



HOKKAIDO UNIVERSITY

Title	Study on the pathological crosstalk between reproduction and immunity leading to abnormal urogenital phenotypes in autoimmune disease model mice
Author(s)	大谷, 祐紀
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第14542号
Issue Date	2021-03-25
DOI	https://doi.org/10.14943/doctoral.k14542
Doc URL	https://hdl.handle.net/2115/82345
Type	doctoral thesis
File Information	Yuki_Otani.pdf



Study on the pathological crosstalk between
reproduction and immunity leading to abnormal
urogenital phenotypes in autoimmune disease model mice

(自己免疫疾患モデルマウスの尿生殖器異常を導く生殖－免疫病態連関)

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Contents

Abbreviations	1
Notes.....	3
Preface	4
Chapter 1: The effect of systemic autoimmune disease on the morpho-function of female reproductive tract.....	7
Introduction	8
Materials and methods	10
Result	13
Discussion.....	17
Summary	21
Figures.....	22
Chapter 2: The effect of systemic autoimmune disease on the morpho-function of male reproductive tract.....	29
Introduction	30
Materials and methods	32
Result	35
Discussion.....	39
Summary	43
Tables and figures.....	44
Chapter 3: The effect of castration on the pathogenesis of systemic autoimmune disease and glomerulonephritis	56
Introduction	57
Materials and methods	59
Results.....	62
Discussion.....	66
Summary	70
Tables and figures.....	71
Conclusion.....	81
Conclusion in Japanese	84
Acknowledgement.....	87
References	88

Abbreviations

A/AR: androgen/androgen receptor

Actb: actin beta

Aire: autoimmune regulator

AR: androgen receptor

ASA: anti-sperm antibody

BTB: blood-testis barrier

BUN: blood urea nitrogen

CB: citrate buffer

cDNA: complementary DNA

Cre: creatinine

dsDNA: double-stranded DNA

EAO: experimental autoimmune orchitis

ECM: extracellular matrix

EMT: epithelial-mesenchymal transition

FSH: follicle-stimulating hormone

GBM: glomerular basement membrane

h: hour

HE: hematoxylin-eosin

IEC: interstitial endocrine cell

IF: immunofluorescence

IHC: immunohistochemistry

IL: interleukin

IL2RG: interleukin 2 receptor subunit gamma

IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

LH: luteinizing hormone

lpr: lymphoproliferation

MPGN: membranous proliferative glomerulonephritis

mRNA: messenger RNA

MRL/+: MRL/MpJ-*Fas*^{+/+}
 MRL/lpr: MRL/MpJ-*Fas*^{lpr/lpr}
 MT: Masson's trichrome
 NDS: normal donkey serum
 NGS: normal goat serum
Nos: nitric oxide synthase
 OSE: ovarian surface epithelium
 PAM: periodic acid methenamine-silver
 PAS-H: periodic acid-Schiff-hematoxylin
 PB: phosphate buffer
 PEC: parietal epithelial cell
 POF: premature ovarian failure
 qPCR: quantitative polymerase chain reaction
 RT: reverse transcription
 S/B: spleen weight to body weight
 SE: standard error
 SEM: scanning electron microscopy
 SLE: systemic lupus erythematosus
 ssDNA: single-stranded DNA
 St.: stage of the seminiferous epithelial cycle
 tACE: testicular isoform of angiotensin-converting enzyme
 TEM: transmission electron microscopy
Tgf, TGF: transforming growth factor
 TLR, *Tlr*: toll-like receptor
 TNF, *Tnf*: tumor necrosis factor
 uACR: urinary albumin creatinine ratio
 X-SCID: X-linked severe combined immunodeficiency
 Yaa: BXSb/MpJ-*Yaa*

Notes

Contents of the thesis were partially published in the following article list. The number of each article is corresponding to chapter number.

List of published articles

1. Otani Y, Ichii O, Otsuka-Kanazawa S, Chihara M, Nakamura T, Kon Y. MRL/MpJ-*Fas*^{lpr} mice show abnormalities in ovarian function and morphology with the progression of autoimmune disease. *Autoimmunity* 48, 402-411, 2015.
2. Otani Y, Ichii O, Masum MA, Kimura J, Nakamura T, Elewa YHA, Kon Y. BXSB/MpJ-*Yaa* mouse model of systemic autoimmune disease shows increased apoptotic germ cells in stage XII of the seminiferous epithelial cycle. *Cell Tissue Res* 381, 203-216, 2020.

Preface

In mammals, reproductive system and immunity regulate mutual functions. Firstly, proper immune homeostasis is necessary to maintain reproductive function in both male and female. Briefly, in healthy individuals, many immune cells are present in the ovaries suggesting functional roles in ovarian function of several species, such as rodents, cows, sheep, pigs, and horses ^{75,114}). For example, the number and type of immune cells are present in corpora lutea, and these localization and populations change depending on the stages of the estrous cycle, suggesting that these leukocytes participate in the development and regression of corpora lutea ^{50,54,79}). In addition to immune cells, a variety of cytokines and their receptors are expressed in the ovaries ⁹⁶). For example, interleukin 6 (IL-6) is produced by granulosa cells, and functions during oogenesis as a regulator of the luteinizing hormone (LH) receptor in rat granulosa cells ³⁹). Additionally, IL-1 signaling via IL-1 β and its receptor are essential for ovarian folliculogenesis and ovulation in the equine ovaries ⁶²). In pigs, tumor necrosis factor alpha (TNF- α) acts as a survival factor for granulosa cells during follicular atresia ⁷²).

In contrast, testes have unique immunological features called as immune privilege – spermatogenic cells are highly segregated from systemic immunity ²⁴). Immunosuppression is essential to maintain germ cells and appropriate spermatogenesis because a variety of testicular antigens start expressing after immune competence is established. These antigens can be easily attacked by the immune system once they are exposed to systemic immune system. Notably, the tight junction, called as the blood-testis barrier (BTB) and formed by adjacent Sertoli cells, partially segregates spermatogenic cells as preventing systemic immune factors from invading the adluminal compartment. In addition, anti-inflammatory factors, such as IL-10 are produced by testicular interstitial cells and resident immune cells, to maintain an immunosuppressive environment ⁸²).

Reproductive factors, mainly sex hormones and sex chromosomes, conversely mediate immune function. Expressions of sex hormone receptors for androgen, estrogen, or progesterone in various immune cells of human and mouse are reported ⁵²) – estrogens and androgens usually show the active and suppressive effects on immune function, respectively. In addition, X chromosome codes much more numbers of immune-related genes compared with Y chromosome, such as Toll-like receptor (TLR) 7 and 8, and regulators of immune cell function in various species ²⁶). Further, several X-linked gene mutations have been determined as causes of immunodeficiency. Briefly, a mutation in IL-2 receptor

subunit gamma (*IL2RG*) coding γ -chain subunit, a receptor component for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, causes X-linked severe combined immunodeficiency (X-SCID) in human. Also, forkhead box P3, regulator of regulatory T-cell, is also identified as a causative gene for immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in humans ²⁶⁾. These evidences support the existence of sex-related disparity in mammalian immune system.

Due to this close functional crosstalk between reproduction and immunity, disorder of either function could cause disease of the other system. Premature ovarian failure (POF) is a serious reproductive disease of women showing amenorrhea, hypoenestrogenism, and elevated serum levels of follicle-stimulating hormone (FSH) by 40 years of age ⁹⁴⁾. Notably, 30% of patients with POF have an autoimmune disease ³¹⁾. As for male reproductive function, anti-sperm antibodies (ASA) are frequently detected in infertile men ²⁸⁾, resulting in decreased sperm concentration and motility ¹⁵⁾. Infertility is commonly serious problem for animals from the viewpoint of biodiversity – continuous reproduction of next generation ensures species survival. In addition, there are clear sex-related disparities in various diseases including tumor, infectious disease, and kidney disease, suggesting the effect of sex chromosome and hormones on their pathogenesis. In veterinary field, however, more than half of domestic dogs and cats are gonadectomized from the viewpoint of animal welfare, and many farm animals are forced to be continuously pregnant, and lactate in order to satisfy food supply for humans. Importantly, gonadectomized dogs were reported to increase the incidence of immune-related diseases such as autoimmune thrombocytopenia, rheumatoid arthritis, and Addison's disease ¹⁰²⁾. However, the biological effect of these procedures interfering their reproductive function to disease development were still unclear. These evidences from both sides of reproductive and immune function in humans and animals imply the bidirectional effects on the pathogenesis of diseases and highlight the necessity for further investigation.

In the thesis, studies focus on the phenotypes of model mouse to elucidate the disease pathogenesis caused by the imbalance of reproduction-immunity crosstalk. The thesis contains three chapters; the first and second chapters examine the effect of systemic autoimmune disease on the morpho-function of female and male reproductive organs, respectively, using disease model mice. The third chapter discusses the function of androgens in the pathogenesis of autoimmune disease and glomerulonephritis by examining the phenotype of castrated disease model mouse. This study accumulates basic evidences

of histological changes in autoimmune-related urogenital disease caused by disturbed interaction between reproduction and immunity in mammals.

Chapter 1

The effect of systemic autoimmune disease on the morpho- function of female reproductive tract

Introduction

In mammals, the female reproductive period is limited due to the finite number of oocytes in the ovaries. In humans, the total number of oocytes gradually decreases with age during physiological processes including ovulation and follicular atresia. However, gonadal dysfunction, such as POF, can cause an unexpected decrease in the number of oocytes stored in the ovaries, resulting in infertility. Notably, an estimated 1.5 million patients are diagnosed with POF each year, and approximately 1% of women worldwide live with this disease ^{31,41}). Several reports have suggested that POF is caused by genetic factors, infectious agents, or iatrogenic causes. Notably, however, approximately one-third of POF cases are attributable to autoimmune disease, such as systemic lupus erythematosus (SLE), autoimmune thyroiditis, and rheumatoid arthritis ³¹). Autoimmune-associated POF is characterized by lymphocytic oophoritis and/or by the presence of ovarian autoantibodies, and, in most cases, it is difficult to reacquire fertility with ovulation of healthy oocytes.

Furthermore, several studies in clinical veterinary medicine also support the notion that altered immune condition may affect reproductive function. Briefly, dogs suffering autoimmune-mediated thyroiditis are anestrus, suggesting a loss of reproductive function ⁷⁶). In laboratory animals, autoimmune regulator (*Aire*)-deficient mice, which show severe autoimmune phenotypes, are infertile, with follicular depletion and lymphocyte infiltration in their ovaries ⁴¹). These evidences suggest possible adverse effect of autoimmune disorder on female reproductive function. However, little is known about the etiology of autoimmune-related ovarian disorder.

MRL/MpJ-*Fas*^{*lpr/lpr*} mice (MRL/*lpr*) are commonly used as autoimmune disease models. MRL/*lpr* showed resemble pathology with human SLE, rheumatoid arthritis, and lupus nephritis ^{74,108}). MRL/*lpr* show a much more severe autoimmune phenotype than their strain of origin, MRL/MpJ-*Fas*^{*+/+*} (MRL/+), resulting from the lymphoproliferation (*lpr*) mutation of *Fas* (TNF receptor superfamily member 6), which causes abnormal survival of auto-reactive T lymphocytes ¹⁰⁴). In addition to the *lpr* mutation, the MRL-background affects the development of autoimmune disease, and is known to contain more than 13 disease susceptibility loci associated with the development of SLE-related diseases ^{38,88}). 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 ¹¹³), supporting their use as indicators of disease severity. Furthermore, the MRL-

background is also related to the manifestation of several unique phenotypes in female reproductive organs. In particular, neonatal MRL/+ show accelerated folliculogenesis associated with the appearance of numerous mast cells in the ovary ^{71,117}). Further, adult MRL/+ frequently develop ovarian cysts, originating from the rete ovarii ⁵⁶). Although MRL/+ show several abnormalities in both immune and reproductive function, the pathological and etiological correlations remain unclear.

