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**MRL/MpJ-*Fas*^{lpr} mice show abnormalities in ovarian function
and morphology with the progression of autoimmune disease**

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Running head: Ovary abnormalities in MRL/MpJ autoimmune mice

Keywords: MRL/lpr mice, autoimmune disease, ovary, estrous cycle, ovarian reserve

Abstract

The immune system is known to affect reproductive function, and maternal-fetal immune tolerance is essential for a successful pregnancy. To investigate the relationship between autoimmune disease and female reproductive function, we performed a comparative analysis of the ovarian phenotypes for C57BL/6 mice, autoimmune disease-prone MRL/MpJ (MRL/+) mice, and congenic MRL/MpJ-*Fas*^{lpr} (MRL/lpr) mice harboring a mutation in the *Fas* gene that speeds disease onset. Both MRL-background strains showed earlier vaginal opening than C57BL/6 mice. The estrous cycle became irregular by 6 and 12 months of age in MRL/lpr mice and mice of the other two strains, respectively. Histological analysis at 3 months revealed that the number of primordial follicles was smaller in MRL-background mice than in C57BL/6 mice after 3 months. In addition, MRL/lpr and MRL/+ mice displayed lower numbers of ovarian follicles and corpora lutea at 3 and 6 months, and 6 and 12 months, respectively, than that in age-matched C57BL/6 mice. MRL/lpr and MRL/+ mice developed ovarian interstitial glands after 3 and 6 months, respectively. In particular, MRL/lpr mice showed numerous infiltrating lymphocytes within the ovarian interstitia, and partially stratified ovarian surface epithelia with more developed microvilli than that observed in

35 C57BL/6 mice at 6 months. No significant differences in serum hormone levels were
36 observed between the strains. In conclusion, MRL/lpr mice display altered ovarian
37 development, morphology, and function consistent with the progression of severe
38 autoimmune disease, as these findings are less severe in MRL/+ counterparts.

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Introduction

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In mammals, the female reproductive period is limited by the finite number of oocytes in the ovaries. In humans, the total number of oocytes gradually decreases with age due to physiological processes such as ovulation and follicular atresia. However, gonadal dysfunction, such as premature ovarian failure (POF), can cause an unexpected decrease in the number of oocytes stored in the ovaries. Patients with POF show amenorrhea, hypoestrogenism, and elevated serum levels of follicle-stimulating hormone (FSH) by 40 years of age [1]. An estimated 1.5 million patients are diagnosed with POF each year, and approximately 1% of women worldwide live with this disease [2, 3]. Several reports have suggested that POF is caused by genetic factors, infectious agents, or iatrogenic causes [1, 2]. However, approximately one-third of POF cases are attributable to autoimmune diseases [2]. Autoimmune-associated POF is characterized by lymphocytic oophoritis and/or by the presence of ovarian autoantibodies and is usually observed in conjunction with autoimmune diseases such as Addison's disease [1, 2, 4]. In most cases, it is difficult for patients with POF to reacquire fertility with ovulation of healthy oocytes [1, 2].

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In healthy individuals, many immune cells are present in the ovaries, suggesting

that they play important roles in ovarian function in several species, such as rodents, cows, sheep, pigs, and horses [5]. For example, many leukocytes, such as lymphocytes, granulocytes, and macrophages, are found in the corpora lutea in rodents, cows, and horses. Furthermore, the number and type of immune cells present in corpora lutea change with the estrous cycle, suggesting that these leukocytes participate in the development and regression of corpora lutea [6, 7, 8]. In addition to immune cells, a variety of cytokines and their receptors are expressed in the ovaries, indicating that they play important roles in the control of ovarian function [9]. For example, interleukin 6 (IL-6) is expressed in granulosa cells, where it regulates the expression of the luteinizing hormone (LH) receptor in rats [10]. Additionally, interleukin-1 (IL-1) signaling via IL-1 β and its receptor are essential for ovarian folliculogenesis and ovulation in equine ovaries [11]. In pigs, tumor necrosis factor alpha (TNF α) acts as a survival factor for granulosa cells during follicular atresia [12]. Furthermore, several studies in clinical veterinary medicine also support the notion that altered immune function may affect reproductive function. Briefly, dogs suffering autoimmune-mediated thyroiditis are anestrus, suggesting a loss of reproductive function [13]. In laboratory animals, autoimmune regulator (*Aire*)-deficient mice, which show severe autoimmune phenotypes, are infertile, with follicular depletion and lymphocyte infiltration in their

ovaries [3]. Furthermore, the pathogenesis of autoimmune disease is influenced by the production of sex steroid hormones in the gonads; in particular, estrogen aggravates systemic lupus erythematosus (SLE) in humans and rodent models [14, 15]. These data suggest a close relationship between immune and reproductive function in mammals.

BXSB/MpJ-*Yaa*, NZBWF1, and MRL/MpJ-*Fas*^{*lpr*} (MRL/*lpr*) mice are used as autoimmune disease models, and their pathology resembles that of human SLE and rheumatoid arthritis [16, 17]. MRL/*lpr* mice show a much more severe autoimmune phenotype than congenic MRL/MpJ (MRL/+) mice, which results from the lymphoproliferation (*lpr*) mutation of *Fas* that causes abnormal survival of auto-reactive T lymphocytes [18]. In addition to the *lpr* mutation, the MRL background is prone to the development of autoimmune diseases, as it carries at least 13 disease susceptibility loci associated with the development of SLE-related diseases [19, 20, 21]. The onset of SLE is usually characterized by splenomegaly, autoantibody production, and glomerulonephritis. Accordingly, spleen weights significantly correlated with the serum autoantibody levels in MRL background strain [22], supporting their use as indicators of disease severity. Interestingly, MRL mice also manifest several unique phenotypes in the female reproductive organs [23, 24]. In particular, neonatal MRL/+ mice show accelerated folliculogenesis associated with the appearance of numerous mast cells in

the ovary [24]. Further, adult MRL/+ mice frequently develop ovarian cysts, originating from the rete ovarii [23]. Although MRL/+ mice show several abnormalities in both immune and reproductive function, the pathological and etiological correlations remain unclear.

In this study, we used MRL-background mice to evaluate the relationship between autoimmune disease and female reproductive function. Female MRL/lpr mice showed abnormalities not only of ovarian function but also of morphology, characterized by early loss of the estrous cycle accompanied by a significant increase in spleen weight. These findings indicate that severe autoimmune disease affects female reproductive function, and provide novel insights into the treatment of infertility derived from immune system disruption.

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Methods

106 **Animal and sample preparation**

107 Female C57BL/6, MRL/+, and MRL/lpr mice were purchased from Japan SLC, Inc.

108 (Hamamatsu, Japan). Mice were maintained according to *The Guide for the Care and*

109 *Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary*

110 *Medicine* (approved by the Association for Assessment and Accreditation of Laboratory

111 Animal Care International). Virgin female mice at 3, 6, 9, and 12 months of age were

112 used at the metestrus stage of the estrous cycle, as determined by monitoring vaginal

113 smears. Body weights were measured, and then blood samples were collected by cutting

114 vena cava under deep anesthesia (Avertin; 2,2,2-tribromoethanol dissolved in

115 2-methyl-2-butanol, 2.4 g/kg, administered intraperitoneally). After mice were

116 euthanized by cervical dislocation, the ovaries and spleen were collected. Spleen weight

117 was measured, and the spleen weight to body weight ratio was calculated as an index of

118 autoimmune disease progression.

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120 **Examination of vaginal opening and estrous cycles**

The vaginal opening status was monitored in all mice as an indicator of the onset of puberty. After vaginal opening, vaginal smears were collected for 10 consecutive days every month until 12 months of age to assess estrous cyclicity. Properties of epithelial cells, leukocytes, and vaginal mucus were used to characterize each stage, as follows: “proestrus” was characterized by the appearance of round and nucleated epithelial cells without leukocytes; “estrus” was characterized by clustered and keratinized squamous epithelial cells; “metestrus” was characterized by a large number of leukocytes; and “diestrus” was characterized by low numbers of leukocytes and the presence of vaginal mucus. Metestrus was included in diestrus based on a previous report [25].

Histological analyses

Collected ovaries were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (PB) overnight, embedded in paraffin, and cut into sections (3 μ m thick). Deparaffinized sections were stained with hematoxylin-eosin (HE) or periodic acid-Schiff (PAS) for evaluation of ovarian morphology. The number of primordial, primary, secondary, and antral follicles was counted in five sections, and only follicles containing oocytes with an apparent nucleus were counted. The number of corpora lutea was also counted in five

sections. Finally, the total number counted in all five sections was used as the value for the respective ovary.

Some sections were immunostained using the following procedure: for antigen retrieval, sections were incubated in 20 mM Tris-HCl (pH 9.0) for 20 min at 105°C (CD3) or 0.05% trypsin/0.01 M phosphate-buffered saline (PBS, pH 7.4) for 5 min at 37°C (B220). The samples were then soaked in methanol containing 0.3% H₂O₂ to block internal peroxidase activity. Sections blocked in 10% normal goat serum for 30 min at room temperature were incubated with rat anti-B220 (1:1,600, Cedarlane, Ontario, Canada) or rabbit anti-CD3 (1:200, Nichirei, Tokyo, Japan) at 4°C overnight. After washing three times in PBS, sections were incubated with biotin-conjugated goat anti-rat IgG antibody (Caltag-Medsystems Limited, Buckingham, UK) for B220, or biotin-conjugated goat anti-rabbit IgG antibody (SABPO Kit, Nichirei, Tokyo, Japan), for 30 min at room temperature, washed again, and incubated with streptavidin-biotin complex (SABPO Kit, Nichirei) for 30 min. The sections were then incubated with 3,3'-diaminobenzidine tetrahydrochloride-H₂O₂ solution. Finally, the sections were lightly counterstained with hematoxylin. The number of CD3-positive cells in each immunostained section was counted, and the total number in five sections was used as the value for the respective ovary.

