FULL PAPER

Anatomy

The histopathological features of the surgical endometriosis model using systemic autoimmune disease-prone mice

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ABSTRACT. Endometriosis is a common gynecological disease that affects women of reproductive age in which the uterine endometrium grows outside the uterus. Origin of the ectopic endometrium is thought to be the retrograde endometrium through the oviducts. However, factors that determine the adherence and proliferation of the ectopic endometrium have not been revealed. Importantly, systemic autoimmune diseases are considered a key factor in the endometriosis onset. Herein, we established a surgical endometriosis rodent model using autoimmune disease-prone MRL/MpJ-$Fas^{lpr/lpr}$ (MRL/lpr) and MRL/+ mice to provide basic evidence of the relationship between autoimmune disease and endometriosis. Endometriosis lesions were successfully induced in two regions after transplanting uterine tissues from donor mice into the peritoneal cavity of recipient mice: the peritoneum or adipose tissue around the transplantation point (proximal lesions) and the gastrosplenic ligament or intestinal mesentery far from the transplantation site (distal lesions). Distal lesions were observed only in MRL/lpr mice, whereas endometriosis lesions showed no genotype- or region-related differences in the histology and distribution of sex hormone receptors and T cells. In contrast, transplanted uterine tissues in donor MRL/lpr mice exhibited a large infiltration of T cells in the lamina propria. Splenomegaly was more common in recipient than that in donor MRL/lpr mice. These results suggest that the infiltration of endogenous T cells in the endometrium alters the growth features of ectopic endometrium, possibly affecting the severity of endometriosis in patients with systemic autoimmune diseases.

KEYWORDS: autoimmune disease, endometriosis, immune cell, surgical model, uterus

Endometriosis is a chronic gynecological disease, defined as a pathological condition in which the uterine endometrium grows outside the uterine cavity in the pelvic or peritoneal organs, such as the peritoneum and ovary [23]. Endometriosis affects approximately 10% of the female population at reproductive age, causing menstrual cramps and pelvic pain [10]. In addition to the clinical symptoms of pain, endometriosis in the oviduct or ovary leads to obstruction of the oviductal lumen and ovulation disorder, resulting in female infertility [4, 15]. The uterine wall is histologically composed of the endometrium, myometrium, and perimetrium [25]. The female endometrium is functionally divided into two layers: functional and basal, the former of which is sloughed off during menstruation [25]. Sampson proposed the theory of endometriosis onset, in which reflux of the sloughed endometrium proliferates ectopically [26]. However, the reflux of the sloughed endometrium is a physiological phenomenon observed in 70–90% of women during menstruation [32], hence it has been estimated that the unidentified pathological factors facilitate the ectopic survival and proliferation of the endometrium in patients.

In mammals, physiological endometrial growth is regulated by hormonal and immunological factors. After ovulation, estradiol promotes endometrial proliferation, whereas progesterone differentiates the endometrium into secretory tissue [6, 22]. In addition to hormonal regulation, remodeling of the endometrium during menstruation, implantation, and the postpartum phase is mediated by a range of immune cells distributed in the endometrium, including macrophages, neutrophils, dendritic cells, and T cells [20]. Importantly, abnormalities of the immune system caused by genetic or epigenetic alterations have been suggested to play an important role in the onset of endometriosis [14]. In healthy women, the retrograde endometrium in the peritoneal cavity undergoes apoptosis without a marked inflammatory reaction. In contrast, in patients with endometriosis, impaired innate immune system and activation of inflammatory responses within the peritoneal cavity have enhanced ectopic endometrium growth by inhibiting apoptosis and promoting angiogenesis [14]. Particularly, the poor phagocytic capacity owing to the scavenger dysfunction in the peritoneal macrophages closely involves in the ectopic growth of endometrium in patients with endometriosis [14]. Genomic studies have reported that several genes

involved in immune regulation contribute to endometriosis development, including interleukin-16, enzyme tyrosine kinase-2, and Fc-receptor like-3 genes [9]. Patients with endometriosis produce autoreactive antibodies including anti-endometrial, anti-ovarian, anti-phospholipid, and anti-histone antibodies [8]. Autoreactive antibodies are not only directed against ectopic endometrial antigens, but also potentially regulate the inflammatory process in the early stage of endometriosis [34]. During the inflammatory process, an imbalance between effector and regulatory T cells results in excessive secretion of inflammatory cytokines, leading to the development of endometriosis [27]. These findings suggest a strong association between endometriosis and systemic autoimmune diseases. However, the exact mechanism underlying endometriosis development in patients with systemic autoimmune abnormalities has not yet been elucidated.