In this chapter, the author used MRL-background mice to evaluate the effect of autoimmune disease on female reproductive function in the reproduction-immunity crosstalk. Female MRL/lpr 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 insight into autoimmune-related infertility of females in both human and animals.

Materials and methods

Animal and sample preparation

Female C57BL/6N, MRL/+, and MRL/lpr were purchased from Japan SLC, Inc. (Shizuoka Japan). Mice were maintained according to *The Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine* (Approval No. 13-0031, 13-0032). This animal experiment program was approved by the Association for Assessment and Accreditation of Laboratory Animal Care International). Virgin female mice at 3, 6, 9, and 12 months of age were used at the metestrus stage of the estrous cycle, as determined by monitoring vaginal smears. Body weights were measured, and then blood samples were collected by cutting caudal vena cava under deep anesthesia (Avertin; 2,2,2-tribromoethanol dissolved in 2-methyl-2-butanol, 2,400 mg/kg, administered intraperitoneally). After mice were euthanized by cervical dislocation, the ovaries and spleen were collected. Spleen weight was measured, and the spleen weight to body weight ratio (S/B) was calculated as an index of autoimmune disease progression.

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, to assess estrous cyclicity, vaginal smears were collected for 10 consecutive days every month until 12 months of age. 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 ¹⁰⁵.

Histological analyses

Collected ovaries were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and cut into sections (3 µm thick). Deparaffinized sections were stained with hematoxylin-eosin (HE) or periodic acid-Schiff-hematoxylin (PAS-H) for evaluation of ovarian morphology. The number of primordial, primary, secondary, and antral follicles was counted in 5 sections, and only follicles

containing oocytes with an apparent nucleus were counted. The number of corpora lutea was also counted in 5 sections. Finally, the total number counted in all 5 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 (for anti-CD3 antibody) or 0.05% trypsin/0.01M phosphate-buffered saline (PBS, pH 7.4) for 5 min at 37°C (for anti-B220 antibody). 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 antibody (1:1,600, Cedarlane, Ontario, Canada) or rabbit anti-CD3 antibody (1:200, Nichirei, Tokyo, Japan) at 4°C overnight. After washing 3 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(R) Kit, Nichirei), for 30 min at room temperature, washed again, and incubated with streptavidin-biotin complex (SABPO(R) 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 5 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 postfixated with 1% osmium tetroxide in 0.1 M phosphate buffer (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/6N and MRL/lpr 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 3,000 rpm for 15 min. After clotted blood was removed, the samples were centrifuged at 12,000 for 5 min, and the supernatant was immediately frozen at -80°C. Serum levels of endogenous mouse FSH and testosterone were measured using an ELISA Kits (Endocrine Technologies Inc., Newark, NJ, 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 ($P < 0.05$).

Result

Autoimmune disease symptoms of mice

S/B was used as an index of autoimmune disease in mice (Fig. 1-1A). At all ages examined, MRL/lpr showed significantly higher values than the other 2 strains. At 9 and 12 months, although MRL/+ showed slightly higher values than C57BL/6N, no significant differences were observed between C57BL/6N and MRL/+.

Onset of puberty and estrous cyclicity of mice

Vaginal opening was observed in C57BL/6N, MRL/+, and MRL/lpr to determine the onset of puberty (Fig. 1-1B). Vaginal opening occurred significantly earlier in MRL/+ (18.8 ± 4.3 days) and MRL/lpr (19.8 ± 1.7 days) than in C57BL/6N (39.6 ± 2.9 days).

Age-related changes in estrous cyclicity were examined next. Figure 1-1C shows the representative pattern of mouse estrous cycle of mice of each of the 3 strains at 3, 6, 9, and 12 months. C57BL/6N and MRL/+ 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), and demonstrated abnormal cyclicity at 12 months (Fig. 1-1C). However, MRL/lpr lost cyclicity after 6 months, characterized by a prolonged diestrus period and a shortened estrus period.

Figure 1-1D and E show the proportion of time spent in estrus and diestrus, respectively, for each of the 3 strains. Estrus was shorter in MRL/lpr than in the other strains at 6 and 9 months; the difference observed at 6 months was significant. At 12 months, C57BL/6N and MRL/+ tended to show shorter and longer periods of estrus, respectively, compared to earlier months; however, no significant differences were observed among strains (Fig. 1-1D). At 6 and 9 months, diestrus was longer in MRL/lpr than in the other strains; the difference observed at 6 months was significant. At 12 months, C57BL/6N and MRL/lpr showed a longer period of diestrus than in earlier months, but no significant differences were observed among strains (Fig. 1-1E).

Age-related histological changes in mouse ovaries

Figure 1-2A to I show the ovarian histology of C57BL/6N, MRL/+, and MRL/lpr at 3, 6, and 12 months. At 3 months, follicles at various stages of development were observed at the ovarian cortices in

all 3 strains (Fig. 1-2A, D, and G); the number tended to decrease with age (Fig. 1-2B, C, E, F, H, and I). In particular, very few follicles were observed in MRL/lpr at 12 months (Figure 1-2I). At all ages examined, a large number of corpora lutea were observed in C57BL/6N and MRL/+ (Fig. 1-2A–F); however, few were observed in MRL/lpr (Fig. 1-2G–I). Further, the ovarian interstitium was larger in MRL/lpr than in the other strains.

Next, the author 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 3 strains. The total number of follicles decreased with age in all strains (Fig. 1-2J–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 showed a smaller number of primordial follicles than C57BL/6N at all ages examined, and significant differences between strains were observed at 3 and 6 months (Fig. 1-2J and K). Further, MRL/+ mouse ovaries contained a significantly larger number of secondary follicles than the other 2 strains at 3 months (Fig. 1-2J). The total number of follicles was significantly lower in MRL/lpr than in the other 2 strains at 3 months, and in both MRL/+ and MRL/lpr than in C57BL/6N at 6 months (Fig. 1-2J and K). In addition, the number of corpora lutea was lowest in MRL/lpr at all ages examined; it was also significantly lower in MRL/+ than in C57BL/6N at 12 months (Fig. 1-2M).

As shown in Figure 1-3, strain-related differences in ovarian morphology were observed at the ovarian interstitium and on the ovarian surface. The ovarian interstitium contains interstitial glands, which consist of interstitial endocrine cells (IECs) (Fig. 1-3A–I). These IECs originate from internal theca cells of atretic follicles and primarily produce testosterone in rodents ⁷⁾. IECs were clearly observed in C57BL/6N at 12 months (Fig. 1-3C) and in MRL/+ after 6 months (Fig. 1-3E and F). However, large numbers of IECs were clearly present in MRL/lpr mouse ovaries from 3 months and were observed at all ages examined (Fig. 1-3G–I).

In addition, the ovarian surface epithelium (OSE) was a squamous monolayer in C57BL/6N and MRL/+ at all ages examined, as shown in Figure 1-3J and K, whereas a partially cuboidal, stratified epithelium was observed in MRL/lpr after 6 months (Fig. 1-3L).

Ultrastructure of the ovarian surface

The morphology of the ovarian surface was compared between C57BL/6N and MRL/lpr at 6

months using electron microscopy. Under SEM observation, the ovarian surface of C57BL/6N appeared smooth (Fig. 1-4A) and was mostly covered with oval- or spindle-shaped epithelial cells with microvilli (Fig. 1-4B). In contrast, the ovarian surface of MRL/lpr showed several dome-shaped bulges (Fig. 1-4C) covered by rounded epithelial cells that were fully covered in microvilli (Fig. 1-4D). The microvillus density of epithelial cells appeared to be higher in MRL/lpr than in C57BL/6N (Fig. 1-4B and D).

Next, the ovarian surface was observed using semi-thin and ultra-thin sections (Fig. 1-4E–H). In C57BL/6N, the OSE consisted of monolayered dark- or pale-colored cells (Fig. 1-4E). 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 (Fig. 1-4F). Likewise, two cell types were observed in the semi-thin OSE sections of MRL/lpr (Fig. 1-4G). However, the OSE cells and ovarian tunica albuginea (lowermost layer of the ovary capsule) were taller and thicker, respectively, in MRL/lpr than in C57BL/6N (Fig. 1-4E and G), which was also accompanied by an increase in the presence of collagen fibrils (Fig. 1-4H). Under TEM, the dark-colored cells in the semi-thin sections showed a highly electron-dense cytoplasm and abundant microvilli in MRL/lpr (Fig. 1-4H). The pale-colored cells in semi-thin sections had a cytoplasm with low electron density and a larger nucleus than the dark-colored cells, but didn't show typical oocyte-like morphology. The microvillus density of cells with a highly electron-dense cytoplasm in MRL/lpr appeared to be higher than that in C57BL/6N (Fig. 1-4F and H), as well as in cells of MRL/lpr with a cytoplasm with low electron density.

Inflammatory cell infiltration in mouse ovaries

Immunohistochemical analysis was used to detect T- and B-cells in the ovaries of C57BL/6N, MRL/+, and MRL/lpr (Fig. 1-5). At 6 months, few CD3-positive T-cells were observed in the ovaries of C57BL/6N and MRL/+, and these tended to localize in or around corpora lutea (Fig. 1-5A and B). However, numerous CD3-positive T-cells were observed in the ovaries of MRL/lpr (Fig. 1-5C). These cells were widely distributed, and mainly localized to the ovarian interstitium. Quantification of CD3-positive T-cells in the ovaries showed that MRL/lpr showed significantly higher values than the other 2 strains at both 3 and 6 months of age (Fig. 1-5D). At 3 months, CD3-positive T-cells were typically observed in or around the follicles such as atretic and primordial follicles of MRL/lpr (Fig. 1-5E and F).

Further, few B220-positive B-cells were observed in the ovaries of C57BL/6N and MRL/+ at any period examined, as shown in Figure 1-5G and H, whereas some MRL/lpr showed numerous positive cells in the ovarian interstitium at 6 months (Fig. 1-5I).

Serum levels of FSH and testosterone in mice

The author next compared the blood serum levels of FSH and testosterone, as indicators of reproductive ability and IEC function, respectively, in C57BL/6N, MRL/+, and MRL/lpr at 6 and 12 months (Fig. 1-6)^{7,12}. Although serum FSH levels tended to be slightly higher in MRL/lpr than in C57BL/6N and MRL/+ at 12 months, no significant differences among strains were observed at any age (Fig. 1-6A). Similar to FSH, serum testosterone levels were higher in MRL/lpr than in C57BL/6N and MRL/+ at 6 and 12 months, but the differences were not significant (Fig. 1-6B).

Discussion

Several studies have indicated the functional crosstalk between the immune and reproductive systems ^{96,114}). This study examined the effect of autoimmune disease on ovarian morphology using MRL-background mouse strains. From 3 months, MRL/lpr showed autoimmune defects, indicated by significantly increased spleen weight compared to MRL/+ and C57BL/6N. At the same age, the total number of follicles and corpora lutea tended to be smaller in MRL/lpr 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 ^{31,44,94}). Although the MRL-background carries several susceptibility loci associated with the development of autoimmune defects ^{38,88}), changes in ovarian morphology and development of autoimmune disease were less pronounced in MRL/+ than in MRL/lpr. Further, some MRL/+ and MRL/lpr developed ovarian cysts, as reported previously ⁵⁶); however, no correlation between cyst development and the parameters evaluated was found in this study (data not shown). Importantly, the present data indicates that splenomegaly observed at 3 months precedes the onset of estrous cycle irregularity at 6 months in MRL/lpr. Therefore, the *lpr* mutation and/or the severe autoimmune defects caused by this mutation may be crucial to the changes in ovarian morphology observed in MRL-background strains.