Ultrastructural analysis

For scanning electron microscopy (SEM), halves of glutaraldehyde-fixed ovaries were placed in 2% tannic acid for 1 h at 4°C and postfixed with 1% osmium tetroxide in 0.1 M PB for 1 h. Specimens were dehydrated through a series of graded alcohols, transferred into 3-methylbutyl acetate, and dried using an HCP-2 critical point dryer (Hitachi, Tokyo, Japan). The dried specimens were sputter-coated using a Hitachi E-1030 ion sputter coater (Hitachi) and then examined on an S-4100 SEM (Hitachi) with an accelerating voltage of 5 kV.

For transmission electron microscopy (TEM), the ovaries of C57BL/6 and MRL/lpr mice at 6 months were fixed with 2.5% glutaraldehyde in 0.1 M PB for 4 h. Tissues were post-fixed with 1% osmium tetroxide in 0.1 M PB for 2 h, dehydrated using a series of graded alcohols, and embedded in epoxy resin (Quetol 812 Mixture; Nisshin EM, Tokyo, Japan). Semi-thin sections (0.5 µm thick) were stained with 1% toluidine blue and examined. Ultra-thin sections (70 nm thick) were double-stained with uranyl acetate and lead citrate and observed under a JEOL TEM (JEM-1210; JEOL, Tokyo, Japan).

Measurement of serum hormone levels

Collected blood was centrifuged at $885 \times g$ for 15 min. After clotted blood was removed, the samples were centrifuged at $22,136 \times g$ for 5 min, and the supernatant was immediately frozen at -80°C . Serum levels of endogenous mouse FSH and testosterone were measured using ELISA kits (Endocrine Technologies Inc., Newark, USA) according to the manufacturer's instructions. All samples were tested in duplicate.

Statistical analysis

The results are expressed as the mean $\pm$ standard error (SE) and were analyzed using non-parametric statistical methods. The Kruskal-Wallis test was used to compare numerical results, and multiple comparisons were performed using Scheffe's method when significant differences were observed ($P < 0.05$).

Results

Autoimmune disease symptoms of mice

The spleen weight to body weight ratio was used as an index of autoimmune disease in mice (Figure 1). At all ages examined, MRL/lpr mice showed significantly higher values than the other two strains ($P < 0.05$). At 9 and 12 months, although MRL/+ mice showed slightly higher values than C57BL/6 mice, no significant differences were observed between C57BL/6 and MRL/+ mice.

Onset of puberty and estrous cyclicity of mice

Vaginal opening was observed in C57BL/6, MRL/+, and MRL/lpr mice to determine the onset of puberty (Figure 2). Vaginal opening occurred significantly earlier in MRL/+ (18.8 ± 4.3 days) and MRL/lpr mice (19.8 ± 1.7 days) than in C57BL/6 mice (39.6 ± 2.9 days) (Figure 2) ($P < 0.05$).

Age-related changes in estrous cyclicity were examined next (Figure 3). Figure 3A shows the representative pattern of mouse estrous cycle in each of the three strains at 3, 6, 9, and 12 months. C57BL/6 and MRL/+ mice showed regular cyclicity, with each cycle lasting 4–7 days until 9 months of age. These strains showed regular cyclicity

until 11 months of age (data not shown). However, MRL/lpr mice lost cyclicity after 6 months, characterized by a prolonged diestrus period and a shortened estrus period.

Figure 3B and C show the proportion of time spent in estrus and diestrus for each of the three strains. Estrus was significantly shorter in MRL/lpr mice than in the other strains at 6 months of age ($P < 0.05$). At 12 months, C57BL/6 and MRL/+ mice tended to show shorter and longer periods of estrus, respectively, compared to earlier months; however, no significant differences were observed among strains (Figure 3B). In addition, diestrus was longer in MRL/lpr mice at 6 months of age than in the other strains ($P < 0.05$). At 12 months, C57BL/6 and MRL/lpr mice showed longer periods of diestrus than in earlier months, but no significant differences were observed among strains (Figure 3C).

Age-related histological changes in mouse ovaries

Figure 4 shows the ovarian histology of C57BL/6, MRL/+, and MRL/lpr mice at 3, 6, and 12 months. At 3 months, follicles at various stages of development were observed at the ovarian cortices in all three strains (Figure 4A, D, and G); the number tended to decrease with age (Figure 4B, C, E, F, H, and I). In particular, very few follicles were observed in MRL/lpr mice at 12 months (Figure 4I). At all ages examined, a large

number of corpora lutea were observed in C57BL/6 and MRL/+ mice (Figure 4A–F); however, few were observed in MRL/lpr mice (Figure 4G–I). Further, the ovarian interstitium was larger in MRL/lpr mice (Figure 4G–I) than in the other strains.

Next, we quantified the number of follicles at each stage of follicular development (primordial, primary, secondary, and antral) and the number of corpora lutea at 3, 6, and 12 months of age in all three strains. The total number of follicles decreased with age in all strains (Figure 4J–L), and the number of follicles observed at each stage differed among strains and at different ages of the mice. Briefly, MRL/+ and MRL/lpr mice showed a smaller number of primordial follicles than C57BL/6 mice at all ages examined, and significant differences between strains were observed at 3 and 6 months (Figure 4J–K) ($P < 0.05$). Further, MRL/+ mouse ovaries contained a significantly larger number of secondary follicles than the other two strains at 3 months (Figure 4J) ($P < 0.05$). The total number of follicles was significantly lower in MRL/lpr mice than in the other two strains at 3 months, and in both MRL/+ and MRL/lpr mice than in C57BL/6 mice at 6 months (Figure 4J–K) ($P < 0.05$). In addition, the number of corpora lutea was lowest in MRL/lpr mice at all ages examined; it was also significantly lower in MRL/+ than in C57BL/6 mice at 12 months (Figure 4M) ($P < 0.05$).

As shown in Figure 5, strain-related differences in ovarian morphology were

observed at the ovarian interstitium and on the ovarian surface. The ovarian interstitium contains interstitial glands comprised of interstitial endocrine cells (IECs) (Figure 5A–I). These IECs originate from internal theca or granulosa cells of atretic follicles and primarily produce testosterone in rodents [26]. IECs were clearly observed in C57BL/6 mice at 12 months (Figure 5C) and in MRL/+ mice after 6 months (Figure 5E–F). However, large numbers of IECs were clearly present in MRL/lpr mouse ovaries at all ages examined (Figure 5G–I). Finally, the ovarian surface epithelium (OSE) was a squamous monolayer in C57BL/6 and MRL/+ mice at all ages examined (Figure 5J–K), whereas a partially cuboidal, stratified epithelium was observed in MRL/lpr mice after 6 months (Figure 5L).

Ultrastructure of the ovarian surface

The morphology of the ovarian surface was compared between C57BL/6 and MRL/lpr mice at 6 months by using electron microscopy. Under SEM observation, the ovarian surface of C57BL/6 mice appeared smooth (Figure 6A) and was mostly covered with oval- or spindle-shaped epithelial cells with microvilli (Figure 6B). In contrast, the ovarian surface of MRL/lpr mice showed several dome-shaped bulges (Figure 6C) covered by rounded epithelial cells that were fully covered in microvilli (Figure 6D).

The microvillus density of epithelial cells appeared to be higher in MRL/lpr mice than in C57BL/6 mice (Figure 6B and D).

Next, the ovarian surface was observed using semi-thin and ultra-thin sections (Figure 6E–H). In C57BL/6 mice, the OSE consisted of monolayered dark- or pale-colored cells (Figure 6E). Under TEM, the dark-colored cells in semi-thin sections showed a highly electron-dense cytoplasm and few microvilli, whereas the pale-colored cells had a cytoplasm with low electron density and a large round nucleus similar to that found in oocytes (Figure 6F). Likewise, two cell types were observed in the semi-thin OSE sections of MRL/lpr mice (Figure 6G). However, the OSE cells and ovarian tunica albuginea (lowermost layer of the ovary capsule) were taller and thicker, respectively, in MRL/lpr mice than in C57BL/6 mice (Figure 6E and G), which was also accompanied by an increase in the presence of collagen fibrils (Figure 6H). Under TEM, the dark-colored cells in the semi-thin sections showed a highly electron-dense cytoplasm and abundant microvilli in MRL/lpr mice (Figure 6H). The pale-colored cells in semi-thin sections had a cytoplasm with low electron density and a larger nucleus than the dark-colored cells (Figure 6H). The microvillus density of cells with a highly electron-dense cytoplasm in MRL/lpr mice appeared to be higher than that in C57BL/6 mice (Figure 6F and H), as well as in cells of MRL/lpr mice with a cytoplasm with low

electron density (Figure 6H).

Inflammatory cell infiltration in mouse ovaries

Immunohistochemical analysis was used to detect T and B cells in the ovaries of C57BL/6, MRL/+, and MRL/lpr mice (Figure 7). At 6 months, few CD3-positive T cells were observed in the ovaries of C57BL/6 and MRL/+ mice, which tended to localize in or around the corpora lutea (Figure 7A and B). However, numerous CD3-positive T cells were observed in the ovaries of MRL/lpr mice (Figure 7C). These cells were widely distributed, and mainly localized to the ovarian interstitium (Figure 7C). Quantification of CD3-positive T cells in the ovaries showed that MRL/lpr mice showed significantly higher values than the other two strains at both 3 and 6 months of age (Figure 7D) ($P < 0.05$). At 3 months, CD3-positive T cells were observed in or around the follicles of MRL/lpr mice (Figure 7E–F). Further, few B220-positive B cells were observed in the ovaries of C57BL/6 and MRL/+ mice at any period examined (Figure 7G–H), whereas some MRL/lpr mice showed numerous positive cells in the ovarian interstitium at 6 months (Figure 7I).