Instead of human endometrial investigations, rodent models of surgically induced endometriosis are currently valid for investigating the detailed and pathohistological pathogenesis of the endometrium [3]. In this study, to clarify the association between autoimmune diseases and endometriosis, we established a surgical endometriosis model using autoimmune disease-prone MRL/MpJ-*Fas^{lpr/lpr}* (MRL/lpr) mice. MRL/lpr mice harbor lymphoproliferative mutations (*lpr*) in the Fas cell surface death receptor (*Fas*) gene, causing severe autoimmune disease symptoms resembling those of systemic lupus erythematosus (SLE), such as arthritis, vasculitis, and glomerulonephritis [1]. Importantly, MRL/lpr mice showed systemic infiltration of autoreactive T cells owing to the dysfunction in Fas-mediated apoptosis and the considerable number of autoantibodies in systemic organs. The identification of these phenotypes in MRL/lpr mice greatly contribute to revealing the pathological role of autoantibodies and autoreactive T cells in endometriosis. The pathohistological features of the endometrium induced in MRL/lpr mice and the association between systemic autoimmune diseases and endometriosis are discussed here.

MATERIALS AND METHODS

Animals

Animal experiments were approved by the Institutional Animal Care and Use Committee of Rakuno Gakuen University (No. VH21A8). Animals were managed in accordance with the Guide for the Care and Use of Laboratory Animals, Rakuno Gakuen University, Japan. Female MRL/lpr mice and MRL/MpJ (MRL/+) mice, as the wild type of MRL/lpr mice, were obtained at 3 and 6 months of age from Japan SLC, Inc. (Hamamatsu, Japan). The mice were housed in groups within plastic cages at 18–26°C in a 12 hr light/dark cycle with free access to a commercial diet and water. All mice were euthanized by cervical dislocation under anesthesia using a combination of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg).

Surgical endometriosis model

Endometriosis was surgically induced in mice as previously described, with minor modifications [5, 18, 21]. A schematic of the experimental design is shown in Fig. 1A. All mice were ovariectomized under anesthesia by peritoneal injection of a mixture of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg) and subcutaneous injection of buprenorphine (0.1 mg/kg) was administered for pain-control. Through a 1 cm incision on the right dorsal abdominal wall the ovaries, oviducts, and cranial section of the uterine horn were extracted. The oviducts were ligated and both ovaries were removed. The ligated female reproductive tract was reinserted into the abdominal cavity, and the abdominal wall and skin incisions were closed. All mice recovered after receiving
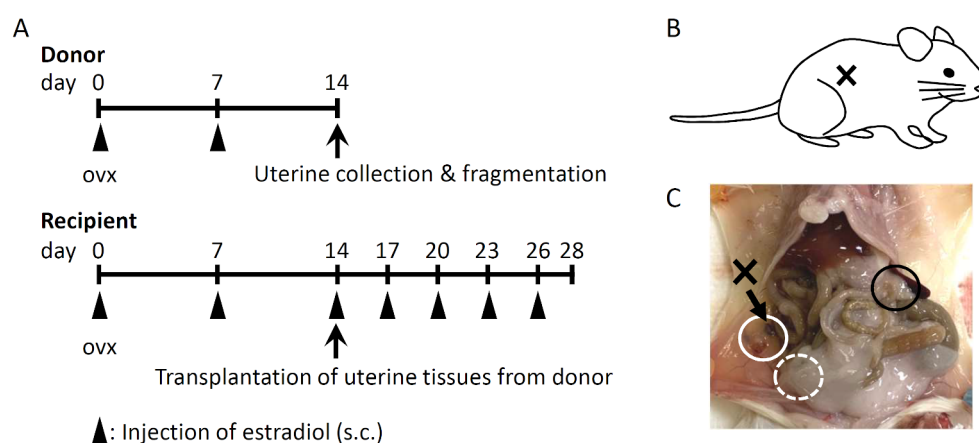