OSE, a type of mesothelium, is thought to be able to produce granulosa cells or oocytes ^{10,67}). In this study, two types of cells, dark- and pale-colored cells, were observed in semi-thin sections of the OSE. The pale cells observed in C57BL/6N were likely oocytes or oogonia, considering their nuclear shape and previous reports ¹⁰). On the other hand, OSE cells showed an altered morphology in MRL/lpr at 6 months. Ultrastructurally, the microvilli were more developed in MRL/lpr than in C57BL/6N, as shown by SEM examination of the OSE and TEM observation of dark-colored cells, and the OSE was partially stratified in MRL/lpr. 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 ⁴³). Further, patients with autoimmune diseases or ovarian cancer show altered amounts or components of ascitic fluid ^{1,55}). Therefore, the features of OSE in MRL/lpr may reflect the altered environment in or around the ovaries.

Further, only MRL/lpr showed a thickened ovarian tunica albuginea. Similar morphological

changes have been reported in patients with polycystic ovarian syndrome and in model mice ¹⁷⁾. Thickening of the tunica albuginea could contribute to infertility by disturbing ovulation. Indeed, MRL/lpr demonstrated smaller number of ovulated oocytes than MRL/+ ³³⁾. Thus, 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 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. These processes are coordinately and regularly mediated by oocytes, follicular epithelial cells/granulosa cells, and lutein cells via the interaction of gonadotropins such as LH and FSH ¹²⁾. Furthermore, loss of the follicle pool and elevated serum FSH levels are pathological characteristics in human patients with POF showing an altered menstrual cycle ³⁶⁾. In this study, no significant differences were observed in serum levels of FSH among strains, but MRL/lpr showed significantly decreased numbers of follicles and corpora lutea from 3 months. 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 showed several common phenotypes, such as earlier vaginal opening and fewer primordial follicles than C57BL/6N, despite the lack of severe autoimmune defects in MRL/+. A previous study suggested that MRL/+ showed more progressed early folliculogenesis than C57BL/6N ^{113,117)}. Particularly, increased mast cells could contribute to early follicular development and oocytic death ⁷¹⁾. Therefore, the earlier onset of puberty and fewer primordial follicles compared to C57BL/6N are unique phenotypes in MRL-background strains. However, the appearance of MRL-derived unique ovarian phenotypes differed between MRL/+ and MRL/lpr in several ways. Briefly, MRL/+ mouse ovaries contained a larger number of secondary follicles than those of MRL/lpr at 3 months. Further, compared to C57BL/6N, the total number of follicles was smaller from 3 and 6 months in MRL/lpr and MRL/+, respectively. These results suggest that the unique phenotypes associated with folliculogenesis in MRL/+ were modified in MRL/lpr. In MRL/lpr, infiltration of T-cells in or around follicles was observed after 3 months. Therefore, these cells or the inflammatory mediators that they produce may affect folliculogenesis in MRL/lpr.

Testosterone-producing IECs originate from internal theca cells of atretic follicles and develop in the ovarian interstitium of rodents, and they primarily produce testosterone ⁷⁾. IECs were abundantly developed in the ovaries of MRL/lpr after 3 months, that was accompanied with the smaller number of corpora lutea, suggesting the increase of follicular atresia and failure of luteinization. Since similar tendency was observed in older MRL/+, the MRL-background may contribute to the development of IECs in both MRL/+ and MRL/lpr. In addition to genetic predisposition, other factors would be involved in the acceleration of these follicular phenotypes in MRL/lpr. Further, the serum testosterone concentration tended to be higher in MRL/lpr than in mice of the other strains, possibly reflecting the development of IECs in MRL/lpr.

In mammalian ovaries, the development and regression of corpora lutea is regulated by cytokines via nuclear factor kappa B signaling, which are produced by immune cells observed in or around corpora lutea ^{96,114)}. In the present study, few lymphocytes were observed in or around the corpora lutea of C57BL/6N and MRL/+. In contrast, a pronounced infiltration of B- and T-cells into the ovarian interstitium was observed in MRL/lpr, suggesting the direct effect of immune cells on ovarian cells, including follicular cells. Furthermore, previous reports have suggested that granulosa cells and theca cells express Fas protein, and that Fas-Fas ligand-mediated apoptosis is important for the regression of corpora lutea ^{87,97)}. Based on these findings, the author suggests that changes in the local immune microenvironment and/or cell death resulting from *lpr* mutation in the *Fas* gene may change the fates of stored oocytes and internal theca cells, resulting in the development of IECs.

The author demonstrated a relationship between ovarian morphology and reproductive function in MRL/lpr autoimmune mice. These data may aid in clarifying the pathogenic mechanism of ovarian dysfunction in autoimmune disease. Briefly, intraovarian infiltration of immune cells, loss of the follicle pool and a disrupted estrous cycle are common pathological features of autoimmune disease between POF patients and MRL/lpr. While MRL/lpr did not clearly exhibit the increased levels of FSH a characteristic of POF, they did display unique ovarian phenotypes including failures in follicle luteinization, increased IECs and altered ovarian surface morphology. Menstrual abnormalities, increased cycle length and decreased serum levels of hormones produced by corpora lutea were observed in patients with SLE ^{64,91)}. Ovarian reserve markers such anti-Müllerian hormone and antral follicle count were decreased in adult patients with childhood-onset SLE ³⁾. In MRL/lpr, early onset of puberty

and altered follicular development were observed at the same time or earlier than the development of autoimmune defects indicated by increased spleen weight. Importantly, it was reported that early age at menarche is associated with an increased risk of SLE in human patients ¹³).

Taken collectively, the author concludes that the *Fas* mutation and subsequent immune defects triggered in the MRL-background likely leads to the abnormal reproductive phenotypes observed in MRL/lpr. This complexity causes the phenotypic similarities and differences observed between MRL/lpr and human clinical cases. In this chapter, these results provide a novel insight into the pathogenesis of autoimmune-related ovarian failure caused by the imbalance of functional crosstalk between female reproduction and immunity in mammals.

Summary

The immune system is known to affect reproductive function, and maternal-fetal immune tolerance is essential for a successful pregnancy. To investigate the effect of autoimmune disease on female reproductive function, the author performed a comparative analysis of the ovarian phenotypes for C57BL/6N, autoimmune disease-prone MRL/+ and congenic MRL/lpr harboring a mutation in the *Fas* gene that accelerate disease onset. Both MRL-background strains showed earlier vaginal opening than C57BL/6N. The estrous cycle became irregular by 6 and 12 months of age in MRL/lpr 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/6N after 3 months. In addition, MRL/lpr and MRL/+ 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/6N. MRL/lpr and MRL/+ developed ovarian interstitial glands after 3 and 6 months, respectively. In particular, MRL/lpr showed numerous infiltrating lymphocytes within the ovarian interstitia, and partially stratified ovarian surface epithelia with more developed microvilli than that observed in C57BL/6N at 6 months. No significant differences in serum hormone levels were observed between the strains. In conclusion, MRL/lpr display altered ovarian development, morphology and function consistent with the progression of severe autoimmune disease, as these findings are less severe in MRL/+ counterparts. Thus, results in this chapter revealed the mechanism how excessive autoimmunity lead to abnormal morpho-function of female reproduction through the crosstalk with immunity.

Figures

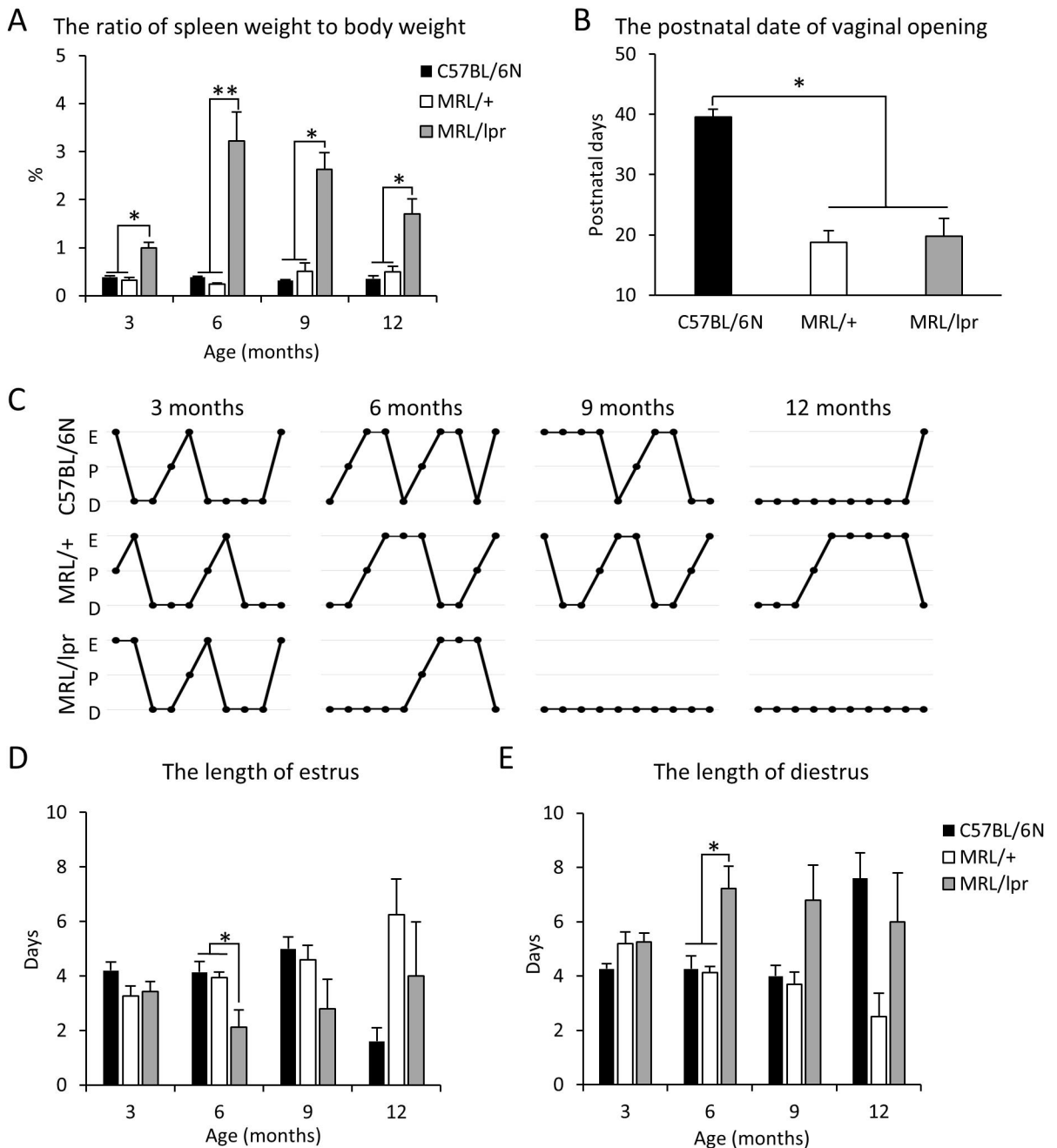


Figure 1-1. Indices of autoimmune disease condition and female reproductive function in mice

(A) The ratio of spleen weight to body weight in mice. (B) The postnatal date of vaginal opening of mice. (C) Estrous cycles monitored for 10 consecutive days in C57BL/6N, MRL/+ and MRL/lpr at 3, 6, 9 and 12 months. Representative cycle traces are shown with days plotted on the X axis. D: diestrus. P: proestrus. E: estrus. (D) The length of estrus, monitored for 10 days, at 3, 6, 9, and 12 months. (E) The length of diestrus, monitored for 10 days, at 3, 6, 9, and 12 months. Each bar represents the mean $\pm$ SE ($n = 4-5$). Significant differences among strains are indicated with an asterisk. *: $P < 0.05$, **: $P < 0.01$ (Kruskal–Wallis test followed by Scheffe’s method).

