4 previously.<sup>20</sup> In brief, for each animal, we used ImageJ ver. 1.32j (NIH, MD, USA) to  
5 measure more than 10 absolute CD3- or B220-positive areas on digital images of the spleen,  
6 which were obtained using a digital camera, and we compared the mean values determined  
7 for the images.

#### 8 9 Real-time reverse transcription-PCR analysis

10 Total RNA was extracted from the kidney, spleen, and thymus tissues of both male and  
11 female GG and SG mice by using the TRIzol reagent (Invitrogen, CA, USA). The purified  
12 total RNA samples were treated with DNase (Nippon Gene, Toyama, Japan) for  
13 contaminated DNA digestion, and the corresponding cDNAs were synthesized using  
14 ReverTra Ace (Toyobo, Osaka, Japan) and oligo(dT) primers (Invitrogen, Carlsbad, CA,  
15 USA). Quantitative real-time PCR analysis was performed on the cDNAs by using the  
16 Brilliant SYBR Green QPCR master mix and a real-time thermal cycler (MX 3000;  
17 Stratagene, Milano, Italy). The specific primers used for each gene have been described  
18 previously.<sup>20</sup> The amplification conditions were as follows: 95°C for 10 min; 40 cycles of  
19 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s; and 1 cycle of 95°C for 10 s, 58°C for 20 s,  
20 and 95°C for 10 s. ROX dye was added to each reaction mixture to normalize the  
21 non-PCR-related fluctuations in the fluorescence signals. The amplification specificity of all  
22 the PCR reactions was confirmed by performing a melting-curve analysis. Nontemplate  
23 controls were included for each primer pair to detect whether any contaminants were  
24 present at significant levels. The quantitative data obtained for the *Fcgr* were normalized to



1 the  $\beta$ -actin expression data. The ratio of *Fcgr3* to *Fcgr2b* was calculated from these  
2 normalized values and expressed in terms of fold increase from the values obtained for the  
3 male SG mice.

4

5 Statistical analysis

6 The results were expressed as the mean  $\pm$  SEM and statistically analysed using the  
7 Student's *t* test ( $p < 0.05$ ).

## 1 **Results**

### 2 Effects of gonadectomy on body weights, organ weights, and clinical parameters

3 The results of the serum sex hormone measurements revealed that the male GG mice  
4 ( $44.75 \pm 2.39$  pg/ml) exhibited lower serum testosterone levels than the male SG mice  
5 ( $340.65 \pm 72.43$  pg/ml), and the female GG mice ( $4.48 \pm 0.34$  pg/ml) exhibited lower serum  
6 estradiol levels than the female SG mice ( $12.76 \pm 6.12$  pg/ml).

7 With regard to body weight in males, the GG mice exhibited a milder tendency for  
8 increase in body weight than the SG mice (Fig. 1). On the other hand, no clear differences  
9 were observed between the female mice of the 2 groups.

10 Table 1 presents the organ weights and clinical parameters of mice. The total kidney  
11 weights/body weights in both male and female GG mice were lower than those in the SG  
12 mice; this difference was significant between the male mice of the 2 groups. The spleen  
13 weights/body weights were significantly higher in the male GG mice than in the male SG  
14 mice. On the other hand, a decreasing tendency was noted in this parameter in the female  
15 GG mice as a result of gonadectomy. The thymus weights were also affected by the  
16 gonadectomy, and the values in the male GG mice were significantly higher than those in  
17 the male SG mice, and the reverse was true in females.

18 To evaluate renal function, we measured BUN concentrations and urinary albumin  
19 excretion (Table 1). No significant differences in the serum BUN concentrations were  
20 detected between the groups or between the sexes. The urinary albumin excretion was  
21 significantly higher in the male GG mice than in the male SG mice. On the other hand,  
22 gonadectomy was not observed to affect this parameter in the female mice.

23 We measured the total IgG and anti-dsDNA Ab concentrations in the serum as indices of  
24 autoimmune disease (Table 1). Although the female mice exhibited higher values than the

1 male mice for both parameters, gonadectomy did not affect these values in either of the  
2 sexes.

#### 3 4 Effects of gonadectomy on glomerulonephritis

5 Figure 2 presents light micrographs illustrating the glomeruli of the GG and SG mice of  
6 both sexes. More membranous and proliferative glomerular lesions were observed in female  
7 SG mice than in male SG mice (Fig. 2a and c). Further, the male GG mice exhibited more  
8 severe glomerular lesions than the male SG mice and developed proliferative lesions  
9 characterized by expansion of the mesangial matrix, proliferation of the mesangial cells, and  
10 infiltration of mononuclear cells, rather than membranous lesions (Fig. 2a and b). On the  
11 other hand, although there was an improvement in the glomerular lesions in some of the  
12 female GG mice, this could not be considered as the general tendency owing to the large  
13 differences among individuals (Fig. 2c and d).