Serum levels of FSH and testosterone in mice

295 We next compared the blood serum levels of FSH and testosterone—indicators of
296 reproductive ability and IEC function, respectively—in C57BL/6, MRL/+, and MRL/lpr
297 mice at 6 and 12 months (Figure 8) [26, 27]. Although serum FSH levels tended to be
298 slightly higher in MRL/lpr mice than in C57BL/6 and MRL/+ mice at 12 months, no
299 significant differences among strains were observed at any age (Figure 8A). Similar to
300 FSH, serum testosterone levels were higher in MRL/lpr mice than in C57BL/6 and
301 MRL/+ mice at 6 and 12 months, but the differences were not significant (Figure 8B).

Discussion and Conclusions

Several studies have indicated a close correlation between the immune and reproductive systems [5, 9]. This study examined the effect of autoimmune disease on ovarian morphology using MRL-background mouse strains. From 3 months, MRL/lpr mice showed autoimmune defects, indicated by significantly increased spleen weight compared to MRL/+ and C57BL/6 mice. At the same age, the total number of follicles and corpora lutea tended to be smaller in MRL/lpr mice than in mice of other strains. Further, numerous IEC and infiltrating lymphocytes were observed in MRL/lpr mouse ovaries. Importantly, lymphocytic oophoritis with loss of the follicle pool has also been reported in autoimmune-mediated POF [1, 2, 4]. Although the MRL background carries several susceptibility loci associated with the development of autoimmune defects [19, 20], changes in ovarian morphology and development of autoimmune disease were less pronounced in MRL/+ mice than in MRL/lpr mice. Further, some MRL/+ and MRL/lpr mice developed ovarian cysts, as reported previously [23]; however, no correlation between cyst development and the parameters evaluated was found in this study (data not shown). Importantly, our data indicates that splenomegaly (3 months) precedes the onset of estrous cycle irregularity (6 months) in MRL/lpr mice. Therefore, the *lpr*

mutation and/or its associated autoimmune defects may be crucial to the changes in ovarian morphology observed in MRL-background strains.

It has been proposed that granulosa cells or oocytes may form from the mesodermal OSE [28, 29]. In this study, both dark- and pale-colored cells were observed in semi-thin sections of the OSE. The pale cells were likely oocytes or their progenitors, considering their nuclear shape and previous reports [28]. The OSE showed a unique morphology in MRL/lpr mice but not in MRL/+ or C57BL/6 mice at 6 months. Ultrastructurally, the microvilli were more developed in MRL/lpr mice than in C57BL/6 mice, as shown by SEM examination of the OSE and TEM observation of dark-colored cells, and the OSE was partially stratified in MRL/lpr mice. Although no clear evidence elucidating the mechanism for altering OSE structures was found, altered morphology of the peritoneal mesothelium has been reported in ascitic conditions—such as intraperitoneal dialysis [30]. Further, patients with autoimmune diseases or ovarian cancer show altered amounts or components of ascitic fluid [31, 32]. Therefore, the features of OSE in MRL/lpr mice may reflect an altered environment in or around the ovaries.

Further, only MRL/lpr mice showed a thickened ovarian tunica albuginea similar to that reported in patients with polycystic ovarian syndrome and in model mice [33, 34].

Thickening of the tunica albuginea could contribute to infertility by disturbing ovulation.

In this study, the ability of MRL/lpr mice to ovulate or become pregnant remains unclear; however, evaluation of the tunica albuginea may be important to elucidate the pathogenesis of ovulation disorders in autoimmune diseases, such as autoimmune-mediated POF.

MRL/lpr mice showed an irregular estrous cycle associated with the significant increase of spleen weights and morphological changes in the ovaries beginning at 6 months of age. The estrous cycle progresses through folliculogenesis, ovulation, and luteinization [35]. These processes are coordinated by oocytes, follicular epithelial cells/granulosa cells, and lutein cells via the interaction of gonadotropins such as LH and FSH [35]. Furthermore, loss of the follicle pool and elevated serum FSH levels are pathological characteristics in human patients with POF showing an altered menstrual cycle [14]. In this study, no significant differences were observed in serum levels of FSH among strains, but MRL/lpr mice showed significantly decreased numbers of follicles and corpora lutea. Therefore, abnormal populations of ovarian functional units—such as follicles and corpora lutea—rather than serum gonadotropin levels, may have greater impact on the cyclicity of the estrous cycle in this murine autoimmune disease model.

In this study, MRL/+ and MRL/lpr mice showed several common phenotypes, such as earlier vaginal opening and fewer primordial follicles than C57BL/6 mice, despite the significant increase of spleen weights observed in MRL/+ mice. A previous study suggested that MRL/+ mice showed enhanced progression through early folliculogenesis than C57BL/6 mice [22, 36]. Therefore, the earlier onset of puberty and fewer primordial follicles compared to C57BL/6 mice are unique phenotypes in MRL-background strains.

However, the appearance of MRL-derived unique ovarian phenotypes differed between MRL/+ and MRL/lpr mice in several ways. Briefly, MRL/+ mouse ovaries contained a larger number of secondary follicles than those of MRL/lpr mice at 3 months. Further, compared to C57BL/6 mice, the total number of follicles was smaller from 3 and 6 months in MRL/lpr and MRL/+ mice, respectively. These results suggest that the unique phenotypes associated with folliculogenesis in MRL/+ mice were altered in MRL/lpr mice. Moreover, MRL/lpr mice exhibited an increased infiltration of T cells in or around follicles after 3 months of age, suggesting that these cells or their derived cytokines may affect folliculogenesis.

Testosterone-producing IECs originate from internal theca cells or granulosa cells of atretic follicles and develop in the ovarian interstitium of rodents [26]. In this study,

although the number of corpora lutea was smaller, the number of IECs in the ovaries of MRL/lpr mice was increased after 3 months, and a similar tendency was observed in older MRL/+ mice. Therefore, the MRL background may contribute to the development of IECs in both MRL/+ and MRL/lpr mice. Further, the serum testosterone concentration tended to be higher in MRL/lpr mice than in mice of the other strains, possibly reflecting the development of IECs in MRL/lpr mice.

In mammalian ovaries, the development and regression of corpora lutea is regulated by cytokines produced by immune cells observed in or around corpora lutea via NF κ B signaling [5, 9]. In the present study, few lymphocytes were observed in or around the corpora lutea of C57BL/6 and MRL/+ mice. In contrast, a pronounced infiltration of B and T cells into the ovarian interstitium was observed in MRL/lpr mice. Furthermore, previous reports have suggested that granulosa cells and theca cells express Fas, and that Fas-Fas ligand-mediated apoptosis is important for the regression of corpora lutea [37, 38]. Based on these findings, we suggest that changes in the local immune microenvironment and/or cell death resulting from *lpr* mutation in the *Fas* gene may affect the differentiation of internal theca cells/granulosa cells into IECs.

In conclusion, we demonstrated a relationship between ovarian morphology and reproductive function in MRL/lpr autoimmune mice. These data may aid in clarifying

392 the pathogenic mechanism of ovarian dysfunction in autoimmune disease. Briefly,
393 intraovarian infiltration of immune cells, loss of the follicle pool, and a disrupted
394 estrous cycle are common pathological features of autoimmune disease between POF
395 patients and MRL/lpr mice. While MRL/lpr mice did not clearly exhibit the increased
396 levels of FSH characteristic of POF, they did display unique ovarian phenotypes
397 including failures in follicle luteinization, increased IECs, and altered ovarian surface
398 morphology. Menstrual abnormalities, increased cycle length, and decreased serum
399 levels of hormones produced by corpora lutea were observed in patients with SLE [39,
400 40]. Ovarian reserve markers such anti-Müllerian hormone and antral follicle count
401 were decreased in adult patients with childhood-onset SLE [41]. In MRL/lpr mice, early
402 onset puberty and altered follicular development were observed for the same time or
403 earlier than the development of autoimmune defects indicated by increased spleen
404 weight. Importantly, it was reported that early age at menarche is associated with an
405 increased risk of SLE in human patients [42]. Taken collectively, we conclude that the
406 *Fas* mutation and subsequent immune defects triggered in the MRL background likely
407 leads to the abnormal reproductive phenotypes observed in MRL/lpr mice. This
408 complexity causes the phenotypic similarities and differences observed between
409 MRL/lpr mice and human clinical cases. This study provides a novel insight into the

410 treatment of infertility resulting from disruption of the immune system.

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Declaration of Interest

The authors declare that they have no competing interests.

References

1. Silva, C. A., L. Y. Yamakami, N. E. Aikawa, D. B. Araujo, J. F. Carvalho, and E. Bonfá. 2014. Autoimmune primary ovarian insufficiency. *Autoimmun. Rev.* 13: 427–430.
2. Goswami, D. and G. S. Conway. 2007. Premature ovarian failure. *Horm. Res.* 68: 196–202.
3. Jasti, S., B. D. Warren, L. K. McGinnis, W. H. Kinsey, B. K. Petroff, and M. G. Petroff. 2012. The autoimmune regulator prevents premature reproductive senescence in female mice. *Biol. Reprod.* 86: 110.
4. Kalantaridou, S. N., D. T. Braddock, N. J. Patronas, and L. M. Nelson. 1999. Treatment of autoimmune premature ovarian failure. *Hum. Reprod.* 14: 1777–1782.