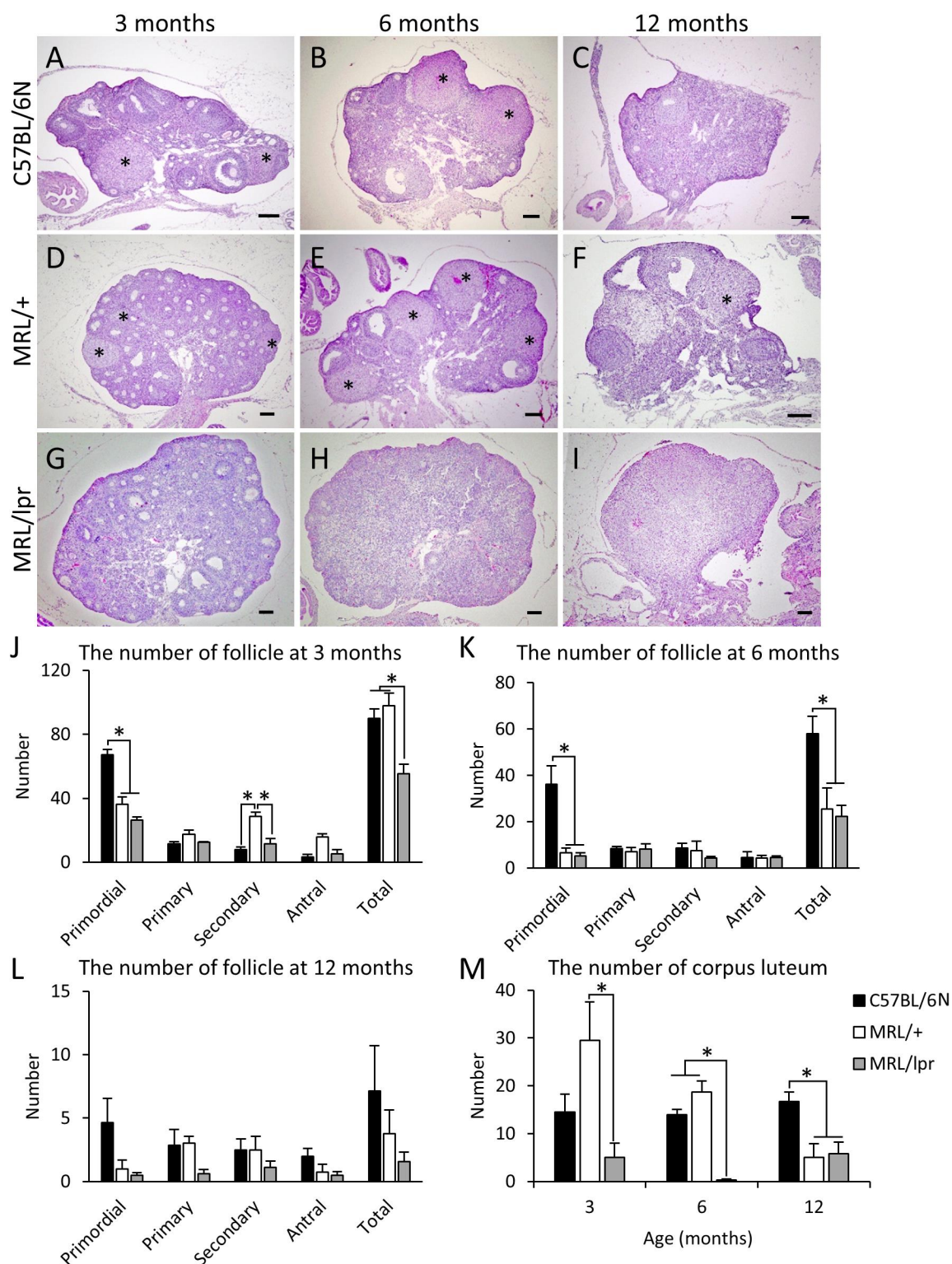


Figure 1-2. Age-related morphological changes in mouse ovaries

(A–I) Whole ovaries of C57BL/6N (A–C), MRL/+ (D–F), and MRL/lpr (G–I) 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. Follicles decrease in number with age in all strains (A–I); the decrease is most severe in MRL/lpr (G–I). Fewer corpora lutea and an ovarian interstitium with a larger area are observed in MRL/lpr (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 corpora lutea at each age (M). The number of follicles and corpora lutea were counted in 5 sections. The total number in all 5 sections was used as the value for that ovary. Each bar represents the mean $\pm$ SE (n = 4). Significant differences among strains are indicated with an asterisk. *: $P < 0.05$ (Kruskal–Wallis test followed by Scheffe's method).

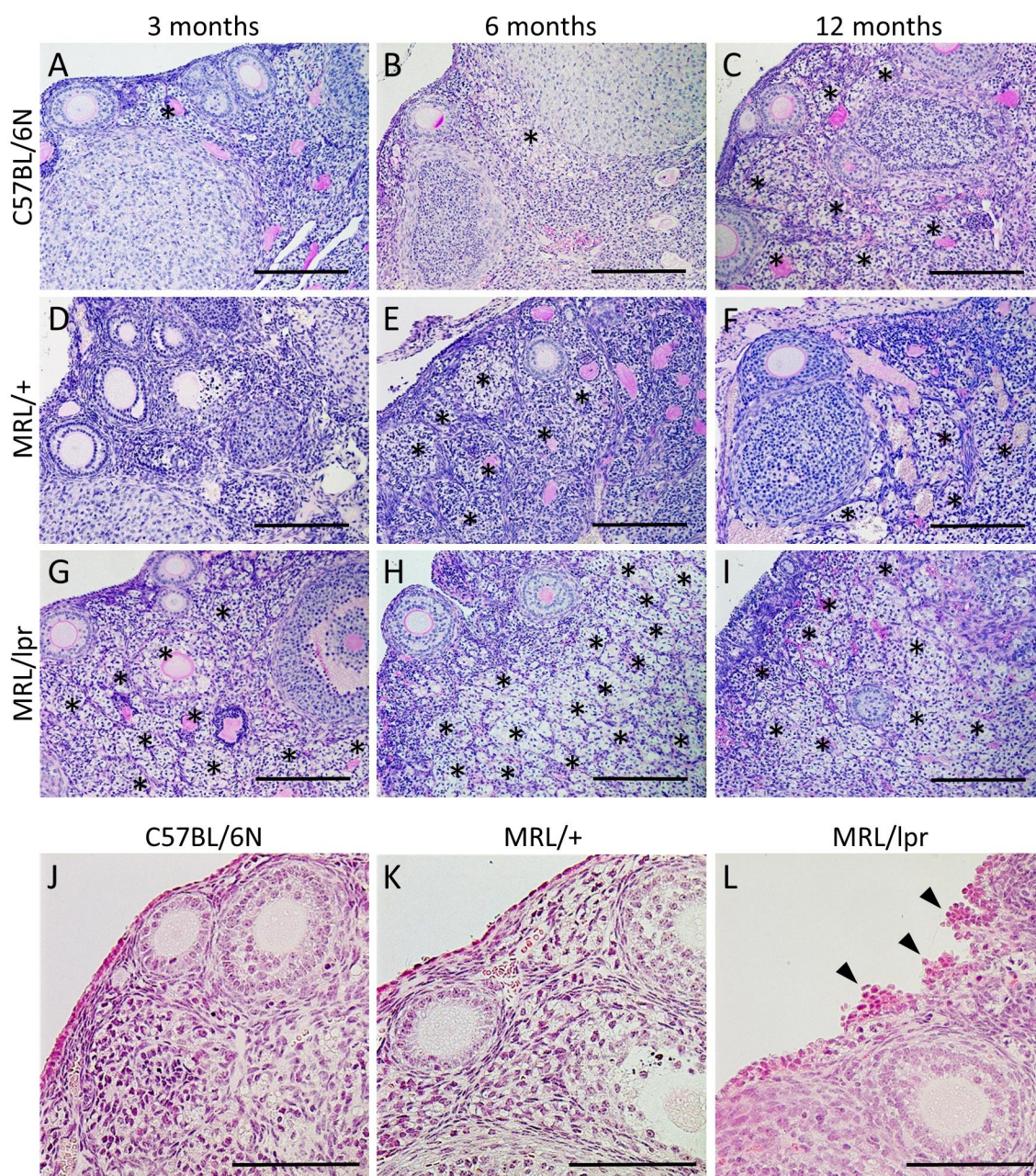


Figure 1-3. Age-related histological changes in the ovarian interstitium and surface epithelium in mice

(A–I) Ovarian cortices of C57BL/6N (A–C), MRL/+ (D–F), and MRL/lpr (G–I) at 3 (A, D, and G), 6 (B, E, and H), and 12 (C, F, and I) months. All sections were stained with PAS-H. Asterisks indicate interstitial glands composed of IECs. Fully developed interstitial glands are observed in C57BL/6N ovaries at 12 months (C), in MRL/+ at 6 (E) and 12 (F) months, and in MRL/lpr at 3 (G), 6 (H), and 12 (I) months. (J–L) Ovarian cortices of C57BL/6N (J), MRL/+ (K), and MRL/lpr (L) at 6 months. All sections were stained with HE. A squamous, monolayered epithelium covers the ovarian surface in C57BL/6N (J) and MRL/+ (K) mice. Cuboidal and stratified surface epithelial cells (arrowheads) are observed in MRL/lpr mice (L). Bars = 100 μ m.