Fig. 1. Scheme showing timeline of the surgical endometriosis model experiment. (**A**) All mice are ovariectomized (ovx) at day 0 and injected estradiol subcutaneously (s.c.) at day 7 and 14. At day 14, the donor mice are euthanized, and their uterine tissues are collected and fragmented. The fragmented uterine tissues are transplanted into the recipient mice. The recipient mice are injected estradiol s.c. on days 17, 20, 23, and 26, and euthanized at day 28. (**B**) The incision and transplantation point on the right dorsal abdominal wall are denoted by the cross mark. (**C**) The regions where the proximal lesions develop around the transplantation point are circled in white line (the peritoneum) and white dashed line (the adipose tissue). The region where the distal lesions develop is circled in black line. The cross mark denotes the sutured incision on the abdominal wall for uterine tissue transplantation.

a intraperitoneal injection of atipamezole (0.3 mg/kg) (day 0). On days 0 and 7, all mice were subcutaneously injected with β-estradiol (0.5 µg/head, FUJIFILM Wako Pure Chemical Co., Osaka, Japan) to facilitate the entopic growth of the endometrium as described in previous studies [18]. Ovariectomized mice were randomly divided into two groups: donor and recipient. Four combinations of donors and recipients were used in this study: (donor/recipient)=(MRL/+ at 3 months of age/MRL/+ at 3 months of age), (MRL/+ at 6 months of age/MRL/+ at 6 months of age), (MRL/lpr at 3 months of age/MRL/lpr at 3 months of age), and (MRL/lpr at 6 months of age/MRL/lpr at 6 months of age) (n=5 per group). Donor mice were euthanized on day 14, and the right uterine horn tissues were collected and fragmented in 400 µL of ampicillin/D-phosphate-buffered saline (PBS) (FUJIFILM Wako Pure Chemical Co.). The left uterine horn was collected, fixed with 4% paraformaldehyde (PFA) overnight at 4°C, and embedded in paraffin. The spleens of donor mice were collected, and the ratio of spleen weight to body weight (S/B) was measured as a marker of systemic autoimmune disease. On day 14, recipient mice were implanted with fragmented uterine tissues, under anesthesia by the peritoneal injection of a combination of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg) and a subcutaneous injection of buprenorphine (0.1 mg/kg) was administered for pain-control. Through a 1 cm incision on the right dorsal abdominal wall, fragmented uterine samples collected from donor mice (400 µL/head) were transplanted into the peritoneal cavity of recipient mice (Fig. 1B and 1C). In this study, we did not suture the implanted uterine tissues with the abdominal wall. The wounds were closed, and the recipient mice were administered an intraperitoneal injection of atipamezole (0.3 mg/kg) for recovery. The recipient mice were subcutaneously injected with β-estradiol (0.5 µg/head) on days 17, 20, 23, and 26 and euthanized on day 28 to enhance ectopic growth of the endometrium and induce endometriosis [18]. The S/B ratio was measured, and the endometriosis lesions were collected, fixed with 4% PFA overnight at 4°C, and embedded in paraffin. The endometriosis lesions developed in two regions: the proximal lesions, which developed on the peritoneum or adipose tissue around the transplantation site (i.e., the incision point on the right dorsal abdominal wall) and the distal lesions, which developed on the gastrosplenic ligament or intestinal mesentery far from the transplantation site (Fig. 1C).

Histological analysis

The embedded right uterine horn collected from the donor and endometriosis lesions collected from the recipient were sliced into 3.5 µm-thick histological sections. Immunohistochemistry (IHC) and hematoxylin and eosin (HE) staining were performed on deparaffinized sections. Detailed information on the antibodies and serum blocking agents used for IHC is presented in Table 1. The sections were then incubated for 15 min at 110°C in 20 mM Tris-HCl (pH 9.0). The sections were soaked in methanol containing 0.3% hydrogen peroxide, incubated with blocking serum for 60 min at room temperature, and then incubated overnight at 4°C with primary antibodies. After washing thrice in 0.01 M PBS, the sections were incubated for 30 min with secondary antibodies before being washed. The sections were incubated for 30 min at room temperature using a streptavidin-biotin complex (SABPRO Kit, Nichirei, Tokyo, Japan), incubated with a 3,3′-diaminobenzidine tetrahydrochloride-hydrogen peroxide solution, and lightly stained with hematoxylin. A Primostar 3 microscope (ZEISS Inc., Oberkochen, Germany) was used to examine the stained sections.