- 430 5. Walusimbi, S. S. and J. L. Pate. 2013. Physiology and Endocrinology Symposium:
431 role of immune cells in the corpus luteum. *J. Anim. Sci.* 91: 1650–1659.
- 432 6. Komatsu, K., N. Manabe, M. Kiso, M. Shimabe, and H. Miyamoto. 2003. Changes
433 in localization of immune cells and cytokines in corpora lutea during luteolysis in
434 murine ovaries. *J. Exp. Zool. A Comp. Exp. Biol.* 296: 152–159.
- 435 7. Lawler, D. F., J. Hopkins, and E. D. Watson. 1999. Immune cell populations in the
436 equine corpus luteum throughout the oestrous cycle and early pregnancy: an
437 immunohistochemical and flow cytometric study. *J. Reprod. Fertil.* 117: 281–290.
- 438 8. Penny, L. A., D. Armstrong, T. A. Bramley, R. Webb, R. A. Collins, and E. D.
439 Watson. 1999. Immune cells and cytokine production in the bovine corpus luteum
440 throughout the oestrous cycle and after induced luteolysis. *J. Reprod. Fertil.* 115:
441 87–96.
- 442 9. Sirotkin, A. V. 2011. Cytokines: Signaling molecules controlling ovarian functions.
443 *Int. J. Biochem. Cell Biol.* 43: 857–861.
- 444 10. Imai, F., H. Kishi, K. Nakao, T. Nishimura, and T. Minegishi. 2014. IL-6
445 up-regulates the expression of rat LH receptors during granulosa cell differentiation.
446 *Endocrinology* 155: 1436–1444.
- 447 11. Martoriati, A. and N. Gérard. 2003. Interleukin-1 (IL-1) system gene expression in

- 448 granulosa cells: kinetics during terminal preovulatory follicle maturation in the
449 mare. *Reprod. Biol. Endocrinol.* 1: 42.
- 450 12. Nakayama, M., N. Manabe, N. Inoue, T. Matsui, and H. Miyamoto. 2003. Changes
451 in the expression of tumor necrosis factor (TNF) α , TNF α receptor (TNFR) 2, and
452 TNFR-associated factor 2 in granulosa cells during atresia in pig ovaries. *Biol.*
453 *Reprod.* 68: 530–535.
- 454 13. Tizard, I. R. 2011. Organ-specific autoimmune disease. In *Veterinary Immunology:*
455 *An Introduction*, 7th ed. T. Tada, S. Furusawa, and M. Yasuda, eds. Interzoo, Tokyo.
456 p. 366–378 (in Japanese).
- 457 14. Hughes, G. C. and D. Choubey. 2014. Modulation of autoimmune rheumatic
458 diseases by oestrogen and progesterone. *Nat. Rev. Rheumatol.* 10: 740–751.
- 459 15. Lee, T. P. and B. L. Chiang. 2012. Sex differences in spontaneous versus induced
460 animal models of autoimmunity. *Autoimmun. Rev.* 11: A422–A429.
- 461 16. Nose, M., M. Terada, M. Nishihara, J. Kamogawa, T. Miyazaki, W. Qu, S. Mori,
462 and S. Nakatsuru. 2000. Genome analysis of collagen disease in MRL/lpr mice:
463 polygenic inheritance resulting in the complex pathological manifestations. *Int. J.*
464 *Cardiol.* 75: S53–S61.
- 465 17. Theofilopoulos, A. N. and F. J. Dixon. 1985. Murine models of systemic lupus

- erythematosus. *Adv. Immunol.* 37: 269–390.
18. Takahashi, T., M. Tanaka, C. I. Brannan, N. A. Jenkins, N. G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 76: 969–976.
19. Ichii, O., A. Konno, N. Sasaki, D. Endoh, Y. Hashimoto, and Y. Kon. 2008. Autoimmune glomerulonephritis induced in congenic mouse strain carrying telomeric region of chromosome 1 derived from MRL/MpJ. *Histol. Histopathol.* 23: 411–422.
20. Santiago-Raber, M. L., C. Laporte, L. Reininger, and S. Izui. 2004. Genetic basis of murine lupus. *Autoimmun. Rev.* 3: 33–39.
21. Gu, L., A. Weinreb, X. P. Wang, D. I. Zack, J. H. Qiao, R. Weisbart, and A. J. Lusis. 1998. Genetic determinants of autoimmune disease and coronary vasculitis in the MRL-lpr/lpr mouse model of systemic lupus erythematosus. *J. Immunol.* 161: 6999–7006.
22. Vidal, S, D. H. Kono, and A. N. Theofilopoulos. 1998. Loci predisposing to autoimmunity in MRL-Fas lpr and C57BL/6-Faslpr mice. *J. Clin. Invest.* 101: 696–702.
23. Lee, S. H., O. Ichii, S. Otsuka, Y. H. Elewa, Y. Namiki, Y. Hashimoto, and Y. Kon.

484 2011. Ovarian cysts in MRL/MpJ mice are derived from the extraovarian rete: a
485 developmental study. *J. Anat.* 219: 743–755.

486 24. Nakamura, T., S. Otsuka, O. Ichii, Y. Sakata, K. Nagasaki, Y. Hashimoto, and Y.
487 Kon. 2013. Relationship between numerous mast cells and early follicular
488 development in neonatal MRL/MpJ mouse ovaries. *PLoS One* 8: e77246.

489 25. Tello, J. A., T. Kohout, R. Pineda, R. A. Maki, R. Scott Struthers, and R. P. Millar.
490 2013. Reproductive physiology of a humanized GnRH receptor mouse model:
491 application in evaluation of human-specific analogs. *Am. J. Physiol. Endocrinol.*
492 *Metab.* 305: E67–E77.

493 26. Manabe, N. and J. Kimura. 2008. Female genital organ. In *Textbook of Veterinary*
494 *Histology*, 4th ed. Japanese Association of Veterinary Anatomists, eds, Gakusousha,
495 Tokyo. p. 19–208 (in Japanese).

496 27. Kinoshita, Y. and T. Yamamoto. 2000. Menopause and ovarian aging. *Nippon Sanka*
497 *Fujinka Gakkai Zasshi*, 52: N-345–348 (in Japanese).

498 28. Celik, O., E. Celik, I. Turkcuoglu, E. Yilmaz, Y. Simsek, and B. Tiras. 2012.
499 Germline cells in ovarian surface epithelium of mammals: a promising notion.
500 *Reprod. Biol. Endocrinol.* 10: 112.

501 29. Mork, L., D. M. Maatouk, J. A. McMahon, J. J. Guo, P. Zhang, A. P. McMahon, and

502 B. Capel. 2012. Temporal differences in granulosa cell specification in the ovary
503 reflect distinct follicle fates in mice. *Biol. Reprod.* 86: 37.

504 30. Jovanović, N., S. Zunić-Bozinovski, D. Trpinac, S. Krstić, D. Oprić, J.
505 Trbojević-Stanković, and B. Stojimirović. 2013. Ultrastructural changes of the
506 peritoneum in a rabbit model of peritoneal dialysis. *Vojnosanit. Pregl.* 70:
507 1023–1028.

508 31. Ahmed, N. and K. L. Stenvers. 2013. Getting to know ovarian cancer ascites:
509 opportunities for targeted therapy-based translational research. *Front. Oncol.* 3:
510 256.

511 32. Lee, C. K., J. M. Han, K. N. Lee, E. Y. Lee, J. H. Shin, Y. S. Cho, Y. Koh, B. Yoo,
512 and H. B. Moon. 2002. Concurrent occurrence of chylothorax, chylous ascites, and
513 protein-losing enteropathy in systemic lupus erythematosus. *J. Rheumatol.* 29:
514 1330–1333.

515 33. Devin, J. K., J. E. Johnson, M. Eren, L. A. Gleaves, W. S. Bradham, J. R. Jr.
516 Bloodworth, and D. E. Vaughan. 2007. Transgenic overexpression of plasminogen
517 activator inhibitor-1 promotes the development of polycystic ovarian changes in
518 female mice. *J. Mol. Endocrinol.* 39: 9–16.

519 34. Irahara, M. 2008. New diagnosis criteria of PCOS in Japan. *Nippon Sanka Fujinka*

- 520 *Gakkai Zasshi*, 60: N-185–190 (in Japanese).
- 521 35. Katagiri, S. 2012. Female reproductive physiology. In *Textbook of Theriogenology*,
522 4th ed. T. Nakao, S. Tsumagari and S. Katagiri, eds. Buneido, Tokyo, p. 71–73 (in
523 Japanese).
- 524 36. Yamashita, Y., T. Nakamura, S. Otsuka-Kanazawa, O. Ichii, and Y. Kon. 2015.
525 Morphological characteristics observed during early follicular development in
526 perinatal MRL/MpJ mice. *Jpn. J. Vet. Res.* in press.
- 527 37. Slot, K. A., M. Voorendt, M. de Boer-Brouwer, H. H. van Vugt, and K. J. Teerds.
528 2006. Estrous cycle dependent changes in expression and distribution of Fas, Fas
529 ligand, Bcl-2, Bax, and pro- and active caspase-3 in the rat ovary. *J. Endocrinol.*
530 188: 179–192.
- 531 38. Sakamaki, K., H. Yoshida, Y. Nishimura, S. Nishikawa, N. Manabe, and S.
532 Yonehara. 1997. Involvement of fas antigen in ovarian follicular atresia and
533 luteolysis. *Mol. Reprod. Dev.* 47: 11–18.
- 534 39. Medeiros, P. B., M. V. Febrônio, E. Bonfá, E. F. Borba, A. D. Takiuti, and C. A.
535 Silva. 2009. Menstrual and hormonal alterations in juvenile systemic lupus
536 erythematosus. *Lupus* 18: 38–43.
- 537 40. Shabanova, S. S., L. P. Ananieva, Z. S. Alekberova, and I. I. Guzov. 2008. Ovarian

538 function and disease activity in patients with systemic lupus erythematosus. *Clin.*
539 *Exp. Rheumatol.* 26: 436–441.

540 41. de Araujo, D. B., L. Y. Yamakami, N. E. Aikawa, E. Bonfá, V. S. Viana, S. G. Pasoto,
541 R. M. Pereira, P. C. Serafin, E. F. Borba, and C. A. Silva. 2014. Ovarian reserve in
542 adult patients with childhood-onset lupus: a possible deleterious effect of
543 methotrexate? *Scand. J. Rheumatol.* 43: 503-511.