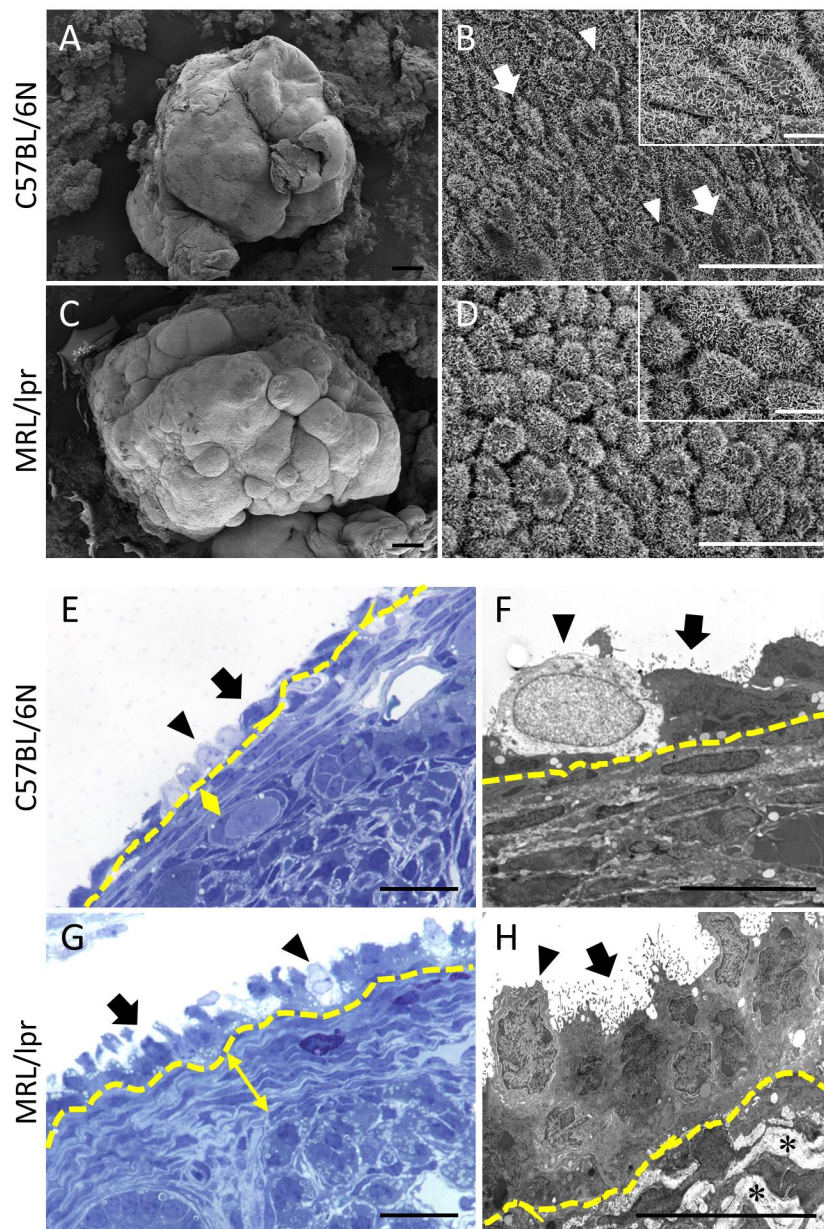
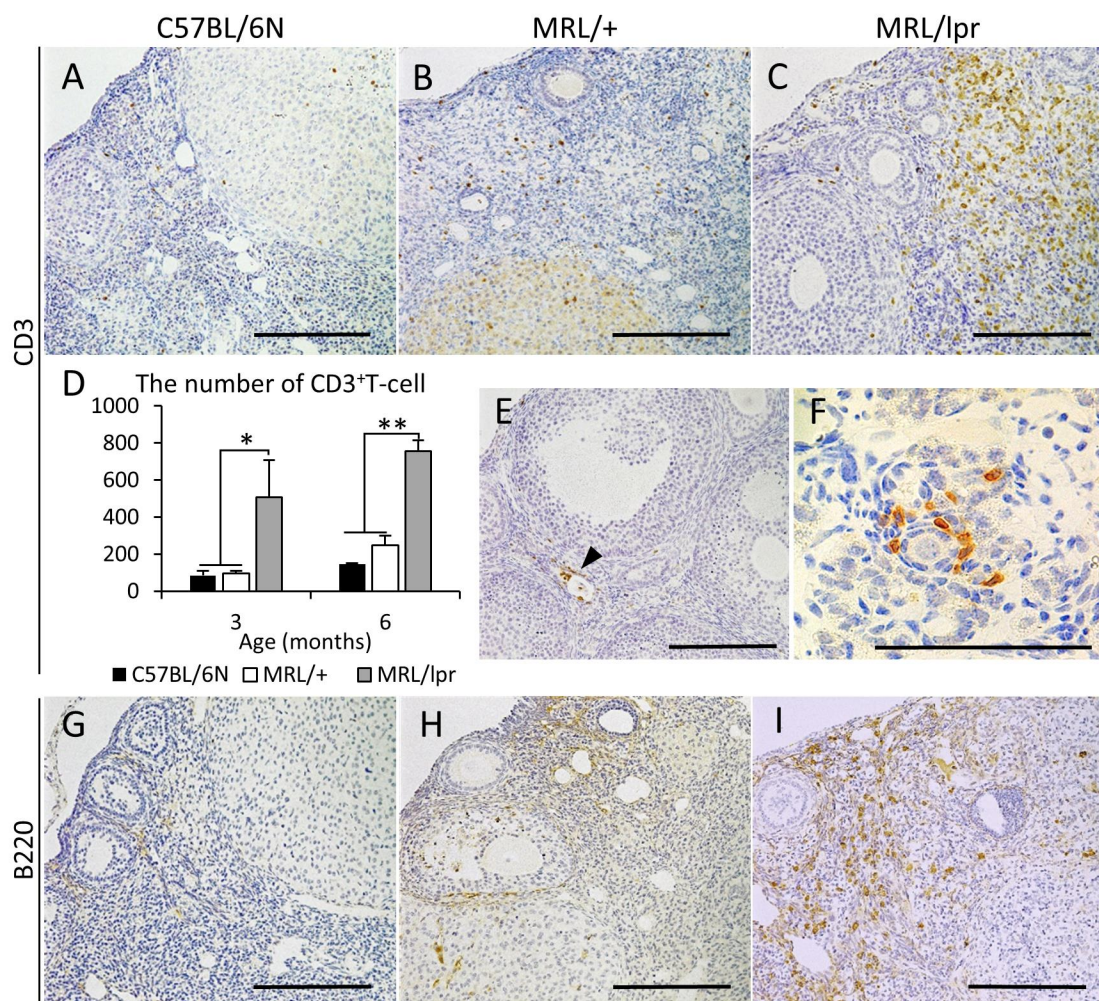


Figure 1-4. Ultrastructure of mouse ovaries

(A–D) Ultrastructure of the ovarian surface under SEM observation in C57BL/6N (A and B) and MRL/lpr (C and D) at 6 months. The ovarian surface of C57BL/6N is smooth (A) and covered with oval epithelial cells (arrowheads) or spindle-shaped cells (arrows) with microvilli (B). The ovarian surface of MRL/lpr shows several dome-shaped bulges (C), and is mostly covered with round epithelial cells with abundant microvilli (D). The insets show ovarian epithelial cells at a higher magnification. Black bars = 200 μm. White bars = 20 μm. Inset bars = 5 μm. (E–H) Ultrastructure of the ovarian surface in C57BL/6N (E and F) and MRL/lpr (G and H) 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 (arrowheads) and black-colored cells (black arrows), are observed in the superficial epithelium of C57BL/6N (E) and MRL/lpr (G). The tunica albuginea (dual-headed yellow arrows) are thicker in MRL/lpr than in C57BL/6N (E and G). In the ovarian surface epithelium of C57BL/6N (F), the round cells contain 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 (H), all cells are taller than those of C57BL/6N (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 contains numerous collagen fibers (H, asterisks). Similar to C57BL/6N, cells with abundant microvilli and an electron-dense cytoplasm (arrows) are observed, and its density appeared to be higher than in C57BL/6N (F and H). Bars = 10 μm.



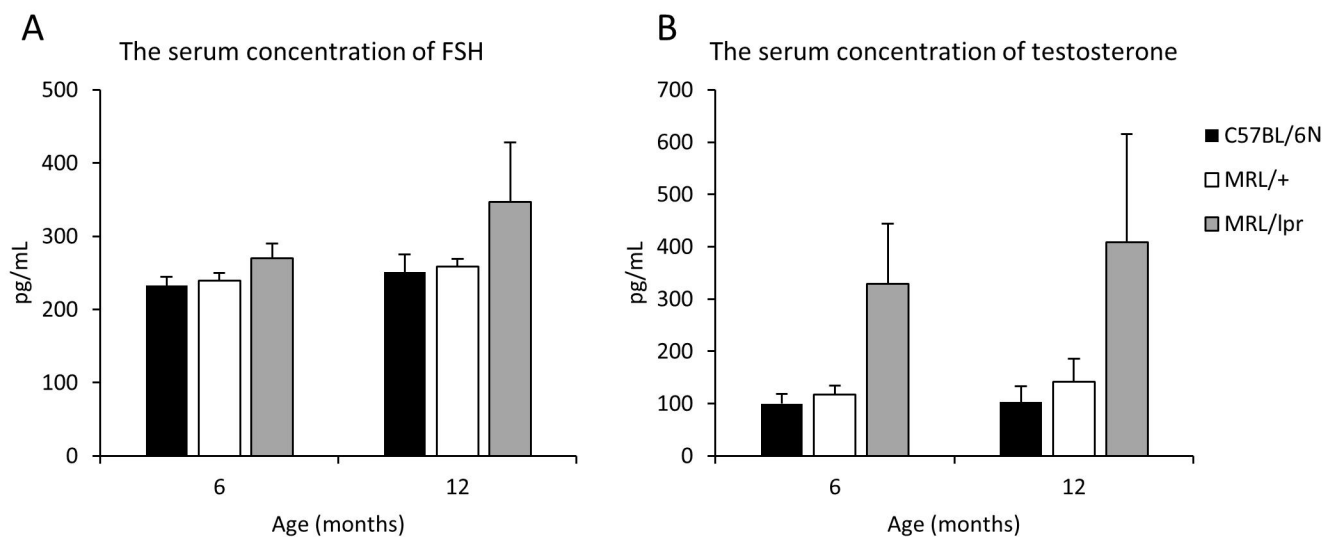


Figure 1-6. The serum concentrations of FSH and testosterone in female mice

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

Chapter 2

The effect of systemic autoimmune disease on the morpho- function of male reproductive tract

Introduction

Histological investigation of female model mouse suggests a direct interaction of immune cells and ovarian cells under autoimmune disease condition in chapter 1. Next, in this chapter, the author investigated pathological phenotype of male reproductive tract of autoimmune model mouse. Unlike female, male germ cells are highly protected from systemic immune reaction as an immune privilege site. BTB and immunosuppressive environment play critical roles in maintaining immune privilege site. Once the environment is disrupted by trauma, infection, and autoimmune disorder, molecules of germ cells could be recognized as an antigen, then would be attacked by immune reaction ²⁴⁾. Furthermore, secondary autoimmune orchitis leading to infertility is reported in patients with systemic autoimmune diseases, such as SLE, Behçet's disease, and rheumatoid arthritis. Furthermore, ASA is present in half of the patients with SLE ⁹⁵⁾. For research purposes, experimental autoimmune orchitis (EAO) can be induced by immunization with testicular homogenate and adjuvant in mice, rats, rabbits, and guinea pigs ⁷⁰⁾. EAO, resulting in infertility, is histopathologically characterized by T-cell-dependent lymphocytic inflammation and damaged seminiferous tubules, with disruption of the BTB ⁴⁹⁾. In these patients and model animals, the abnormality of systemic or local immune systems causes reproductive system-related phenotypes, but detailed pathogenesis is unknown.

In this chapter, the author examined the pathological features of the male reproductive system in a male-dominant systemic autoimmune disease model mouse to reveal the pathological mechanism of autoimmune-related male reproductive disorder. The BXSB strain is derived from an intercross of C57BL/6J and SB/Le mouse ²⁾. It has been found that some mutants in the BXSB strain are characterized by autoantibody production, splenomegaly, and severe lupus nephritis-like features due to the excessive proliferation of autoreactive lymphocytes ¹⁰³⁾. These mutants carry a Y-linked autoimmune accelerating mutation, a translocation of some genes on the X chromosome telomere region to that of the Y chromosome. A Y-linked autoimmune accelerating mutation has been identified as the most potent causative molecule to mediate autoimmune disorder in these mutants, designated as BXSB/MpJ-*Yaa* mice (*Yaa*) ⁶⁸⁾. Although previous studies revealed the BXSB genome is also suspected of forming autoimmune disease-prone phenotypes, as aged female BXSB/MpJ mice (BXSB, without Y-linked autoimmune accelerating mutation) manifested autoantibody production and glomerulonephritis ^{5,46)}, male *Yaa* showed much more severe systemic autoimmune disease.