Statistical analysis

The results are expressed as mean ± standard error (s.e.). Data from three or more groups were compared using Tukey's test ($P<0.05$).

RESULTS

The gross features and appearance rate of endometriosis lesions

As mentioned in Materials & Methods, the endometriosis lesions developed in two regions: the proximal lesions and the distal lesions (Fig. 2A). Proximal lesions were observed in all mice, whereas distal lesions were observed only in MRL/lpr mice at 3 and 6 months of age. The appearance rates of endometriosis lesions were 80% (one of the five recipient mice did not develop any lesions) and 100% (all examined recipient mice developed distal and/or proximal lesions) in MRL/+ and MRL/lpr mice, respectively (Fig. 2B). Recipient MRL/+ and MRL/lpr mice with endometriosis lesions in more than two regions were also observed. Moreover, the appearance rates of proximal lesions were 80% in both mice at 3 months of age, 100% in MRL/+ at 6 months of age, and 40% in MRL/lpr at 6 months of age (Fig. 2C). Finally, the appearance rate of the distal lesions in MRL/lpr mice was 40% at 3 months of age and 100% at 6 months of age (Fig. 2D).

Table 1. Primary antibody information in immunohistochemistry

| Antigen	Cat. No	Source	Host	Dilution	Blocking serum	Biotinylated secondary antibody for immunohistochemistry
Estrogen receptor	Ab32603	Abcam Inc., Cambridge, UK	Rabbit	1:400	10% goat normal serum	Goat anti-rabbit IgG antibody, 426012, undiluted (Nichirei)
Progesterone receptor	MA1-410	Invitrogen, Carlsbad, CA, USA	Mouse	1:1,000	10% rabbit normal serum	Rabbit anti-mouse IgG+IgA+IgM antibody, 426031, undiluted (Nichirei)
CD3	413591	Nichirei, Tokyo, Japan	Rabbit	No need	10% goat normal serum	Goat anti-rabbit IgG antibody, 426012, undiluted (Nichirei)
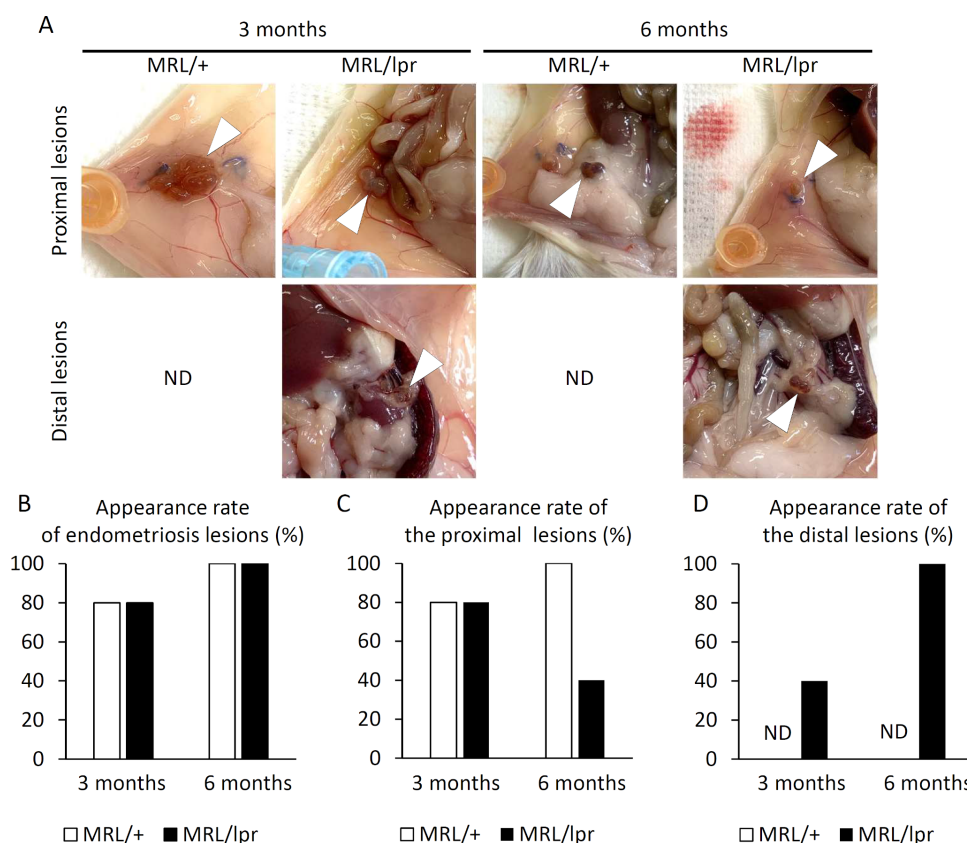
Fig. 2. Endometriosis lesions in MRL/MpJ (MRL/+) and MRL/MpJ-*Fas^{lpr/lpr}* (MRL/lpr) mice. (**A**) The gross feature of the endometriosis lesions (white arrowheads). (**B**) The appearance rate of endometriosis lesions. (**C**) The appearance rate of the proximal lesions. (**D**) The appearance of the distal lesions. ND: not detected. n=5 for each group.