544 42. Costenbader, K. H., D. Feskanich, M. J. Stampfer, and E. W. Karlson. 2007.
545 Reproductive and menopausal factors and risk of systemic lupus erythematosus in
546 women. *Arthritis Rheum.* 56: 1251–1262.

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Figure Legends

550 **Figure 1. The ratio of spleen weight to body weight in mice.**

551 Each bar represents the mean $\pm$ SE ($n \geq 4$), and multiple comparisons were performed
552 using a non-parametric Kruskal–Wallis test (Scheffe’s method). *: $P < 0.05$, **: $P <$
553 0.01.

554

555 **Figure 2. The postnatal date of vaginal opening of mice.**

556 Each bar represents the mean $\pm$ SE ($n \geq 4$), and multiple comparisons were performed
557 using a non-parametric Kruskal–Wallis test (Scheffe’s method). *: $P < 0.05$.

558

559 **Figure 3. Age-related changes in the estrous cycle in mice.**

560 (A) Estrous cycles monitored for 10 consecutive days in C57BL/6, MRL/+, and
561 MRL/lpr mice at 3, 6, 9 and 12 months. Representative cycle traces are shown with days
562 plotted on the X axis. D: diestrus. P: proestrus. E: estrus.

563 (B) The length of estrus, monitored for 10 days, at 3, 6, 9, and 12 months.

564 (C) The length of diestrus, monitored for 10 days, at 3, 6, 9, and 12 months. Each bar
565 represents the mean $\pm$ SE ($n \geq 5$), and multiple comparisons were performed using a

non-parametric Kruskal–Wallis test (Scheffe’s method). *: $P < 0.05$.

Figure 4. Age-related morphological changes in mouse ovaries.

(A–I) Whole ovaries of C57BL/6 (A–C), MRL/+ (D–F), and MRL/lpr (G–I) mice at 3 (A, D, and G), 6 (B, E, and H), and 12 (C, F, and I) months. All sections were stained with HE. Asterisks represent corpora lutea (CL). Follicles decreased in number with age in all strains (A–I); the decrease was most severe in MRL/lpr mice (G–I). Fewer corpora lutea and an ovarian interstitium with a larger area were observed in MRL/lpr mice (G–I) compared to the other strains. Bars = 200 μ m.

(J–M) The number of follicles at each stage at 3 (J), 6 (K), and 12 (L) months, and the number of CL at each age (M). The number of follicles and corpora lutea was counted in five sections. The total number in all five sections was used as the value for that ovary. Each bar represents the mean $\pm$ SE ($n = 4$), and multiple comparisons were performed using a non-parametric Kruskal–Wallis test (Scheffe’s method). *: $P < 0.05$.

Figure 5. Age-related histological changes in the ovarian interstitium and surface epithelium in mice.

(A–I) Ovarian cortices of C57BL/6 (A–C), MRL/+ (D–F), and MRL/lpr (G–I) mice at 3

(A, D, and G), 6 (B, E, and H), and 12 (C, F, and I) months. All sections were stained with PAS stain. Asterisks indicate interstitial glands composed of interstitial endocrine cells. Fully developed interstitial glands were observed in C57BL/6 mice ovaries at 12 months (C), in MRL/+ mice at 6 (E) and 12 (F) months, and in MRL/lpr mice at 3 (G), 6 (H), and 12 (I) months.

(J–L) Ovarian cortices of C57BL/6 (J), MRL/+ (K), and MRL/lpr (L) mice at 6 months. All sections were stained with HE. A squamous, monolayered epithelium covered the ovarian surface in C57BL/6 (J) and MRL/+ (K) mice. Cuboidal and stratified surface epithelial cells (arrowheads) were observed in MRL/lpr mice (L). Bars = 100 μ m.

Figure 6. Ultrastructure of mouse ovaries.

(A–D) Ultrastructure of the ovarian surface under SEM observation in C57BL/6 (A and B) and MRL/lpr (C and D) mice at 6 months. The ovarian surface of C57BL/6 mice was smooth (A) and covered with oval epithelial cells (arrowheads) or spindle-shaped cells (arrows) with microvilli (B). The ovarian surface of MRL/lpr mice showed several dome-shaped bulges (C), and was mostly covered with round epithelial cells with abundant microvilli (D). The insets show ovarian epithelial cells at a higher magnification. Black bars = 200 μ m. White bars = 20 μ m. Inset bars = 5 μ m.

(E–H) Ultrastructure of the ovarian surface in C57BL/6 (E and F) and MRL/lpr (G and H) mice at 6 months. Semi-thin sections under light microscopy (E and G) and ultra-thin sections under TEM (F and H) are shown. The yellow dotted lines indicate the basal membrane (E–H). Two cell types, pale-colored cells (black arrows) and black-colored cells (arrowheads), were observed in the superficial epithelium of C57BL/6 (E) and MRL/lpr (G) mice. The tunica albuginea (dual-headed yellow arrows) were thicker in MRL/lpr mice than in C57BL/6 mice (E and G). In the ovarian surface epithelium of C57BL/6 mice (F), the round cells contained large nuclei, a few microvilli, and a cytoplasm with a low electron density (arrowhead), whereas squamous cells had abundant microvilli and an electron-dense cytoplasm (arrows). In the ovarian surface epithelium of MRL/lpr mice (H), all cells were taller than those of C57BL/6 mice (F and H), and cells with few microvilli and a cytoplasm with a low electron density bulged toward the peritoneal cavity (arrowheads). Thickened tunica albuginea in MRL/lpr mice contained numerous collagen fibers (H, asterisks). Similar to C57BL/6 mice, cells with abundant microvilli and an electron-dense cytoplasm (arrows) were observed, and its density appeared to be higher than in C57BL/6 mice (F and H). Bars = 10 μ m.

Figure 7. Characteristics of cell infiltration in mouse ovaries.

(A–C) The localization of CD3-positive T cells in the ovaries of C57BL/6 (A), MRL/+ (B), and MRL/lpr (C) mice at 6 months. A few positive cells were observed in or around the corpora lutea of C57BL/6 (A) and MRL/+ (B) mice. Numerous positive cells were observed in the ovaries of MRL/lpr mice; these were widely distributed but were mainly localized to the ovarian interstitium (C).

(D) The number of CD3-positive T cells at 3 and 6 months in the ovaries of C57BL/6, MRL/+, and MRL/lpr mice. The number of positive cells was counted in five sections. The total number in all five sections was used as the value for that ovary. Each bar represents the mean $\pm$ SE ($n \geq 3$), and multiple comparisons were performed using a non-parametric Kruskal–Wallis test (Scheffe’s method). *: $P < 0.05$, **: $P < 0.01$.

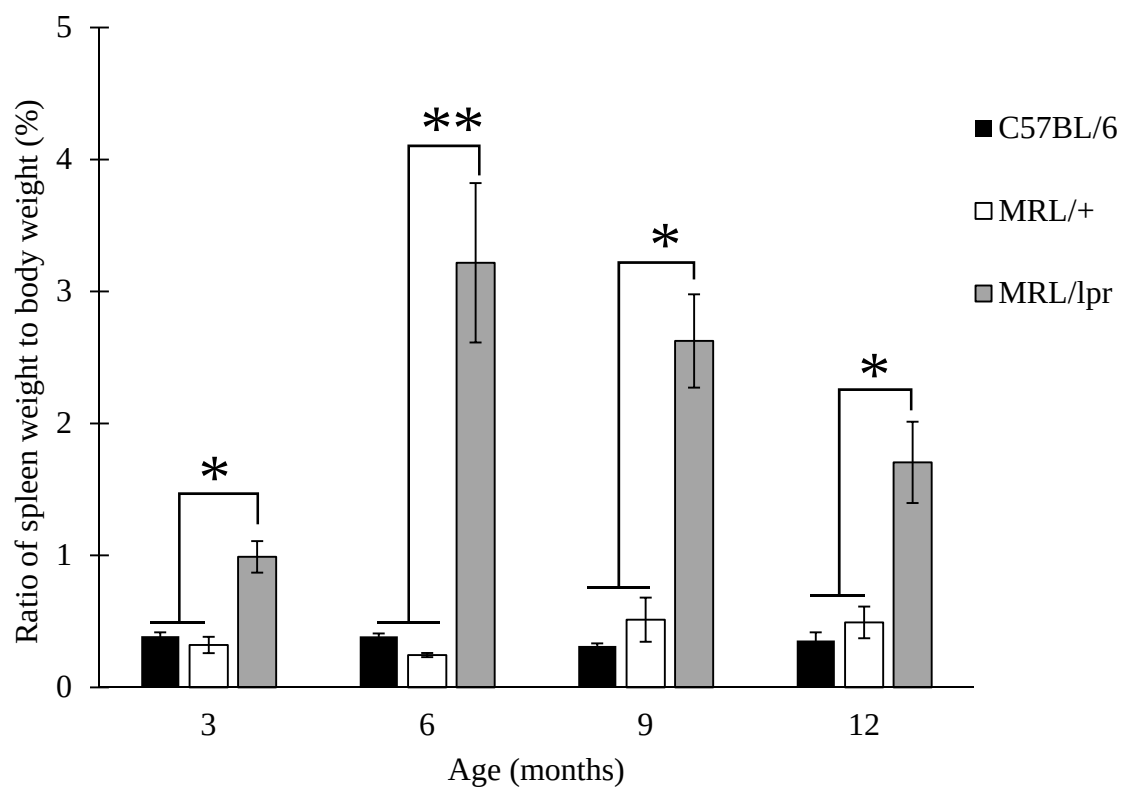
(E–F) The characteristic localization of CD3-positive cells in the ovaries of MRL/lpr mice at 3 months of age. Positive cells were mainly localized in or around atretic (E, arrowhead) and primordial follicles (F).