14 Further, the glomerular damage in the GG and SG mice was histometrically assessed (Fig.  
15 2e and f). With regard to the number of nuclei in the glomeruli, which is an index of  
16 glomerular proliferative lesions, significantly higher values were recorded for the male GG  
17 mice than for the male SG mice (Fig. 2e). Similarly, with regard to the diameter of the renal  
18 corpuscles, which is an index of glomerular hypertrophy, significantly higher values were  
19 recorded for the male GG mice than for the male SG mice (Fig. 2f). Both values tended to  
20 decrease in the female GG mice; however, no statistically significant differences were  
21 observed in this regard.

22 We assessed the degree of glomerular immune-complex deposition by performing  
23 immunohistochemical staining for the IgG2b subtype. In a previous study with aged  
24 B6.MRLc1(82–100) mice, the glomerular immune complex depositions of this subtype were

1 more predominant in females than in males.<sup>19</sup> IgG2b-positive reactions were mainly  
2 detected in the glomerular basement membrane (Fig. 3a–d). The percentage of  
3 IgG2b-positive glomeruli was significantly higher in the male GG mice than in the male SG  
4 mice (Fig. 3e). This parameter was unaffected by the gonadectomy in female mice.

5 Immunohistochemical staining for CD3 and B220 revealed infiltration of CD3-positive cells  
6 into the glomeruli in case of the male GG and female SG mice (Fig. 4a–d). However, no  
7 B220-positive cells were observed in the glomeruli of any of the animals (Fig. 4e–h). Male  
8 GG mice exhibited a higher percentage of CD3-positive glomeruli than male SG mice (Fig.  
9 4i). On the other hand, this percentage tended to decrease in the female GG mice as  
10 compared to the SG mice (Fig. 4i).

#### 11 12 Effects of gonadectomy on the immune system

13 In the spleen, CD3- and B220-positive areas were observed in the periarteriolar lymphatic  
14 sheath and the splenic lymphoid follicles, respectively (Fig. 5a–h). In the CD3-positive areas,  
15 although significantly higher values were observed in the male GG mice than in the male SG  
16 mice, gonadectomy did not affect the values in the female mice (Fig. 5i). On the other hand,  
17 the female SG mice exhibited slightly larger B220-positive areas than the other mice, but  
18 this difference was not significant (Fig. 5j).

19 Although CD3-positive cells were observed in the thymus of all the animals, their  
20 localizations differed. In the case of the male SG mice, these cells were mainly localized in  
21 the thymic medulla (Fig. 6a), while in the other mice, they were diffusely localized in both the  
22 thymic cortex and medulla (Fig. 6b–d).

#### 23 24 Effects of gonadectomy on Fcgr expression in kidneys

1 Quantitative real-time PCR analysis revealed that the highest renal *Fcgr2b* expression  
2 was observed in the female SG mice. This parameter was significantly decreased by  
3 gonadectomy in female and the reverse was true in the males (Fig. 7a). A similar tendency  
4 was also noted in the expression of *Fcgr3*, the male GG mice exhibited significantly higher  
5 *Fcgr3* expression levels than the male SG mice (Fig. 7b). The *Fcgr3-Fcgr2b* expression ratio  
6 was significantly higher for the male GG mice than for the male SG mice. In contrast,  
7 gonadectomy did not affect this ratio in the female mice.

## 1 Discussion

2 The serum concentrations of testosterone and estradiol decreased in the castrated male  
3 and female B6.MRLc1(82–100) mice, respectively. Renal pathological analysis revealed  
4 that the glomerular damage scores and albuminuria increased in the male GG mice. In  
5 previous studies, the state of disease worsened in castrated male NZB/WF1 mice but was  
6 ameliorated with testosterone medication.<sup>6, 7, 11</sup> Furthermore, it has been reported that some  
7 male SLE patients exhibit reduced serum androgen concentrations.<sup>6, 7, 21</sup> Our reports  
8 suggest that reduced androgen levels promoted the development of AGN in  
9 B6.MRLc1(82–100) mice. On the other hand, several previous studies on mouse models  
10 have revealed that oestrogen aggravates the disease, while castration ameliorates it in  
11 females.<sup>3, 6, 7, 11, 22</sup> However, in our study on B6.MRLc1(82–100) mice, histoplanimetric  
12 analysis revealed that the glomerular lesions were slightly ameliorated in some, albeit not in  
13 a statistically significant number, of the castrated female mice. These results strongly  
14 suggested that the male sex hormone, rather than the female one, was closely related to the  
15 sex differences in glomerular damage in the B6.MRLc1(82–100) mice.