Here the author showed the histopathological abnormalities of the Yaa testis, characterized by increases of residual bodies and apoptotic germ cells in stage XII of the seminiferous epithelial cycle, which were closely correlated with systemic autoimmune abnormalities. The author further demonstrated the change of Sertoli cell number was associated with the progression of an autoimmune condition, which likely caused subsequent morphological changes in Yaa testes. These results suggest the etiology of autoimmune-related male reproductive dysfunction, and would provide a novel insight into the pathological crosstalk between male reproduction and systemic immunity.

Materials and methods

Animals and sample preparation

Male C57BL/6N, BXSb, and Yaa were purchased from Japan SLC, Inc.. Three- and six-month-old mice were used in all experiments. Mice were maintained according to *The Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine* (Approval No. 15-0079, 16-0124). This animal experiment program was approved by the Association for Assessment and Accreditation of Laboratory Animal Care International). Body weights were measured, followed by the collection of blood samples by cutting the carotid artery under deep anesthesia (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol). After euthanasia by cervical dislocation, the testes, epididymides, and spleen were collected. The weights of the spleen and testes were measured, and the ratio to body weight was compared in each group, respectively.

Evaluation of serum autoantibodies

To evaluate the systemic autoimmune conditions, serum anti-double-stranded DNA (dsDNA) antibody levels were measured using the Mouse Anti-dsDNA antibody ELISA Kit (Shibayagi, Gunma, Japan) according to the manufacturer's instructions.

Histological analyses

Collected organs were fixed in 4% paraformaldehyde (for immunohistochemistry) or Bouin's fluid overnight, were embedded in paraffin, then cut into sections (2-3 μ m thick). Deparaffinized sections were stained with PAS-H to determine the stages of the seminiferous epithelial cycle (St.).

The immunodetection of cell markers was performed as done in chapter 1. Primary antibodies used in this chapter is listed in Table 2-1. In addition to immune cells, apoptotic cells and residual bodies in seminiferous tubules were detected using anti-single-stranded DNA (ssDNA) antibody and anti-testicular isoform of angiotensin-converting enzyme (tACE) antibody, respectively. For antigen retrieval, sections were incubated in buffered citrate (pH 6.0) for 15 min at 110°C. Finally, the sections were counterstained with PAS-H staining. When the St. in more than 80% of the seminiferous tubules could not be classified due to morphological abnormalities, these testis specimens were excluded from the histoplanimetric analysis. The detailed information is described in Table 2-1.

To detect accumulated RNA in seminiferous tubules, deparaffinized sections were stained with methyl green-pyronin solution (Nacalai Tesque, Kyoto, Japan) for 10 min at room temperature, then washed with distilled water, and cleared with butanol. For further analysis, deparaffinized sections were treated with 0.01% RNase A (Nacalai Tesque) for 20 minutes at 37 °C before stained with methyl green-pyronin to confirm the existence of RNA

Histoplanimetry

Histoplanimetric analysis for the testes was performed as follows:

- (a) *The number and area of the seminiferous tubules*: Histological sections were converted into digital images by scanning with NanoZoomer 2.0-RS and observed with NDP.view2 program (Hamamatsu Photonics K.K., Shizuoka, Japan). In a PAS-H-stained section, each St. of the seminiferous tubules was classified based on the morphological characteristics of the seminiferous tubules ⁶⁵. The number and area of the seminiferous tubules were quantified at each St. with NDP.view2. These values were also used in the following measurements.
- (b) *Analysis of residual bodies in seminiferous tubules*: Sections stained with PAS-H were used as digital images converted by NanoZoomer 2.0-RS. The numbers of residual bodies with more than 10 μm of the minor axis were counted at each classified St. according to analysis (a). The appearance ratio of residual bodies was calculated as the values of each St. was divided by the sum value of residual body number in all St..
- (c) *The number of ssDNA-positive cells in seminiferous tubules*: Sections stained with immunohistochemistry for ssDNA were used as digital images converted by NanoZoomer 2.0-RS. The number of ssDNA-positive cells in a seminiferous tubule was counted at each classified St. according to analysis (a).
- (d) *The number of Sertoli cells in seminiferous tubules*: Sections digitally imaged with NanoZoomer 2.0-RS were used. The number of Sertoli cells in a seminiferous tubule was counted at each classified St. according to analysis (a). The Sertoli cell was histologically identified by its basal localization and morphology with apparent nucleoli.

For the calculation of the number per unit area, counted numbers of residual bodies, ssDNA-positive cells, and Sertoli cells in all or in each St. tubule were divided by the corresponding areas

of the seminiferous tubules in a section.

Reverse transcription and quantitative PCR (qPCR)

Total RNA was isolated from the testes using the TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total RNA by reverse transcription (RT) using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Gene expression levels were examined by using synthesized cDNA, THUNDERBIRD SYBR qPCR Mix (TOYOBO), and a real-time thermal cycler (CFX Connect; BIO-RAD, Hercules, CA, USA), according to the manufacturer's instructions. Gene expression in the testes was normalized to the expression of actin beta (*Actb*). The details of primers are shown in Table 2-2.

Statistical analysis

The results are expressed as mean $\pm$ SE and were analyzed using non-parametric statistical methods. Data among strains of the same age were compared using the Kruskal-Wallis test, and multiple comparisons were performed using Scheffe's method when significant differences were observed ($P < 0.05$). Data between different ages in the same strain were compared using the Mann-Whitney *U*-test ($P < 0.05$). Spearman's correlation coefficient ($P < 0.05$) was used to analyze the correlation between two parameters.

Result

Indices of the autoimmune condition and reproductive function of mice

Figure 2-1A shows the body weight of animals. BXSB and Yaa showed significantly smaller values compared to C57BL/6N at 3 and 6 months of age, indicating early and late disease stages, respectively. An age-related increase was observed in C57BL/6N and Yaa, but not in BXSB. The testis weight was compared as an index of male reproductive function ⁶⁾. At both ages, the testis weight was significantly smallest in Yaa, and BXSB also showed significantly lower values compared with C57BL/6N (Fig. 2-1B). No significant age-related change was observed in their testis weights. For their ratio to body weight, there was no significant strain difference at both ages, but age-related significant decreases were observed in C57BL/6N and Yaa (Fig. 2-1C).

S/B and serum anti-dsDNA antibody levels were used as indices for the severity of systemic autoimmune disease in mice (Fig. 2-1D and E). Yaa showed significantly higher values of both indices compared with the other two strains at both stages. In these parameters, a significant age-related increase was observed only in the S/B of Yaa. Furthermore, the data of testis weight and spleen weight in all examined Yaa revealed a significant negative correlation ($\rho = -0.600$; $P = 0.001$, $n = 26$) (Fig. 2-1F), indicating the close relationships between male reproductive function and autoimmune disease.

Histopathological features of mouse testes

Figure 2-2 shows representative histological images of mouse testes at 3 months of age. Dilated seminiferous tubules with enlarged lumens were observed in some BXSB (3 and 6 months of age: 40%) and Yaa (3 months of age: 67%; 6 months of age: 50%), but not in C57BL/6N at both ages (Fig. 2-2A–C). As correlated with the enlargement of seminiferous tubules, the epididymal ducts at the tail portion in the C57BL/6N contained numerous spermatozoa, but not in some of the mice belonging to the other two strains at 3 months of age (Fig. 2-2D–F). These findings in the seminiferous tubules and the epididymal ducts were similarly observed at 6 months of age. Furthermore, multinucleated cells were observed in some seminiferous tubules of Yaa testis (Fig. 2-2G), and some of their rete testis were dilated and filled with sperm at both ages (Fig. 2-2H). However, these features were not observed in C57BL/6N and BXSB at any age.

On the other hand, residual bodies, globular bodies comprising redundant organelles and RNA shed

from elongating spermatid during spermiation ²⁵⁾, were observed in the seminiferous tubules of all mice at both ages (Fig. 2-3A–D). Some residual bodies were present in the seminiferous tubules throughout St. I to XII in Yaa or at most stages except for St. II to V in BXSB, whereas they were mostly found at St. VIII or IX in C57BL/6N (Fig. 2-3E). The numerical analysis confirmed that Yaa showed higher numbers of residual bodies in total tubular area compared to that of C57BL/6N at both ages, and the difference was significant at 3 months (Fig. 2-3F). In each stage comparison, significant differences among strains were observed in St. VIII, but not in other St.s (Fig. 2-3G). A significant age-related increase was observed only in C57BL/6N at St. VIII. Furthermore, in Yaa, some residual bodies with atypical morphologies, characterized by larger sizes and apoptotic like bodies ¹⁴⁾, were observed. These abnormal residual bodies showed immunopositive reaction for tACE, a membrane molecule expressed in the elongated spermatids used as a marker of residual bodies ¹¹⁰⁾, with ring shape around the body (Fig. 2-3H). These atypical structures were observed on the luminal side of the tubules at several St.s in Yaa at both ages (Fig. 2-3H and I) ¹¹⁶⁾. These atypical residual bodies produced during failure of spermiation are reported to accumulate RNA ⁸⁶⁾ Larger residual bodies observed in Yaa seminiferous tubules were confirmed to contain abundant RNA stained with pyronin (Fig. 2-3J). The reaction of pyronin was confirmed to be eliminated with pre-treatment of RNase (Fig. 2-3K).

Comparison of immune-related phenotypes in mouse testes

Resident immune cells in testes and cytokines released from these cells or testicular cells are essential to maintain normal testicular function, such as the proliferation and apoptosis of germ cells, and establishment of BTB ¹⁰⁷⁾. First, the infiltration and distribution of immune cells were analyzed by immunohistochemistry, since infiltrated immune cells as testicular inflammation affect spermatogenesis (Fig. 2-4). As a result, no CD3-positive T-cells and B220-positive B-cells were found in the testes of all examined strains at both ages (Fig. 2-4A–F). Iba1-positive macrophages were observed in the interstitium in all examined strains (Fig. 2-4G–I), but their distribution and appearance frequency did not alter among strains and ages (data not shown).

The mRNA expression levels of pro-/anti-inflammatory genes [*Il1a*, *Il1b*, nitric oxide synthase 2 (*Nos2*), *Il6*, *Tnf*, transforming growth factor (*Tgf*), and *Il10*] in mice testes were evaluated by qPCR. There was no significant strain- or age-related difference among the examined strains (Fig. 2-4J).

Apoptotic cells in mouse testes

Apoptotic cell death is an essential process during spermatogenesis, in particular, for the removal of abnormal germ cells and maintaining the appropriate germ cell to Sertoli cell ratio ³⁰⁾. As a pathological condition, toxic, chemical, and genetic factors could induce the apoptosis of germ cells ⁹²⁾. Furthermore, in the testes with autoimmune orchitis, apoptotic germ cells are observed frequently, resulting in infertility ⁷⁰⁾. Immunohistochemical analysis was performed to detect apoptotic cells in the mouse testes, and Figure 2-5A to C show the representative images of ssDNA-positive apoptotic germ cells at 3 months, and they were observed in the seminiferous tubules of all strains at both ages. In numerical comparison, the mean number of ssDNA-positive germ cells in all examined areas of total tubules was highest in Yaa compared with other strains at both ages (Fig. 2-5D). Notably, in the stage comparison, ssDNA-positive apoptotic germ cells were frequently observed in St. XII seminiferous tubules of all strains (Fig. 2-5E). Furthermore, significant differences among strains and ages were observed at St. XII as well as St. IV. For St. XII, Yaa showed the highest values in both ages, and significances were observed with two other strains and C57BL/6N at 3 and 6 months of age, respectively. On the other hand, at 6 months, these positive cells in BXSB and Yaa were significantly lower than in C57BL/6N at St. IV, and Yaa showing the significant age-related decrease at this stage.