The index of systemic autoimmune disease

Donor and recipient MRL/lpr mice had a higher S/B than MRL/+ mice at 3 and 6 months of age (Fig. 3A and 3B). At 3 months of age, the recipient MRL/lpr mice had more severe splenomegaly than the donor MRL/lpr mice at the same age (Fig. 3A and 3B).

Histology of the uterine horn in the donor

At 3 and 6 months of age, the distribution of uterine glands in the lamina propria of the endometrium was lower in MRL/lpr mice than in MRL/+ mice. Estrogen and progesterone receptors were localized to the epithelial cells of the uterine glands and interstitial cells of the lamina propria in all mice. The expression and distribution of sex hormone receptors in the uterine horn did not differ across age and strain groups. CD3-positive T cells were scattered in the lamina propria of the uterine horn of MRL/+ mice at both 3 and 6 months of age and MRL/lpr mice at 3 months of age, whereas they were infiltrated in MRL/lpr mice at 6 months of age (Fig. 4).

Histology of the endometriosis lesions developed in the recipient

All mice had uterine-gland-like structures, blood vessels, and cysts in both proximal and distal endometriosis lesions. Estrogen and progesterone receptors are localized in the epithelium of the uterine-gland-like structures and interstitial cells. In all endometriosis lesions, CD3-positive T cells were scattered in the interstitium, with no differences in the expression and distribution among genotype or age groups (Fig. 5).