16 The renal glomeruli express sex hormone receptors that participate in the extracellular  
17 matrix turnover.<sup>23, 24</sup> It has been reported that testosterone promotes the development of  
18 glomerular sclerosis, which is characterized by sclerotic lesions due to dysbolism of  
19 extracellular matrices such as mesangial matrices, while oestrogen has inhibitory effects on  
20 this disease.<sup>23</sup> However, in the present study using B6.MRLc1(82–100) mice, we did not  
21 obtain results similar to these previous reports. Therefore, we considered that the sex  
22 differences on glomerular damage in the B6.MRLc1(82–100) mice could not be estimated  
23 as the direct effect of sex hormones.

1 The sex of an individual greatly influences the immunological system. Females have been  
2 found to exhibit stronger immune responses against infectious diseases and greater  
3 susceptibility to autoimmune diseases than males.<sup>1</sup> We evaluated the immunological effects  
4 of gonadectomy on the spleen and thymus in B6.MRLc1(82–100) mice: the weights of both  
5 organs were found to be greater in females than in males, and the organ weights increased  
6 after gonadectomy in the males. Interestingly, we found that the sex differences in the  
7 effects of gonadectomy on the immune organs were related to the T cells rather than the B  
8 cells. In brief, larger areas of T cells were found in the spleens of the female mice than in  
9 those of the male mice, and these areas increased significantly in the male mice after  
10 gonadectomy. CD3-positive cells were localized on the thymic medulla in the male SG mice.  
11 In contrast, these cells were diffusely localized in both the cortex and medulla in the other  
12 mice. Among humans, males are reported to have a lower number of T cells than females,  
13 and the percentage of IL-2-producing T cells in males were lower than those in females.<sup>25</sup>  
14 Further, previous studies on mice have reported that the inhibitory effects of androgens on  
15 autoimmune diseases depend on the thymus and that testosterone inhibits the proliferation  
16 of T cell lines.<sup>11, 26</sup> In addition, the production of Th1 cytokines such as IL-2 and IFN- $\gamma$  has  
17 been reported to increase in castrated male C57BL/6 mice.<sup>27</sup> These reports suggest that T  
18 cell functions clearly differ between sexes and that testosterone has special inhibitory  
19 effects on their survival and function. On the basis of these reports, we considered that the T  
20 cells might have been activated in response to reduced androgen activity in the  
21 B6.MRLc1(82–100) mice and that the observed increase in T-cell infiltration into the  
22 glomeruli of the male GG mice might have been due to the elevated expression of local  
23 inflammatory mediators such as Th1 cytokines.

1 Autoantibodies to nuclear components such as histones, DNA, and RNA are detected in  
2 the serum of patients or model animals with autoimmune diseases. Sex hormones affect the  
3 production of these autoantibodies; in particular, oestrogen and testosterone function as  
4 aggravating and inhibitory factors, respectively.<sup>6, 7, 28</sup> In our study, although the female  
5 B6.MRLc1(82–100) mice exhibited significantly higher serum levels of total IgG and  
6 autoantibodies than the males, gonadectomy was not found to affect these parameters.  
7 These results suggested that the sex differences on autoantibody production in the  
8 B6.MRLc1(82–100) mice were not influenced by sex hormones, but possibly influenced by  
9 the sex chromosomes. In humans, the appearance of anti-tubular basement membrane Abs  
10 is associated with the X chromosome.<sup>29</sup> In Turner's syndrome characterized by monosomy  
11 of the X chromosome in females, the genes susceptible to thyroiditis, which is caused by  
12 autoantibody production, are localized on the X chromosome (p11.2–p22.1).<sup>30</sup> Further, the  
13 pathological differences between male and female BXSB mice have been confirmed to be  
14 closely related to the *Yaa* gene, which is generated by duplication of the *Tlr7* gene localized  
15 on the X chromosomes.<sup>13</sup> These reports indicate the existence of a relationship between  
16 sex chromosomal abnormalities and autoimmune diseases. In B6.MRLc1(82–100) mice, the  
17 sex chromosomes are derived from those of normal C57BL/6 mice. Although it is possible  
18 that interactions between the *Mag* locus and some genes on the X chromosomes might  
19 influence the sex differences with respect to autoantibody production in the  
20 B6.MRLc1(82–100) mice, further detailed analyses are required to clarify whether this is  
21 true.