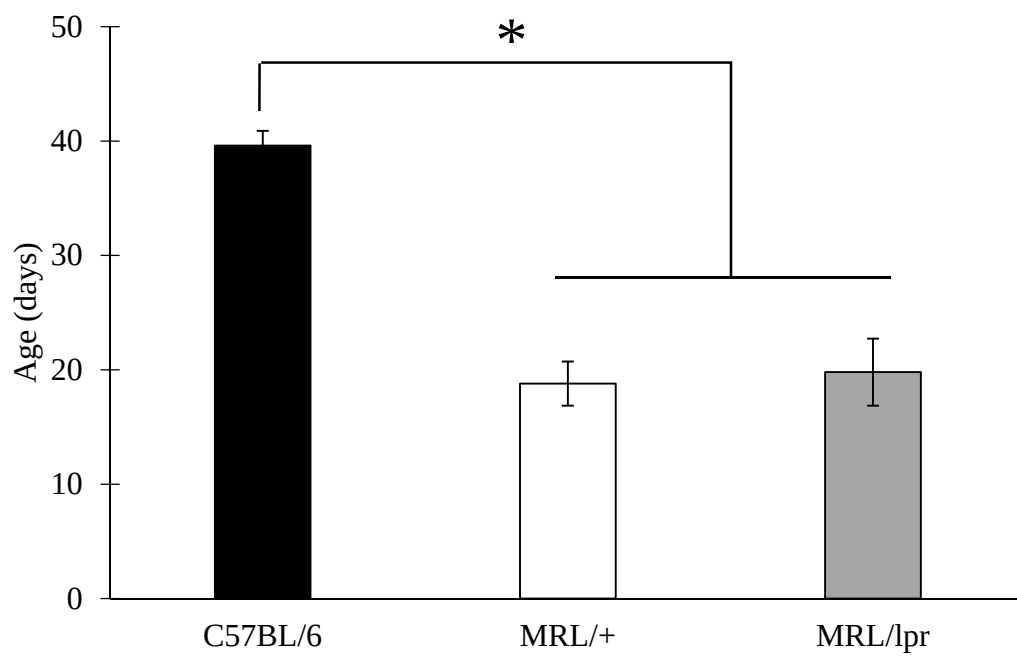
(G–I) The localization of B220-positive cells in the ovaries of C57BL/6 (G), MRL/+ (H), and MRL/lpr (I) mice at 6 months. Numerous positive cells were observed in the ovarian interstitium of MRL/lpr mice (I). Bars = 50 μ m.

638 **Figure 8. The serum concentrations of FSH and testosterone in female mice.**

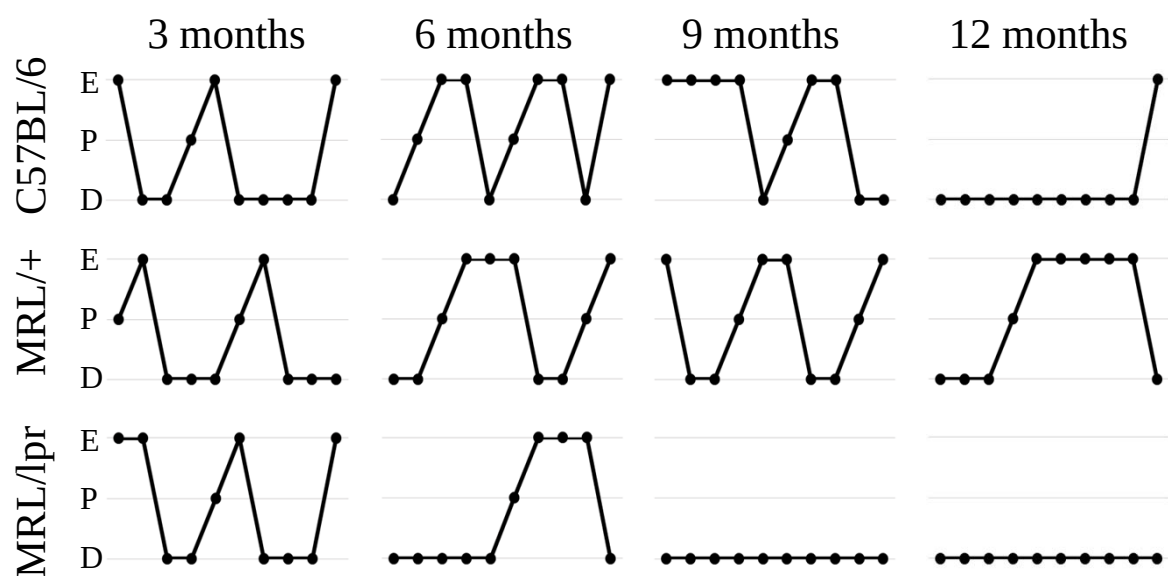
639 **(A)** The serum concentration of FSH in female C57BL/6, MRL/+, and MRL/lpr mice at
640 6 and 12 months.

641 **(B)** The serum concentration of testosterone in female C57BL/6, MRL/+, and MRL/lpr
642 mice at 6 and 12 months. Each bar represents the mean $\pm$ SE ($n \geq 4$).