DISCUSSION

In this study, we established a surgical endometriosis model in autoimmune disease-prone mice. The endometriosis lesions developed in MRL-strain mice consist of uterine gland-like structures and interstitial cells expressing sex hormone receptors, indicating that these lesions originated from the uterine fragments transplanted from the donor. The gross and histological features of the endometriosis lesions observed in MRL-strain mice mirror those in previously established murine models of endometriosis [6, 19, 20]. Furthermore, uterine gland development, angiogenesis, and cystic lumen in MRL-strain mice also mirror the ones in humans [12]. In addition to previously established rodent models of endometriosis [5, 18, 19], the model established in this study presents a valid research approach to understand the relationship between autoimmune disease and endometriosis. The previously established surgical endometriosis models [21, 31], in which the endometrial fragments were sutured on the peritoneum or intestinal mesentery, could not determine
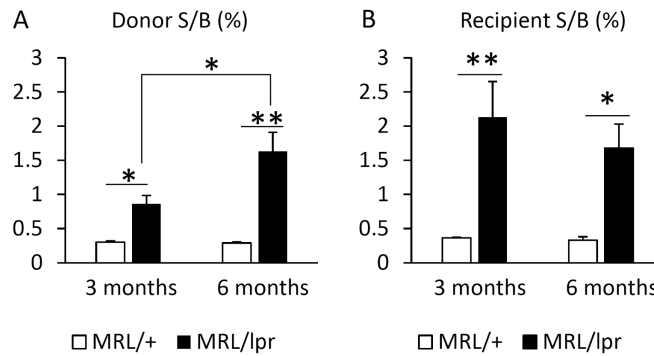
Fig. 3. The ratio of spleen-to-body weight ratio (S/B) in the donor (**A**) and the recipient (**B**). *$P<0.05$, **$P<0.01$ (Tukey's test, n=5 for each group). MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-$Fas^{lpr/lpr}$.
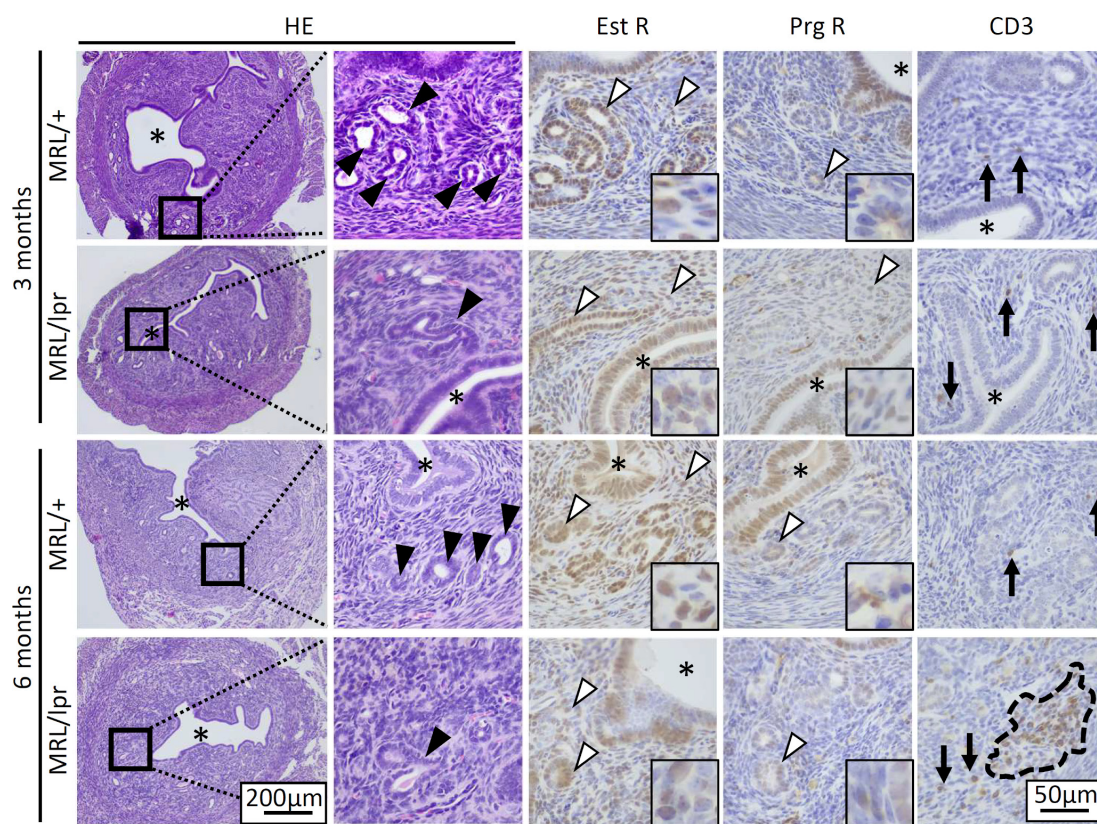

Fig. 4. Histology of the uterine horn in donor. Asterisks: uterine lumen, black arrowheads: uterine glands, white arrowheads: immunoreactive positive cells, arrows: CD3-positive T cells. The dashed line shows the infiltration of CD3-positive T cells. HE: hematoxylin-eosin staining, Est R: immunohistochemistry of estrogen receptor, Prg R: immunohistochemistry of progesterone receptor, CD3: immunohistochemistry of CD3-positive T cells. MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-$Fas^{lpr/lpr}$.

the transfer properties of the endometrium from the transplantation point to the distal regions of the peritoneal cavity. However, in the present and previously established models [5, 18, 19], mice were injected with fragmented uterine tissues, which was useful for examining the ectopic engraftment and proliferation properties of the endometrium. However, there have been no detailed reports on these properties in the latter models. In this study, we observed the differences in the regions of lesion development among mice genotypes.