22 The *Mag* locus carries genes of the *Fcgr* family that encode immune-complex receptors.  
23 In the previous study, we established that an imbalance between inhibitory and active *Fcgr*  
24 is closely related to the pathogenesis of AGN in B6.MRLc1(82–100) mice.<sup>20</sup> In the present



1 study, sex differences and the effects of gonadectomy on the *Fcgr* balance were assessed  
2 in B6.MRLc1(82–100) mice. We observed that the renal *Fcgr3* expression and  
3 *Fcgr3-Fcgr2b* ratio was elevated in the female mice of both groups and in the male GG mice.  
4 Among humans, females exhibit a greater number of CD16 (FcγRIII)-positive monocytes  
5 than males.<sup>31</sup> Furthermore, the FcγRII expression is reported to be lower in male SLE  
6 patients than in normal males and in female SLE patients.<sup>32</sup> Thus far, no report has  
7 discussed sex-related differences in the expression of *Fcgr*, and our results are interesting  
8 in that they implicate an *Fcgr* imbalance as a potential factor responsible for sex differences  
9 in the pathogenesis of AGN. Nevertheless, we consider that factors other than the *Mag*  
10 locus may also affect these sex differences. In a previous study, the results of a quantitative  
11 trait locus (QTL) analysis performed using F2 generations derived from MRL-lpr and  
12 C57BL/6 mice suggested that chromosome 7 in the C57BL/6 genome carries AGN- and  
13 splenomegaly-resistant genes regulated by the male sex.<sup>18</sup> These previous findings and our  
14 present data indicate that the sex differences on incidence of AGN in B6.MRLc1(82–100)  
15 mice might be influenced by the *Mag* locus and/or by these disease-associated genes in the  
16 C57BL/6 genome.

17 In conclusion, the sex differences and the effects of gonadectomy on the pathogenesis of  
18 AGN were analysed in B6.MRLc1(82–100) mice. In particular, the male GG mice exhibited  
19 exacerbation of the disease. Our results strongly suggested that the sex differences in the  
20 pathogenesis of AGN in B6.MRLc1(82–100) mice are primarily due to the inhibitory effects  
21 of the male sex hormone.

22

1 **Figure Legends**

2 Fig. 1. Body weights, serum levels of sex hormones, and kidney weights. (a) Body weights  
3 recorded along a time course. Male SG mice (diamond), male GG mice (triangle), female  
4 SG mice (square), and female GG mice (circle). Each value represents the mean  $\pm$  SEM. n  
5 = 3.

6 Fig. 2. Comparison of glomerular damage between the SG and GG groups. (a–d) Light  
7 micrographs of the glomeruli. Male SG mice (a), male GG mice (b), female SG mice (c), and  
8 female GG mice (d). Glomerular damage is more severe among the females than the males  
9 (panels a and c). The glomeruli of female SG mice exhibit severe proliferative and  
10 membranous lesions (panel c). GG males exhibit more severe proliferative lesions (rather  
11 than membranous lesions) than SG males. The proliferative lesions are characterized by  
12 expansion of the mesangial matrix, proliferation of the mesangial cells, and infiltration of  
13 mononuclear cells (panels a and b). Among the females, glomerular damage was  
14 ameliorated in some of the GG mice (panel d). Scale bar, 50  $\mu$ m. (e) Number of nuclei in the  
15 glomeruli. (f) Diameter of the renal corpuscles. Each value represents the mean  $\pm$  SEM.  
16 \*Significantly different from the values recorded for the SG mice of the same sex ( $p < 0.05$ ).  
17 n = 3.

18 Fig. 3. Comparison of the SG group and the GG group with regard to immune-complex  
19 deposition in the glomeruli. (a–d) Immunohistochemical staining for IgG2b. Male SG mice  
20 (a), male GG mice (b), female SG mice (c), and female GG mice (d). IgG2b  
21 immunoreactions are clearly observed in the glomerular basement membranes of the male  
22 GG, female SG, and female GG mice (panels b–d). However, tissue samples of the male SG  
23 mice are minimally stained (panel a). Scale bar, 50  $\mu$ m. (e) Number of IgG2b-positive  
24 glomeruli. Each value represents the mean  $\pm$  SEM. \*Significantly different from the values

1 recorded for SG mice of the same sex ( $p < 0.05$ ).  $n = 3$ . %: the number of IgG2b-positive  
2 glomeruli in 100 renal corpuscles.