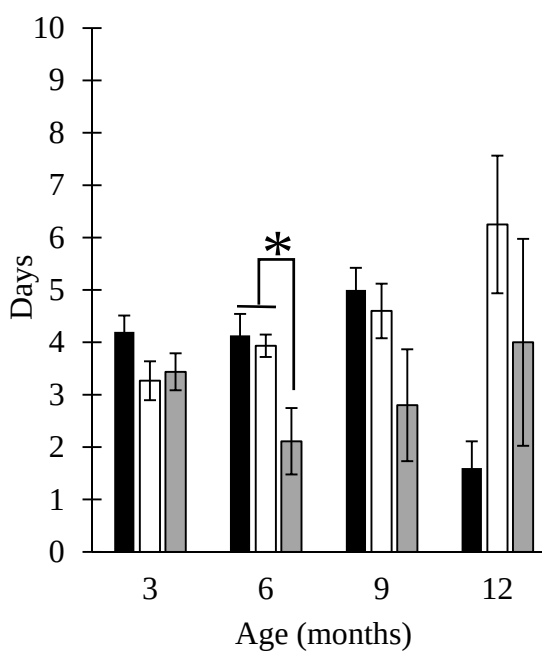




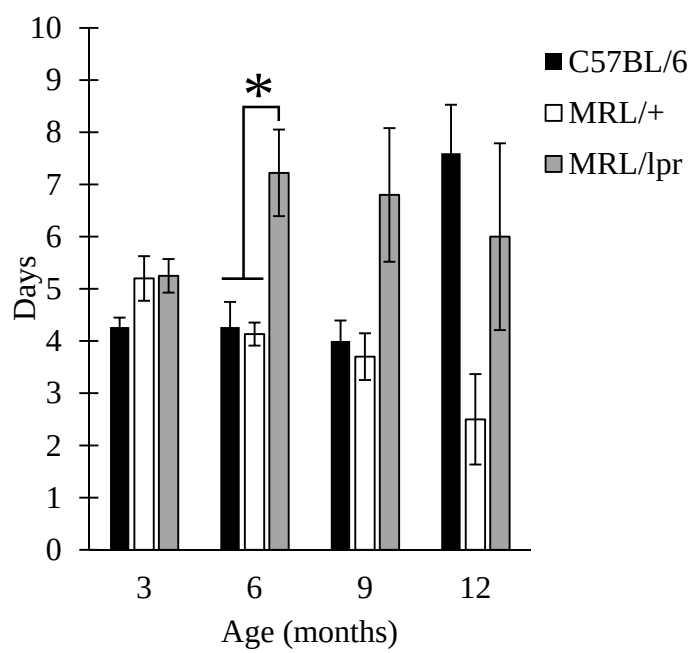
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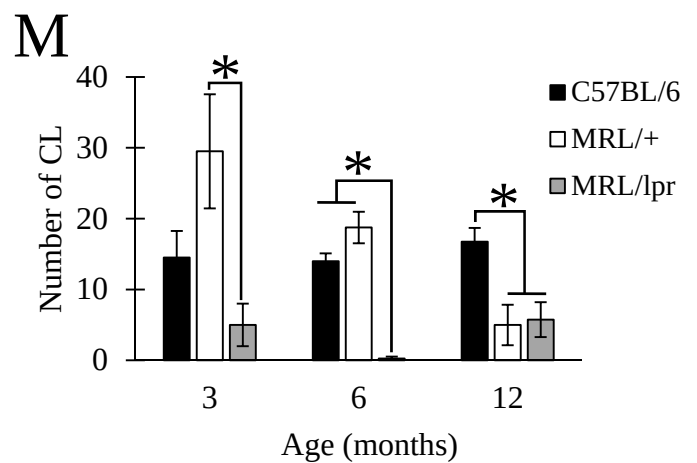
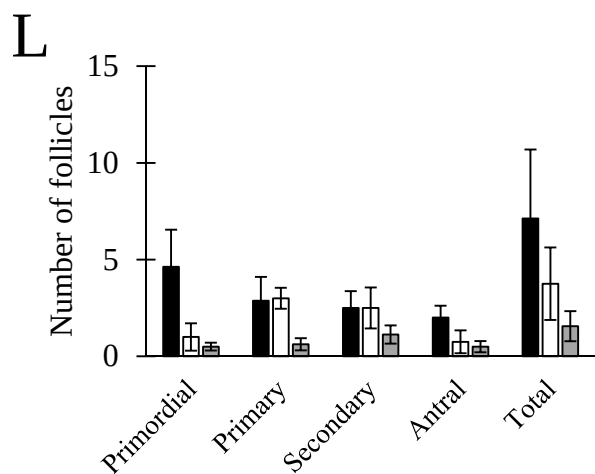
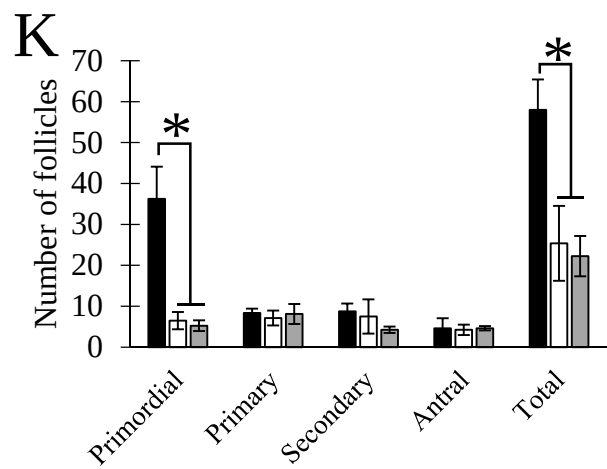
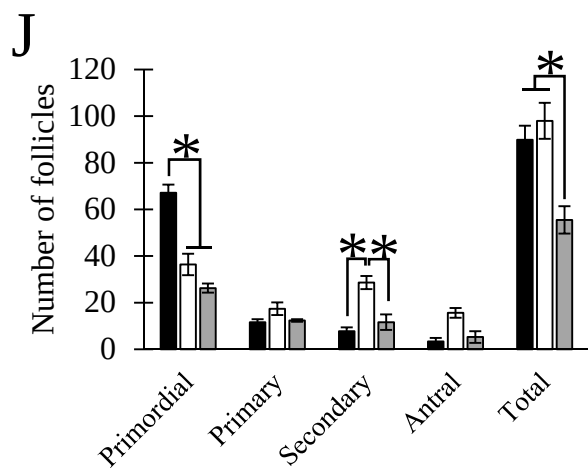
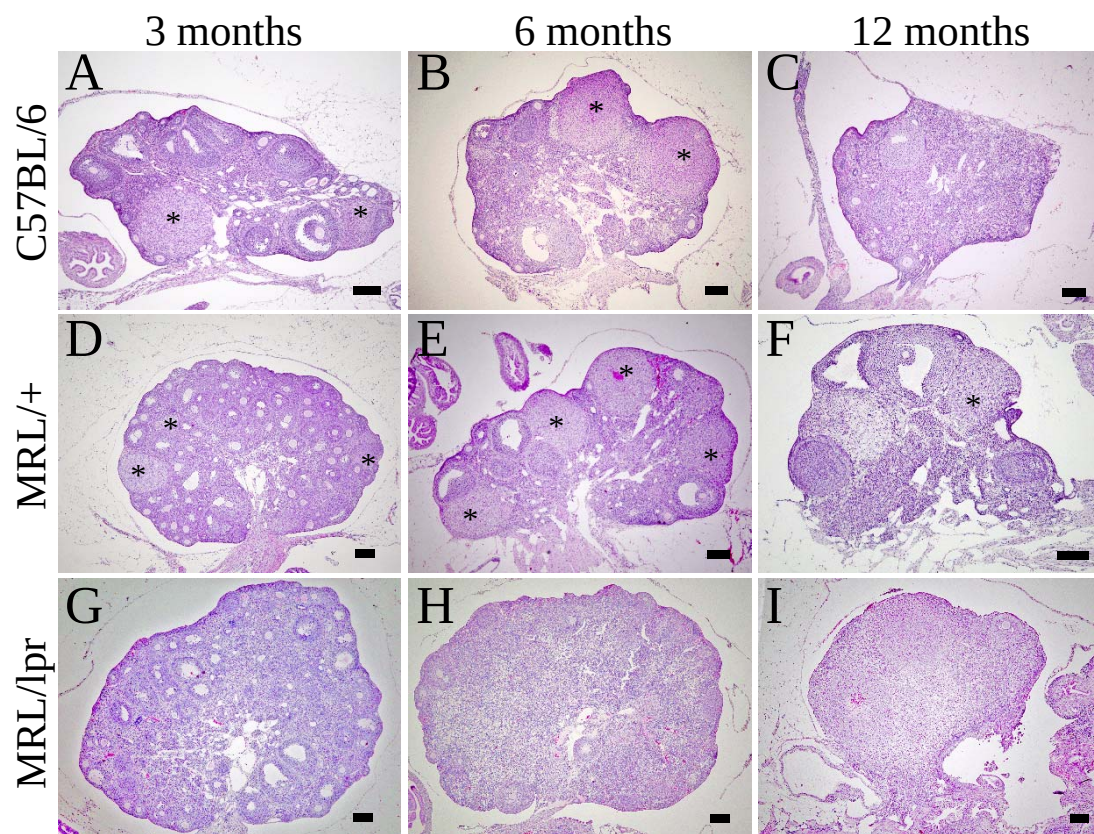


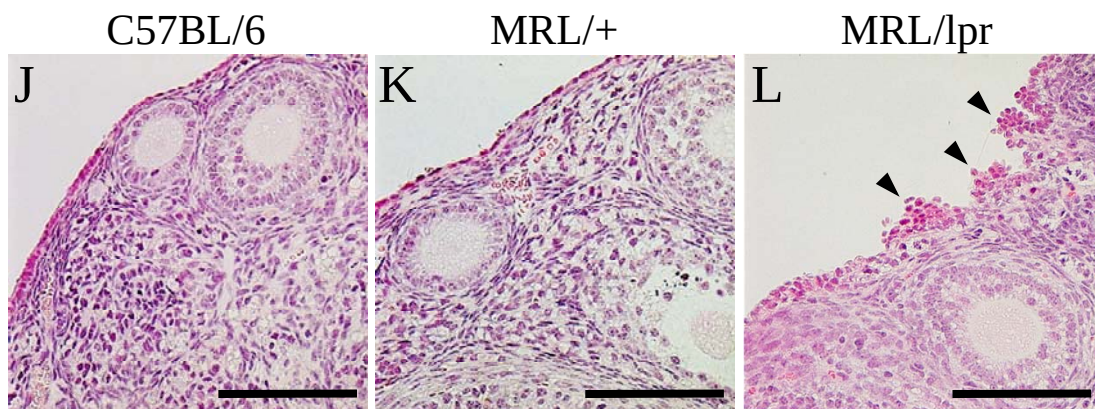
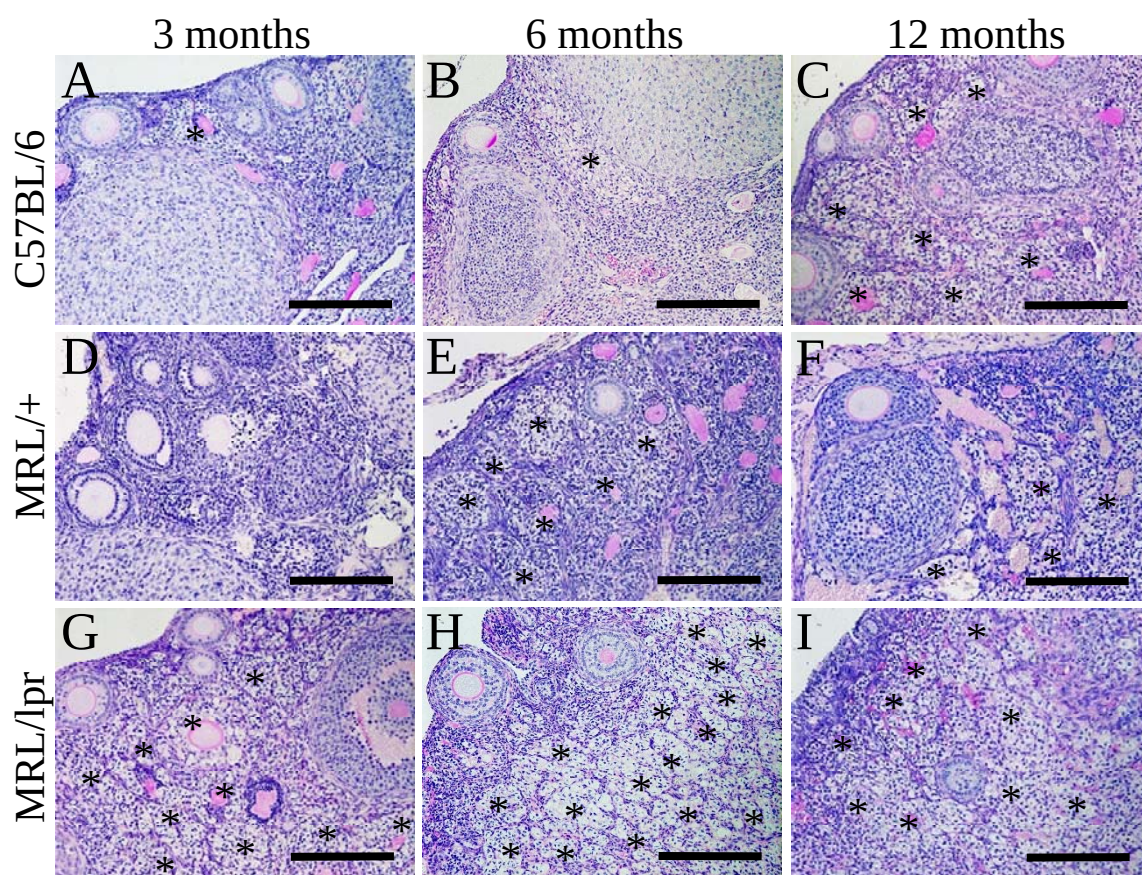
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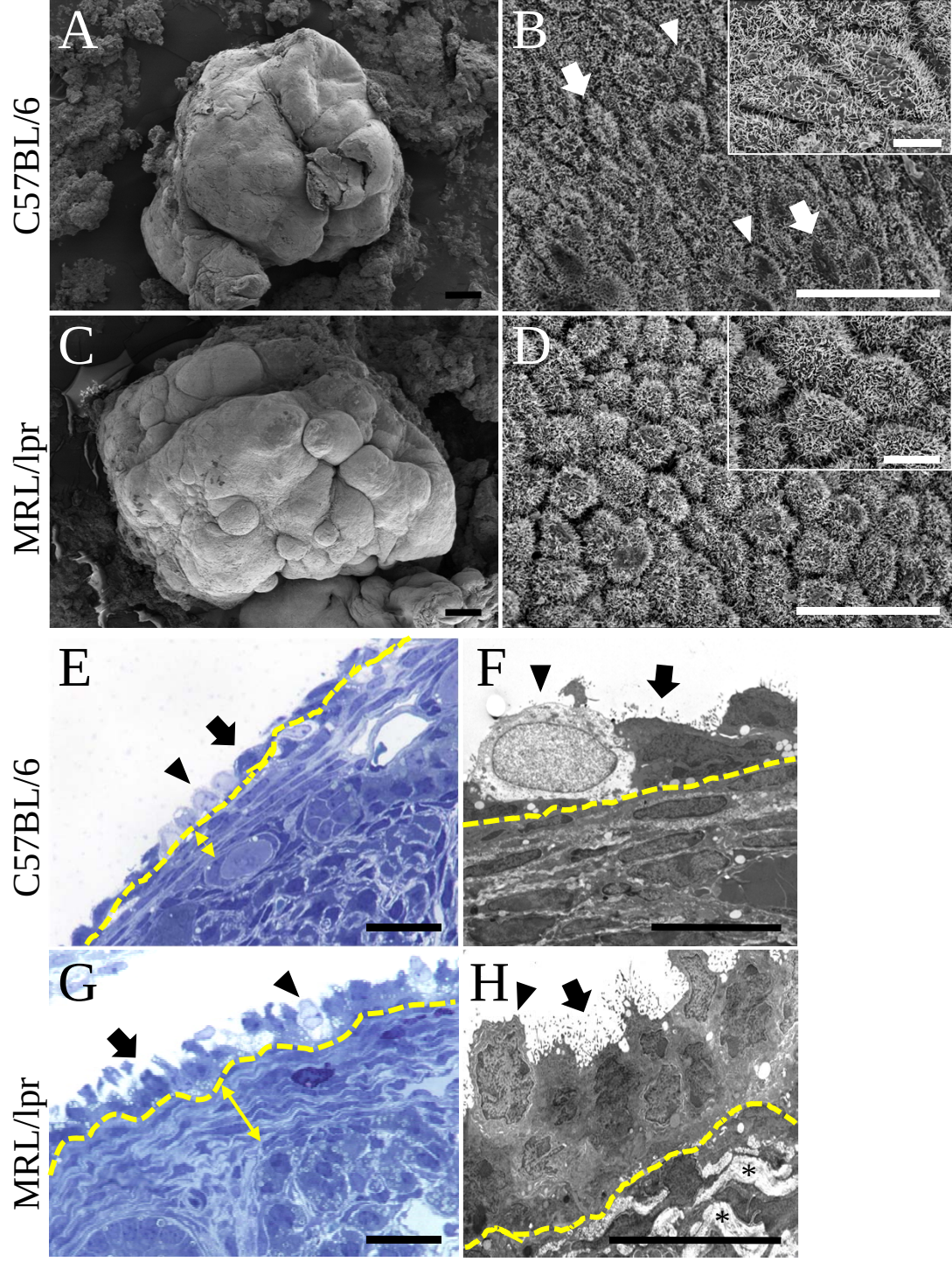