As reported in previous studies [16, 17], MRL/lpr mice showed significant splenomegaly at both 3 and 6 months of age and exacerbated severe systemic autoimmune disease at 6 months of age when compared to MRL/+ mice. Although MRL/lpr mice exhibited systemic autoimmune abnormalities, the appearance rate of endometriosis in MRL/lpr mice at 6 months of age showed no differences between those in MRL/+ mice and MRL/lpr mice at 3 months of age. In contrast, the endometriosis lesions in MRL/lpr mice tend to develop distally from the transplantation site. These results suggest that systemic autoimmune abnormalities in mice
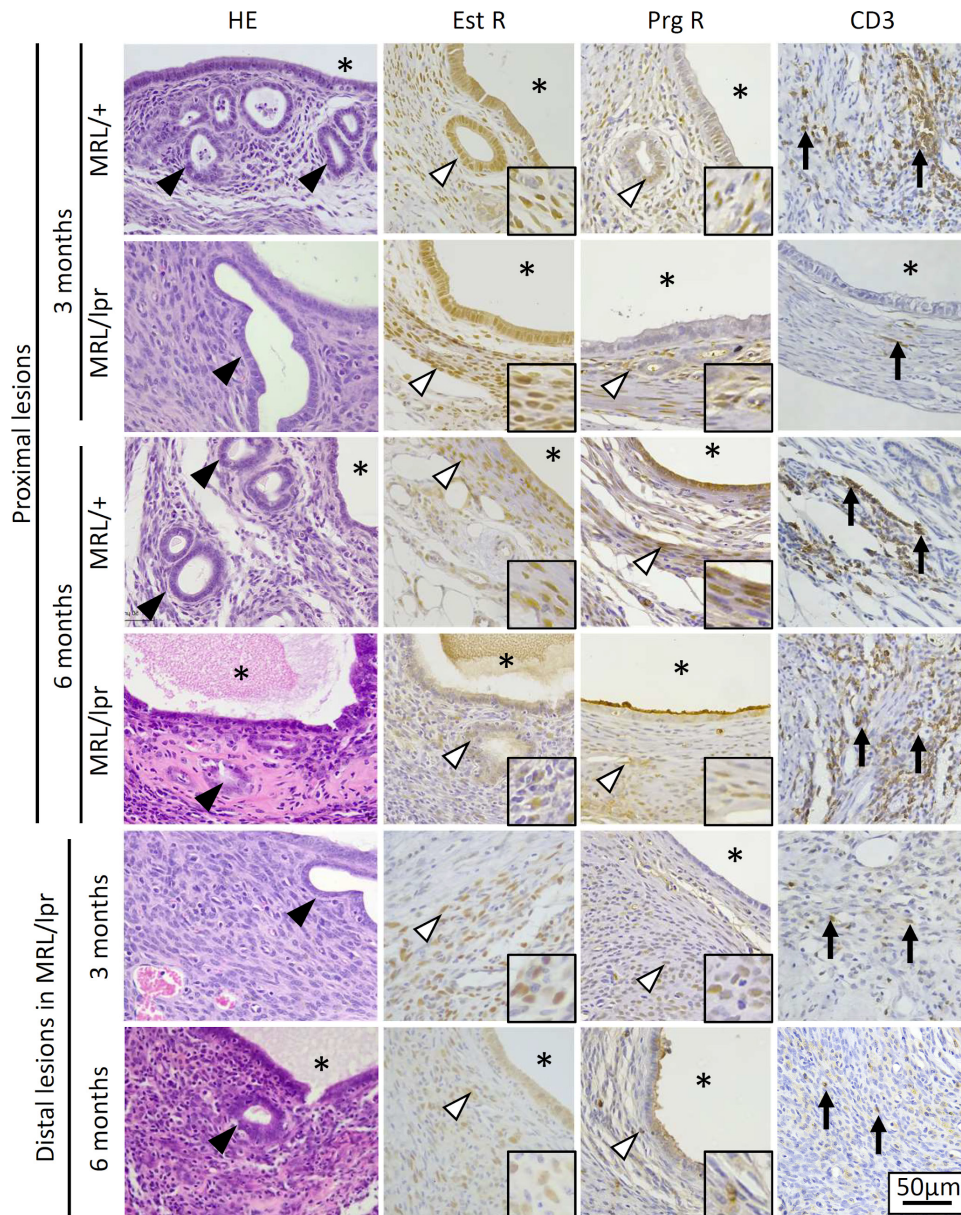
Fig. 5. Histology of the endometriosis lesions developed in recipient. Asterisks: cysts, black arrowheads: uterine glands-like structures, white arrowheads: immunoreactive positive cells, arrows: CD3-positive T cells. HE: hematoxylin-eosin staining, Est R: immunohistochemistry of estrogen receptor, Prg R: immunohistochemistry of progesterone receptor, CD3: immunohistochemistry of CD3-positive T cells. MRL/+: MRL/MpJ, MRL/lpr: MRL/MpJ-*Fas^{lpr/lpr}*.