3 Fig. 4. Comparison of the SG and GG groups with regard to lymphocyte infiltration into the  
4 glomeruli. (a–d) Immunohistochemical staining for CD3. (e–h) Immunohistochemical  
5 staining for B220. Male SG mice (a and e), male GG mice (b and f), female SG mice (c and  
6 g), and female GG mice (d and h). Infiltration of CD3-positive mononuclear cells is observed  
7 in the glomeruli of the male GG, female SG, and female GG mice (panels b–d). However,  
8 this infiltration is minimal in the case of the male SG mice (panel a). Infiltration of  
9 B220-positive mononuclear cells is not observed in the glomeruli of any of the mice. Instead,  
10 B220-positive cells are observed in the capillary lumen of the interstitium (arrow). Scale bar,  
11 50  $\mu\text{m}$ . (e) Number of CD3-positive glomeruli. Each value represents the mean  $\pm$  SEM.  
12 \*Significantly different from the values recorded for the SG mice of the same sex ( $p < 0.05$ ).  
13  $n = 3$ . %: number of IgG2b-positive glomeruli in 100 renal corpuscles.

14 Fig. 5. Comparison of the areas of T and B cells in the spleen. (a–d)  
15 Immunohistochemical staining for CD3. (e–h) Immunohistochemical staining for B220. CD3-  
16 and B220-positive areas are mainly observed in the periarteriolar lymphatic sheath and  
17 splenic lymphoid follicles, respectively, and these findings do not differ between the groups.  
18 Scale bar, 200  $\mu\text{m}$ . (i) Histoplanimetric analysis for CD3-positive areas in the spleen. (j)  
19 Histoplanimetric analysis for B220-positive areas in the spleen. Each value represents the  
20 mean  $\pm$  SEM. \*Significantly different from the values recorded for the SG mice of the same  
21 sex ( $p < 0.05$ ).  $n = 3$ .

22 Fig. 6. Localization of T cells in the thymus. (a–d) Immunohistochemical staining for CD3.  
23 CD3-positive cells are mainly observed in the thymic medulla in all the mice (panels a–d).  
24 However, except in the case of the male SG mice (panel a), these cells are also diffusely

1 localized in the thymic cortex of the mice (panels b–d). Scale bar, 200  $\mu$ m. C: cortex. M:  
2 medulla.

3 Fig. 7. Comparison of the relative expression levels of *Fcgr2b* and *Fcgr3* mRNA in the  
4 kidney, as determined by quantitative real-time PCR analysis. (a) *Fcgr2b*, (b) *Fcgr3*, and (c)  
5 *Fcgr3-Fcgr2b* ratio. The raw values obtained for *Fcgr2b* and *Fcgr3* are normalized to those  
6 obtained for  $\beta$ -*actin* and expressed as fold increases from the values recorded for the male  
7 SG mice. Each value represents the mean  $\pm$  SEM. \*Significantly different from the values  
8 recorded for the SG mice of the same sex ( $p < 0.05$ ). n = 3.

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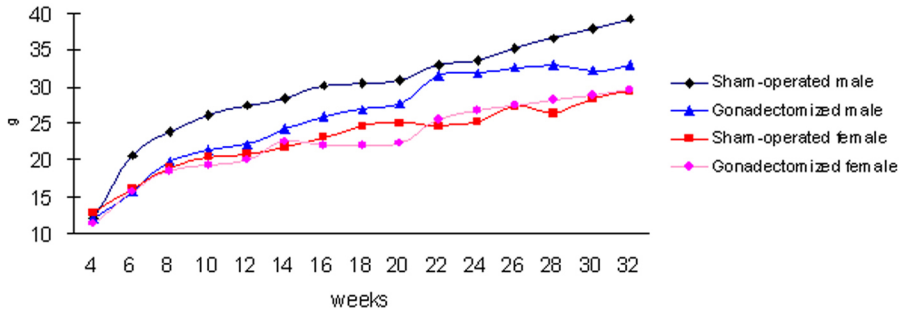
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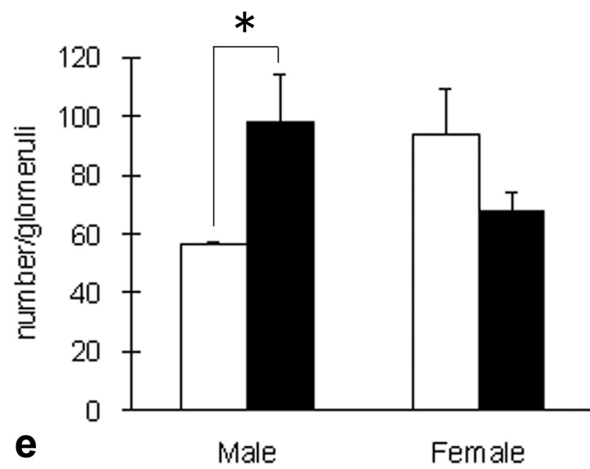
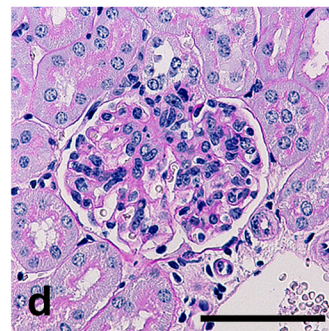
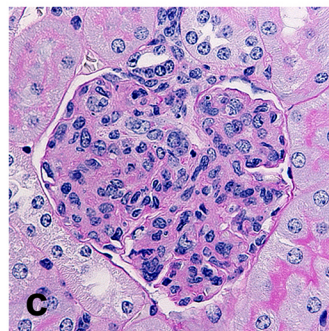
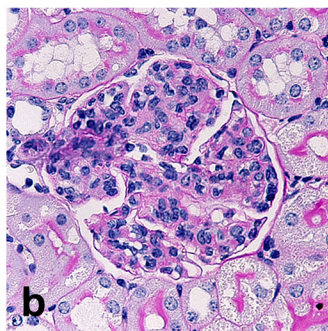
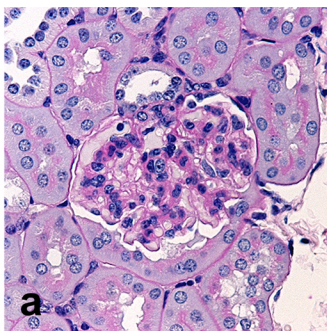
2  
3 Table 1. Summary of clinical parameters in B6.MRLc1(82–100) mice aged 8 months.