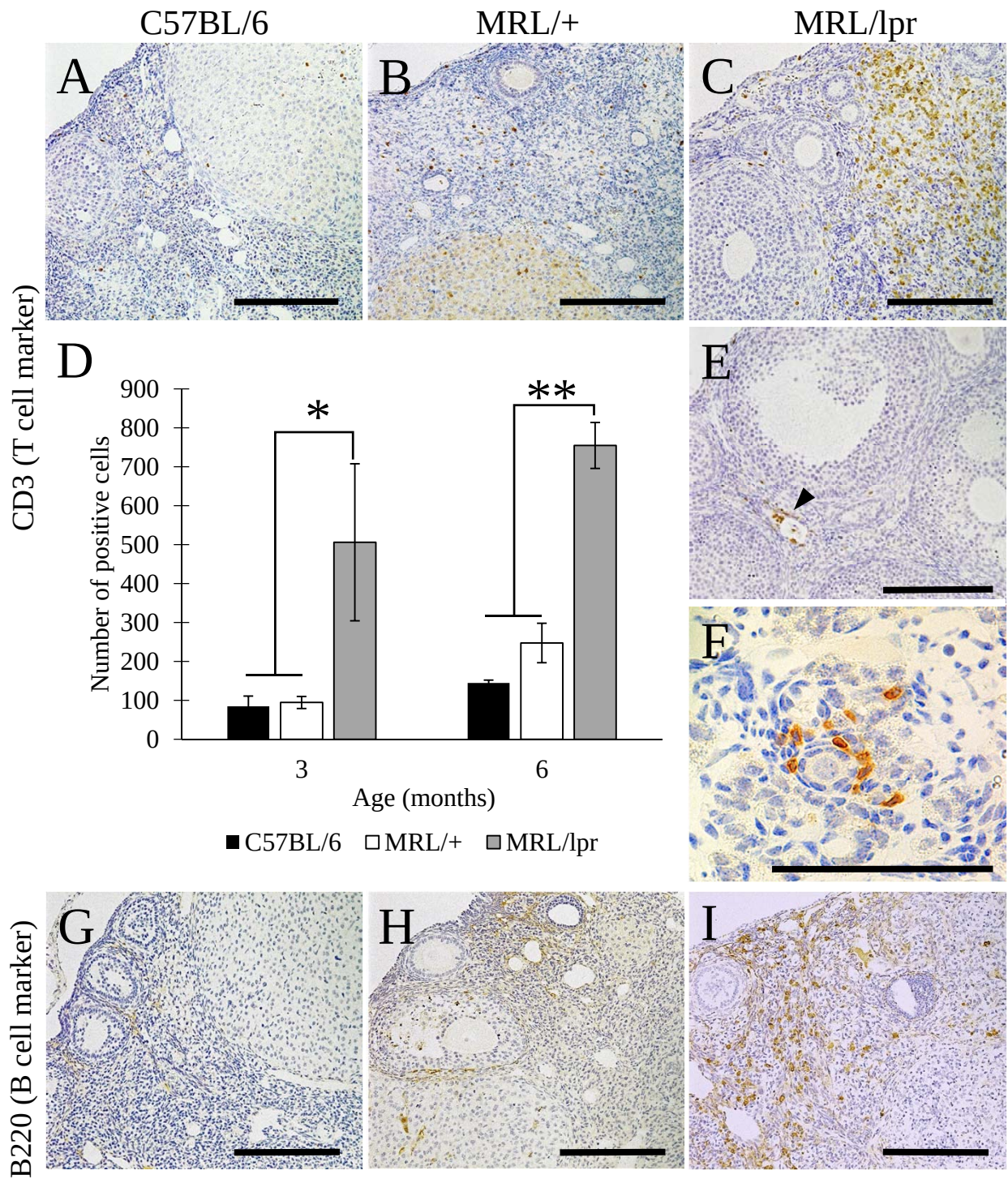
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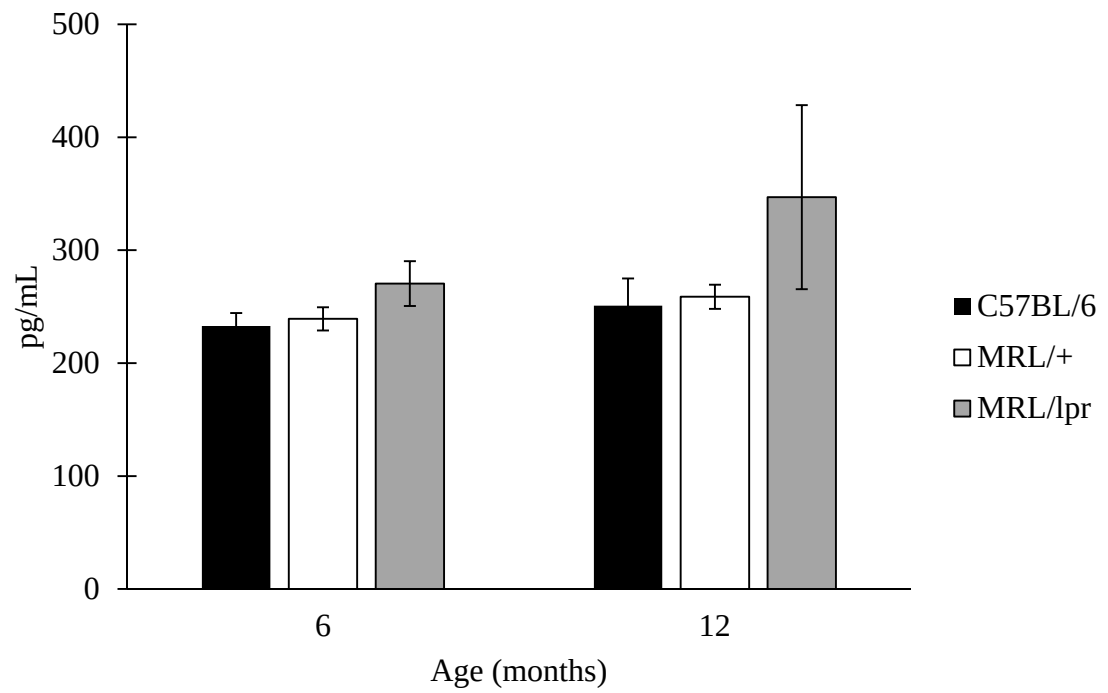








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