do not affect the incidence of endometriosis but do affect the growth of ectopic endometrium. In humans, the common regions of endometriosis development are the ovaries, uterine serosa, and recto-uterine pouch. The clinical symptoms and fertile condition in patients vary depending on where the endometriosis lesions develop [33]. Systemic autoimmune abnormalities, such as SLE and rheumatoid arthritis, are associated with more severe endometriosis in female patients [13]. In humans, systemic autoimmune diseases might alter the clinical symptoms of endometriosis, owing to their effect on the region of retrograde endometrium development. MRL/lpr mice with surgically induced endometriosis potentially contribute to the elucidation of the pathogenesis of endometriosis lesions that develop far from the peritoneal opening of the fallopian tube (i.e., recto-uterine pouch and peritoneum) in humans.

The histology and distribution of sex hormone receptors and T cells in endometriosis lesions did not differ across mice of different ages and genotypes. In contrast, uterine tissue collected and transplanted from the donor showed significant infiltration of T cells and lower distribution of uterine glands in MRL/lpr mice compared to MRL/+ mice. The uterine glands express a range of molecules that have been thought to be involved in endometriosis development, such as interleukin-11/its receptors [28] and distal-less [2]. These molecules are downregulated in endometriosis patients compared to healthy women and are involved in steroid production and endometriosis development [2, 7]. Therefore, the lower distribution of the uterine glands in the uterine lamina propria of MRL/

lpr mice is estimated to affect the expression levels of molecules regulating endometrial survival and proliferation. Furthermore, regulatory T cells in MRL/lpr mice have been reported to have a reduced capacity to suppress the secretion of inflammatory cytokines from effector T cells [24]. The decrease in activated regulatory T cell counts results in activation of effector T cells in endometriosis lesions in both humans and mice, indicating that dysregulation of regulatory T cells plays a role in the onset of endometriosis [29]. Therefore, in MRL/lpr mice, the activation of effector T cells originating from transplanted uterine tissues and the inactivation of regulatory T cells in the recipient peritoneal cavity exacerbated the inflammatory cytokine secretion, progressed the ectopic graft, and proliferated the endometrium, all of which contributed to the endometrium survival at the distal site from the transplantation point.

In MRL/lpr mice, the *lpr* mutation in *the Fas* gene impairs the Fas-mediated apoptosis of immune cells [35]. In women with endometriosis, the ectopic endometrium evades scavenging by effector T cells, natural killer cells, and macrophages owing to the inhibition of Fas-mediated apoptosis, which is thought to be involved in the development of endometriosis [11, 30]. Although the autoreactive immune cells, including effector T cells, natural killer cells, and macrophages lose Fas activity in MRL/lpr mice and are regarded to have a high ability to eliminate the ectopic endometrium, the distal endometriosis lesions observed in MRL/lpr mice might be caused by prolonged survival of ectopic endometrial cells. The aforementioned mechanisms, including the molecular alterations in uterine glands and activation of effector T cells in the transplanted uterine tissues under the dysregulated regulatory T cells in the recipient mice, seem to be more essential for the proliferation of ectopic endometrium in MRL/lpr mice than the scavenging ability of autoreactive immune cells.

In MRL/lpr mice at 3 months of age, the recipients showed a higher spleen-to-body weight ratio than the donors, indicating that endometriosis exacerbates systemic autoimmune disease in mice. When combined with the acute inflammation of ectopic endometrial cells in the peritoneal cavity that transitions to the chronic phase, where autoreactive antibodies are highly produced in women with endometriosis [8], these results provide basic evidence that endometriosis triggers the progression of systemic autoimmune diseases. In conclusion, the established surgical endometriosis model using MRL/lpr mice revealed an interactive relationship between endometriosis and systemic autoimmune diseases. These findings expand the basic research and understanding of endometriosis pathogenesis.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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