4

Parameters	Male		Female	
	Sham-operated	Gonadectomized	Sham-operated	Gonadectomized
5 Total kidney weights/ 6 Body weights (%)	1.14 ± 0.01	0.87 ± 0.05*	1.25 ± 0.19	0.94 ± 0.03
7 Spleen weights/ 8 Body weights (%)	0.32 ± 0.05	0.63 ± 0.13*	0.67 ± 0.03	0.46 ± 0.09*
9 Thymus weights/ 10 Body weights (%)	0.23 ± 0.03	0.42 ± 0.08*	0.46 ± 0.01	0.34 ± 0.03*
11 Serum BUN (mg/dl)	26.87 ± 1.36	27.71 ± 1.99	29.62 ± 2.91	41.45 ± 8.55
12 Albuminuria (mg/dl)	2.26 ± 0.39	4.83 ± 1.12*	1.89 ± 0.54	2.55 ± 0.31
13 Serum total IgG (mg/ml)	3.25 ± 0.27	3.42 ± 0.18	5.47 ± 0.88	7.19 ± 3.61
Serum anti-dsDNA (mg/ml)	0.12 ± 0.01	0.15 ± 0.02	0.42 ± 0.12	0.44 ± 0.15

Each value represents the mean ± SEM. \*: Significantly different from the values recorded for animals of the same sex in the sham-operated group. n = 3. Statistical analysis: Student's t-test ( $p < 0.05$ ). The normal values (female C57BL/6 mice; age, 6 months) of values of blood urea nitrogen (BUN), albuminuria, total IgG, and anti-dsDNA antibody are 16.94mg/dl, 0.48mg/dl, 3.29mg/ml, and 0.11mg/ml, respectively.

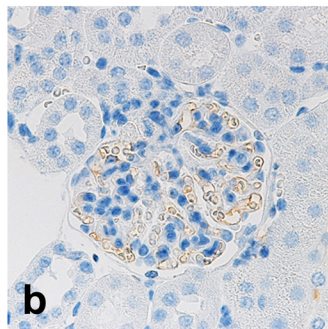
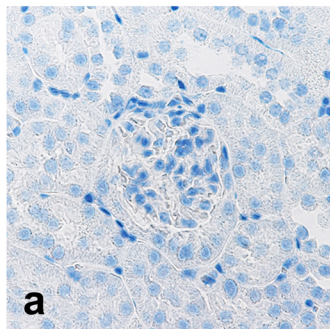