Next, the phenotype of Sertoli cells in each stage of seminiferous tubules were evaluated to examine the effect of Sertoli cells on the number of apoptotic germ cells in the seminiferous tubules. The morphology of the Sertoli cells differed among St., and obvious strain- or age-related differences were not identified (Fig. 2-5F–H). In the numerical analysis, there was no strain- or significant age-related differences in the mean number of Sertoli cells in all examined areas of tubules, but Yaa tended to show smaller values compared with C57BL/6N and BXSB at 3 months (Fig. 2-5I). Furthermore, at 3 months, Yaa tended to show a smaller number of Sertoli cells at all St., and significances with BXSB were observed at St. IV, V, and IX (Fig. 2-5J). These decreased phenotypes of Sertoli cells were not observed in 6-month-old Yaa testes. An age-related increase was detected at St. IX in Yaa.

Correlation between testicular phenotype and autoimmune disease indices in mice

Table 2-3 summarizes the statistical correlation between the examined indices for testicular phenotypes and autoimmune diseases in all examined mice or Yaa. In all examined mice, the ratio of

testis weight to body weight showed no significant correlation with other examined parameters. For histological phenotypes, the mean number of residual bodies in all examined areas of tubules positively correlated with the serum level of the anti-dsDNA antibody in the groups, including 3 months and both ages. That of the ssDNA-positive cells in all examined areas of the tubules also positively correlated with S/B in all age groups and with the serum levels of the anti-dsDNA antibody in the groups, including 6 months and both ages. In contrast, the number of Sertoli cells in all examined areas of the tubules negatively correlated with the serum levels of anti-dsDNA antibody in the 3-month-old groups. In the analysis using Yaa, testis weight to body weight significantly correlated with S/B in both age groups. The mean number of residual bodies in all examined areas of the tubules also positively correlated with S/B in the 3-month-old group. On the other hand, there was no correlation in the numbers of ssDNA-positive cells and Sertoli cells in all examined areas of the tubules.

Table 2-4 shows the statistical correlation of the indices of autoimmune diseases with histological phenotypes that showed significant differences in the strain comparison at each St.. In the analysis of all examined mice, the number of residual bodies in tubules at St. VIII positively correlated with the serum levels of the anti-dsDNA antibody in the groups, including 3 months and both ages. As for the ssDNA-positive cells, there were positive correlations in St. XII tubules with S/B in the groups, including 3 months and both ages and with the serum levels of the anti-dsDNA antibody in all age groups. That of the Sertoli cells in tubules at St. IV and V negatively correlated with S/B and the serum levels of the anti-dsDNA antibody in the 3-month-old groups. In tubules at St. IX, the Sertoli cell number positively correlated with S/B in the groups including both ages, but the coefficient was low. In the analysis using Yaa, there was no definite correlation in residual bodies at St. VIII and Sertoli cells at St. IV and IX, although the number of Sertoli cells at St. V tubules positively correlated with S/B of 3 months. As for the number of ssDNA in tubules at St. IV, there was a positive correlation with S/B of 6 months. Furthermore, the number of positive cells in tubules at St. XII clearly showed a positive correlation with S/B and the serum levels of the anti-dsDNA antibody in the 3-month-old groups. For other St. analysis, definite correlations were not shown (Table 2-5 and 2-6).

Discussion

Yaa manifested the autoimmune disease phenotypes from 3 months of age, which became more severe at 6 months. The testis to body weight ratio decreased with disease progression and significantly correlated with the autoimmune index and spleen size in Yaa. These data suggest that autoimmune abnormality due to a Y-linked autoimmune accelerating mutation can affect the male genital function. In general, the changes in testis weights reflect its histological changes, especially those of seminiferous tubules. Briefly, germ cell apoptosis inversely correlated with testis weight in mice ⁷⁸⁾. Indeed Yaa increased the number of apoptotic germ cells. On the other hand, Yaa and BXSB, but not C57BL/6N showed wider lumens of seminiferous tubules. This reasoning may be accurate as the increased luminal diameter of the seminiferous tubules was diagnosed as a dilation with increased fluid in the seminiferous tubules, which usually increases testis weight ¹⁴⁾. Since significant differences in the ratios of testis to body weight were not observed between healthy controls and Yaa, the fluid accumulation in the testicular tubules could make testis weight loss obscure in Yaa. Notably, nearly 95% of the seminiferous tubular fluid is reabsorbed in the efferent duct, and the failure of reabsorption causes fluid back pressure into seminiferous tubules, resulting in increased luminal diameter ³²⁾. Further, the dysfunction of cilia in the efferent duct has been reported to cause male infertility with dilated seminiferous tubules and rete testis, which likely results from the failure to propel sperms from rete testis into the epididymis ^{106,119)}. Importantly, inflammation could disrupt the morphology and function of ciliated epithelial cells ^{109,111)}. Thus, the dilated seminiferous tubules would reflect the altered function of the efferent duct epithelium related to the fluid reabsorption or flow, and the inflammatory condition by the progression of systemic autoimmune disease in Yaa might affect the epithelial cell function.

In mammal testes, 75% of germ cells die during spermatogenesis through the process of apoptosis ³⁰⁾. In this study, Yaa showed the increased apoptosis of St. XII at both disease stages but a decrease in that of St. IV with aging. Previous study reported that St. IV and St. XII were checkpoint stages of pachytene and metaphase, respectively, and abnormal germ cells were eliminated via apoptosis to maintain normal spermatogenesis, and the mouse genetic factors could affect apoptotic process ⁷⁸⁾. Therefore, the imbalanced checkpoint system between St. IV and St. XII might be involved in the quantitative alternations of apoptotic germ cells in Yaa.

Importantly, St. XII showed the highest percentage among stages in the number of apoptotic germ

cells of all examined mice. As for mouse St. XII, many germ cells undergo meiotic division. During meiosis, cells undergo complicated processes, such as chromosome replication, DNA double-strand break formation, spindle fiber formation, and meiotic cell division ¹⁰⁰). Each process is regulated precisely and complexly; thus, some germ cells with errors undergo apoptosis at this stage ⁶¹). Notably, similar increases of dead cells at St. XII were reported in male MRL/+ and MRL/lpr, but not in C57BL/10, CBA/J, C3H/He, BALB/c, DBA/2, NJL, and SL mice ⁵¹). Furthermore, the present histoplanimetry revealed that the apoptotic cells at St. XII increased from the early disease stage and most strongly correlated with the autoimmune disease indices among examined parameters in Yaa. Therefore, these results indicate that germ cells in St. XII tubules would have high susceptibility to altered immune conditions in mice and could be recognized as cells with errors.

Yaa also showed the highest values in residual body numbers, in particular at St. VIII, among strains at both ages, and significantly correlated with the autoimmune disease index at the early disease stage. A residual body is an aggregate of discarded organelles and RNA from spermatogenic cells. Residual bodies are usually observed at St. VIII and IX, as they are phagocytosed by Sertoli cells immediately after spermiation and then migrate to the basal cytoplasm of Sertoli cells ¹¹⁶). Furthermore, the abnormally large size of residual bodies containing apoptotic like bodies and their appearance in St. where residual bodies are not present normally, were sometimes observed in cases of spermiation failure, caused by chemical injection ¹⁴). Oral administration of one single chemical substance causes the decrease of Sertoli cells and increase of residual bodies, which characterizes larger sizes and the accumulation of RNA in rats ⁸⁶). From these reports, it might be possible that the change of testicular microenvironment caused by a systemic autoimmune condition in Yaa directly affects the process of spermiation. Further, as the number of residual bodies at St. VIII tended to increase with aging in all examined mice, Yaa would accelerate its age-related functional loss of testicular cells.

The increased number of apoptosis of meiotic cells and abnormal residual bodies in Yaa strongly suggested impairment of Sertoli cell function because both structures are usually phagocytized by Sertoli cells ^{14,42}). Importantly, proper clearance of apoptotic germ cells and residual bodies by Sertoli cells would be necessary to prevent an autoimmune reaction against spermatogenic cells induced by autoantigens ¹¹⁵). Further, Sertoli cells are highly attributed toward maintaining the immune privilege of mammalian testes, establishing BTB and immunosuppressive environments to segregate germ cells

from systemic autoimmunity ²⁴⁾. Conversely, the current study revealed that testicular antigens, which meiotic germ cells express, egress into interstitial space through Sertoli cells, contributing toward maintaining immune tolerance ¹¹⁰⁾. Contradictory to immune privilege, systemic autoimmune diseases, such as SLE, Behçet's disease, and rheumatoid arthritis, are diagnosed as associated diseases of autoimmune orchitis leading to male infertility ⁹⁵⁾. Although the pathogenesis remains unclear, the data for male patients with SLE suggest a dysfunction in Sertoli cells. ¹⁰¹⁾. The present histoplanimetric results revealed that Sertoli cells at St. IV-V and IX were significantly decreased in Yaa compared with BXSB at the early disease stage. Importantly, in Yaa, St. V and St. IX were the stages just after the spermatogenesis checkpoint at St. IV and the frequent occurrence of residual bodies at St. VIII, respectively, suggesting the relationship between Sertoli cells and germ cell alternations. On the other hand, a significant decrease of apoptotic germ cells at St. IV was observed in Yaa at the late but not the early stage. In addition, these mice showed the age-related increase of Sertoli cells at St. IX. Although the pathogenesis associating with Sertoli cells was still unclear in Yaa, the negative correlation between Sertoli cell numbers at St. IV-V and autoimmune disease severity at the early stage indicated the effect of autoimmune abnormalities on Sertoli cells. Taken together, further studies focusing on the direct effects of autoimmunity to germ cells as found in increased apoptosis at St. XII and the indirect effects via Sertoli cell injuries at the other stages would be beneficial.

From the genetic perspective, X-linked genes contribute to male reproductive function including meiosis ^{118,122)}. Yaa possesses excessive X-linked genetic factors, known as the genes on the Y-linked autoimmune accelerating locus ⁶⁸⁾, which is considered to be responsible for systemic autoimmune disease. Among the genes on this responsible locus, *Tlr7* and *8* are considered important for the development of autoimmune disease condition ⁸⁰⁾. Tlr is a pattern-recognition receptor expressed on the cell or endosome membrane that plays a role in innate immunity. Notably, the expression of *Tlr* members, including *Tlr7*, in mouse testis has been identified, and the roles of the *Tlr* family in spermatogenesis have been discussed in a previous study ¹¹⁵⁾. Briefly, an *in vitro* study revealed that TLR3 activation by polyinosinic-polycytidylic acid, induced apoptosis of spermatogonia ³⁵⁾. In addition, a study using *Tlr2*- and *Tlr4*-deficient mice showed that *Tlr2* and *Tlr4* contribute to the formation of autoimmune orchitis ⁵⁹⁾. However, there has been no report on the involvement of *Tlr7* and *Tlr8* in the male reproductive system. Therefore, these genes should be further examined as candidate molecules that might be

instrumental in connecting autoimmunity and the male reproductive function.

In conclusion, these results highlight the effect of systemic autoimmune disease on spermatogenesis, characterized with decreased testis weight, increased numbers of residual bodies, and apoptotic germ cells, particularly in St. XII seminiferous tubules without typical inflammation observed in ovaries of autoimmune disease mouse. In addition, the author provided evidence for the novel possible pathogenesis of autoimmune-related testicular disorder that systemic autoimmunity condition could interfere Sertoli cell phenotype, which can subsequently cause the failure of spermatogenesis. Further investigation of the mechanism of testicular phenotypes in Yaa would reveal pathogenesis of spermatogenesis disorder accompanying systemic autoimmune disease.