**Male****Female****Sham-operated****Gonadectomized****Sham-operated****Gonadectomized**

## Male

### Sham-operated

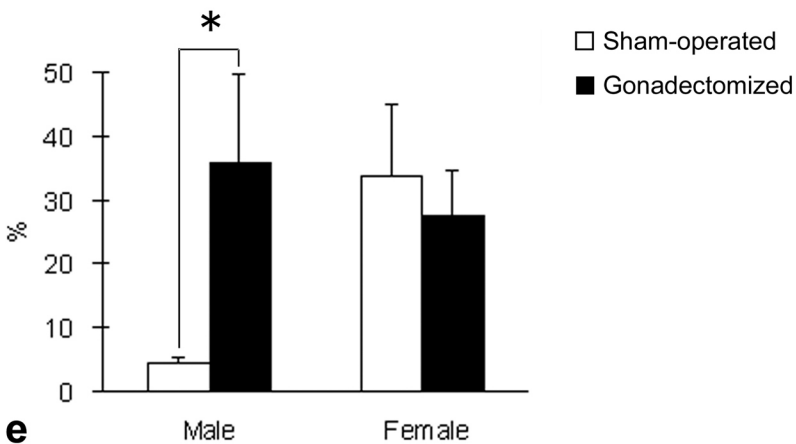
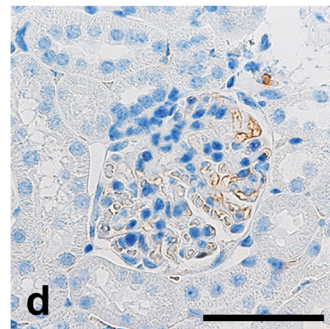
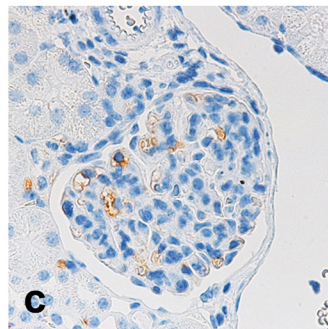
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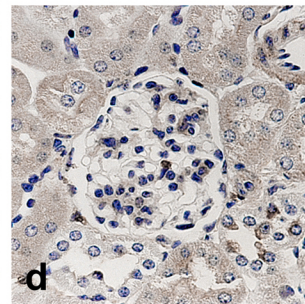
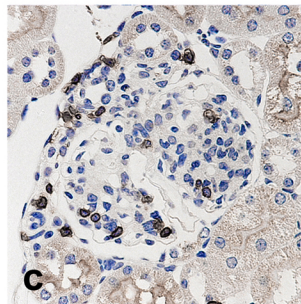
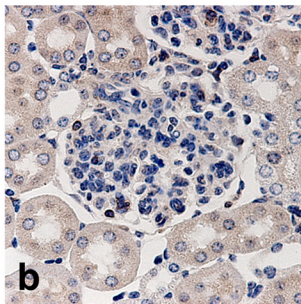
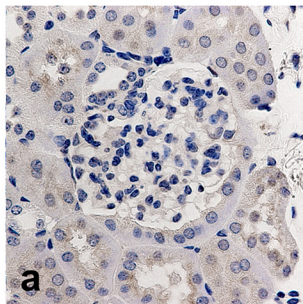
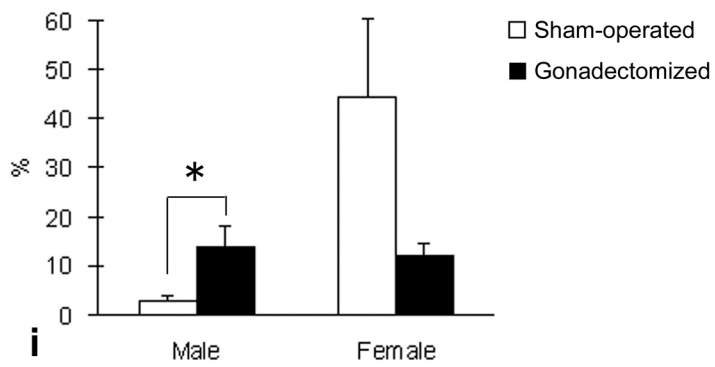
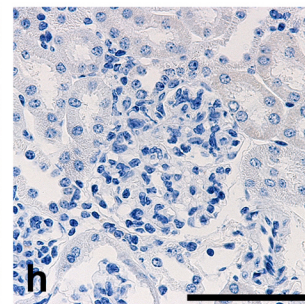
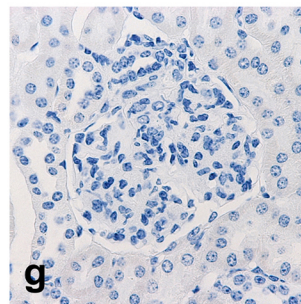
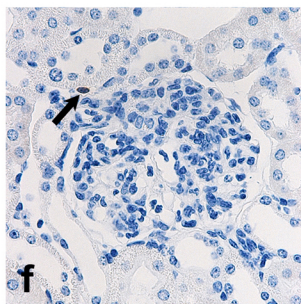
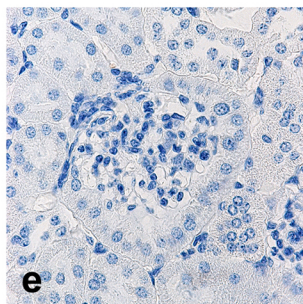


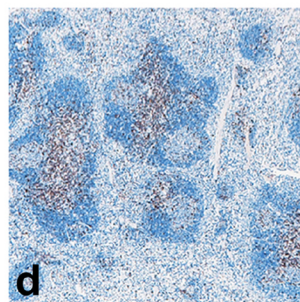
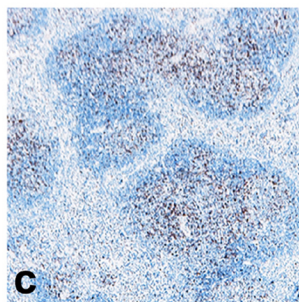
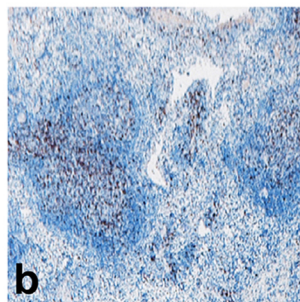
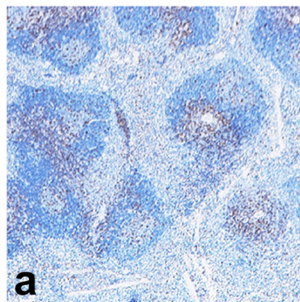
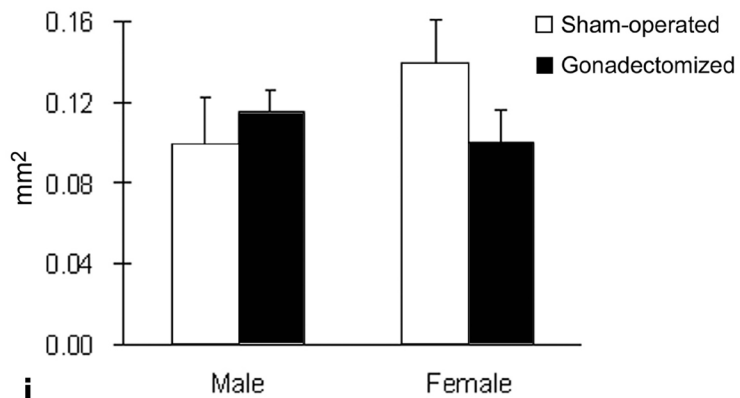
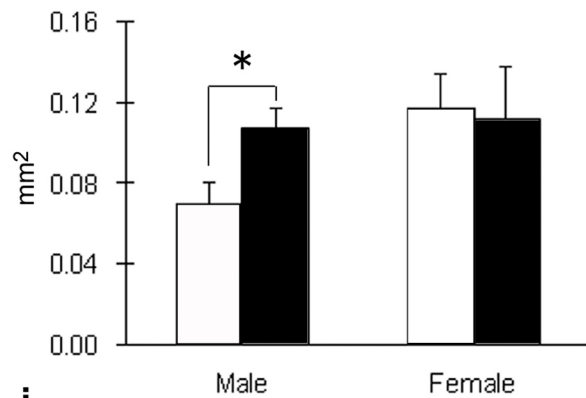
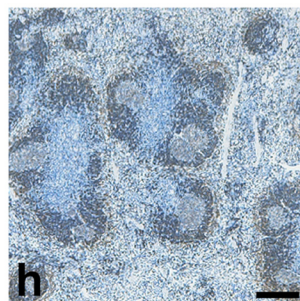
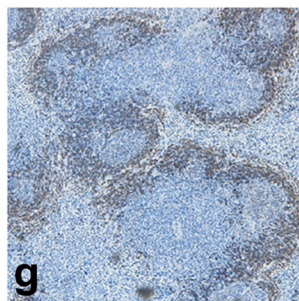
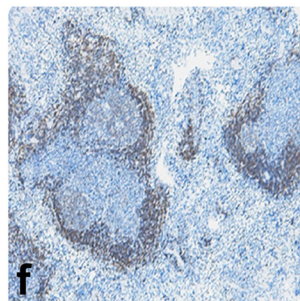
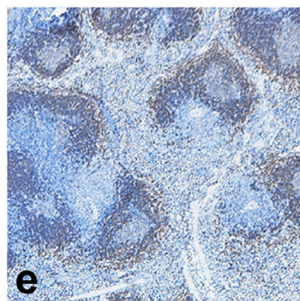
## Female

### Sham-operated

### Gonadectomized



**Male****Female****Sham-operated****Gonadectomized****Sham-operated****Gonadectomized****CD3****B220**

**Male****Female****Sham-operated****Gonadectomized****Sham-operated****Gonadectomized****CD3****B220**

# Male

# Female

Sham-operated

Gonadectomized

Sham-operated

Gonadectomized

**CD3**