Summary

Similar to female infertility, systemic autoimmune diseases are thought to cause male reproductive disorder, even though immune privilege site is established in mammalian testes. In this chapter, the morpho-function of the testes in Yaa was analyzed as a representative model mouse for systemic autoimmune diseases to investigate the effect of excessive autoimmunity on spermatogenesis. At 3 and 6 months of age, Yaa showed splenomegaly and increased levels of serum autoantibodies, whereas no controls showed similar autoimmune condition. In histological analysis, the enlarged lumen of the seminiferous tubules accompanied with scarce spermatozoa in the epididymal ducts were observed in some of Yaa and BXSB, but not in C57BL/6N. Histoplanimetric analysis revealed significantly increased residual bodies and apoptotic germ cells in the seminiferous tubules in Yaa testes without apparent inflammation. Notably, in stage XII of the seminiferous epithelial cycles, the apoptotic germ cell number was remarkably increased, showing a significant correlation with the indices of systemic autoimmune disease in Yaa. Furthermore, the Sertoli cell number was reduced at the early disease stage, which likely caused subsequent morphological changes in Yaa testes. Thus, the present histological study revealed the altered morphologies of Yaa testes, which were not observed in controls, and statistical analysis suggested the contribution of an autoimmune condition to this phenotype, particularly the apoptosis of meiotic germ cells, without cellular inflammation. In conclusion, the unique immunosuppressive environment of mammalian testes might be established strictly, but it could be interfered by systemic immunological condition via altered phenotypes of testicular cells. Therefore, results in this chapter suggested the effect of excessive autoimmunity on abnormal morpho-function of male reproduction through the crosstalk with systemic autoimmunity.

Tables and figures

Table 2-1. Antibodies

Primary Antibody	Source	Detection	Dilution	Blocking	Antigen retrieval	Secondary Antibody
Rabbit anti-CD3	Nichirei (Tokyo, Japan)	T-cell	1:200	10% NGS (SABPO(R) Kit, Nichirei)	10 mM CB (pH 6.0) 110°C, 15 min	Goat anti-rabbit IgG antibody (SABPO(R) Kit, Nichirei)
Rat anti-B220	Cedarlane (Ontario, Canada)	B-cell	1:1600	5% NDS (Sigma-Aldrich, St Louis, MO USA)	10 mM CB (pH 6.0) 110°C, 15 min	Donkey anti-rat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA)
Rabbit anti-Iba1	Wako (Osaka, Japan)	Macrophage	1:1200	10% NGS (SABPO(R) Kit, Nichirei)	10 mM CB (pH 6.0) 110°C, 15 min	Goat anti-rabbit IgG antibody (SABPO(R) Kit, Nichirei)
Rabbit anti-ssDNA	IBL (Gunma, Japan)	Apoptotic cell	1:400	10% NGS (SABPO(R) Kit, Nichirei)	-	Goat anti-rabbit IgG antibody (SABPO(R) Kit, Nichirei)-
Mouse anti-tACE	MBL (Aichi, Japan)	Residual body	1:50	10% NGS (SABPO(R) Kit, Nichirei)	10 mM CB (pH 6.0) 110°C, 15 min	Goat anti-mouse IgG antibody (Southern Biotech, Birmingham, AL, USA)

CB: citrate buffer. ssDNA: single-stranded DNA. tACE: Testicular isoform of angiotensin-converting enzyme. NGS: normal goat serum. NDS: normal donkey serum.

Table 2-2. Primers

Gene name (accession no.)	Official symbol	Primer sequence (5'-3')	Product size (bp)
Actin, beta (NM_007393)	<i>Actb</i>	F: TGTTACCAACTGGGACGACA R: GGGGTGTTGAAGGTCTCAAA	165
Interleukin 1 alpha (NM_010554)	<i>Il1a</i>	F: AGATGACCTGCAGTCCATAACC R: GACAAACTTCTGCCCTGACGAG	121
Nitric oxide synthase 2, inducible (NM_010927)	<i>Nos2</i>	F: AGCTGATGGTCAAAGATCCAGAG R: GTGCATACCACCTTCAACCCGA	116
Interleukin 1 beta (NM_008361)	<i>Il1b</i>	F: TTCCAGGATGAGGACATGAGC R: AATGGGAACGTCAACACACCAG	111
Tumor necrosis factor (NM_013693)	<i>Tnf</i>	F: TCTTCTCATTCCTGCTTGTGGC R: CATAGAACTGATGAGAGGGAGGC	119
Interleukin 6 (NM_031168)	<i>Il6</i>	F: CAACGATGATGCACCTTGCAGA R: GGTACTCCAGAAAGACCAGAGGA	128
Interleukin 10 (NM_010548)	<i>Il10</i>	F: GCATTTGAATTCCCTGGGTGAG R: TTGTAGACACCTTGGTCTTGGAG	147
Transforming growth factor, beta 1 (NM_011577)	<i>Tgfb1</i>	F: ATGCTAAAGAGGTACCCCGC R: TGCTTCCCAGAAATGTCTGACG	119

Table 2-3. Correlation of autoimmune indices with examined parameters of the mouse testes

Parameter	T/B	Number of residual bodies in total	Number of ssDNA- positive cells in total	Number of Sertoli cells in total
	All / Yaa	All / Yaa	All / Yaa	All / Yaa
3-month-old	S/B	ρ	-.357 / -.400	.414 / .900*
		P	.191 / .505	.125 / .037
	Anti-dsDNA	ρ	-.071 / .000	.767** / .800
		P	.800 / 1.000	.001 / .104
	antibody	ρ	.111 / -.800	.489 / .100
		P	.694 / .104	.064 / .873
6-month-old	S/B	ρ	.011 / -.500	.446 / -.200
		P	.970 / .391	.095 / .747
	Anti-dsDNA	ρ	-.127 / -.758*	.296 / .200
		P	.504 / .011	.112 / .580
	antibody	ρ	.350 / .328	.621** / .212
		P	.350 / .328	.000 / .556
3- and 6-month-old	S/B	ρ	.713** / -.539	.711** / -.700
		P	.000 / .108	.003 / .188
	Anti-dsDNA	ρ	.400* / -.273	.295 / -.600
		P	.028 / .446	.286 / .285
	antibody	ρ	-.057 / .552	.650** / -.500
		P	.766 / .098	.009 / .391
3- and 6-month-old	S/B	ρ	-.155 / .406	.221 / .100
		P	.414 / .244	.428 / .873
	Anti-dsDNA	ρ	-.600* / .300	.575* / .100
		P	.018 / .624	.025 / .873
	antibody	ρ	.321 / .700	.243 / .188
		P	.243 / .188	.243 / .188

* $P < 0.05$, ** $P < 0.01$. ρ : Spearman's rank correlation coefficient, $N = 14-30$ (all strains), 5-10 (Yaa). All: C57BL/6N, BXSB, and Yaa.

T/B: ratio of testis weight to body weight; S/B: ratio of spleen weight to body weight; dsDNA: double-stranded DNA.

Table 2-4. Correlation of autoimmune indices with examined parameters of the mouse testes

Parameter		Number of residual bodies at St. VIII	Number of ssDNA-positive cells at St. IV	Number of ssDNA-positive cells at St. XII	Number of Sertoli cells at St. IV	Number of Sertoli cells at St. V	Number of Sertoli cells at St. IX
		All / Yaa	All / Yaa	All / Yaa	All / Yaa	All / Yaa	All / Yaa
3-month-old	S/B	ρ	.157 / -.300	.487 / .300	.470 / .900*	-.736** / .100	-.600* / .900*
		P	.576 / .624	.065 / .624	.077 / .037	.002 / .873	.018 / .037
6-month-old	Anti-dsDNA	ρ	.597* / -.100	.030 / .100	.567* / 1.000**	-.522* / .200	-.636* / .700
	antibody	P	.019 / .873	.914 / .873	.027 / .000	.046 / .747	.011 / .188
3- and 6-month-old	S/B	ρ	.371 / .354	-.338 / .894*	.657** / -.100	.175 / .200	-.011 / -.100
		P	.173 / .559	.219 / .041	.008 / .873	.533 / .747	.970 / .873
3- and 6-month-old	Anti-dsDNA	ρ	.482 / -.200	-.433 / -.112	.739** / .200	.236 / .200	.204 / .700
	antibody	P	.069 / .747	.107 / .858	.002 / .747	.398 / .747	.467 / .188
3- and 6-month-old	S/B	ρ	.290 / .406	.062 / -.485	.543** / .442	-.239 / .406	-.253 / .539
		P	.121 / .244	.745 / .156	.002 / .200	.204 / .244	.178 / .108
3- and 6-month-old	Anti-dsDNA	ρ	.567** / .042	-.249 / -.301	.624** / .527	-.174 / .333	-.255 / .564
	antibody	P	.001 / .907	.185 / .339	.000 / .117	.359 / .347	.173 / .090

* $P < 0.05$, ** $P < 0.01$. ρ : Spearman's rank correlation coefficient, $N = 14-30$ (all strains), 5-10 (Yaa). All: C57BL/6N, BXSB, and Yaa. St.: stage of the seminiferous epithelial cycle; S/B: ratio of spleen weight to body weight; dsDNA: double-stranded DNA.

Table 2-5. Correlation of autoimmune indices with examined parameters in all mice

Parameter		Number of residual bodies	Number of ssDNA-positive cells	Number of Sertoli cells
3-month-old	S/B	ρ	I 0.624*; VIII 0.640*	IV -0.757**; V -0.600*
	Anti-dsDNA antibody	ρ	VIII 0.597*; IX 0.625* XII 0.567*	IV -0.522*; V -0.636*; VI; -0.665**; VIII -0.617*
	S/B	ρ	VI 0.524*; X -0.608*; XII 0.650**	-
6-month-old	Anti-dsDNA antibody	ρ	XI -0.603*, XII 0.739**	-
	S/B	ρ	I 0.387*; VI 0.454*; XI -0.370*; XII 0.543**	-
3- and 6-month-old	Anti-dsDNA antibody	ρ	VIII 0.567**; IX 0.563**; X 0.430*	-
	S/B	ρ	III 0.433*; IX 0.392*	-

* $P < 0.05$, ** $P < 0.01$. ρ : Spearman's rank correlation coefficient, $N = 15-30$. All: C57BL/6N, BXSB, and Yaa. Roman numerals indicate the stage of the seminiferous epithelial cycle. S/B: ratio of spleen weight to body weight; dsDNA: double-stranded DNA.

Table 2-6. Correlation of autoimmune indices with examined parameters in Yaa

Parameter		Number of residual bodies	Number of ssDNA-positive cells	Number of Sertoli cells
3-month-old	S/B	ρ	VI 0.900*; VII 0.975**	XI 0.895*; XII 0.900* V 0.900*
	Anti-dsDNA antibody	ρ	IX 0.900*	XII 1.000** -
6-month-old	S/B	ρ	-	-
	Anti-dsDNA antibody	ρ	-	VI 0.900* -
3- and 6-month-old	S/B	ρ	III 0.701*	III -0.725* VIII 0.661*
	Anti-dsDNA antibody	ρ	-	- VII 0.697*

* $P < 0.05$, ** $P < 0.01$. ρ : Spearman's rank correlation coefficient, $N = 5-10$ (Yaa). Roman numerals indicate the stage of the seminiferous epithelial cycle. S/B: ratio of spleen weight to body weight; dsDNA: double-stranded DNA.

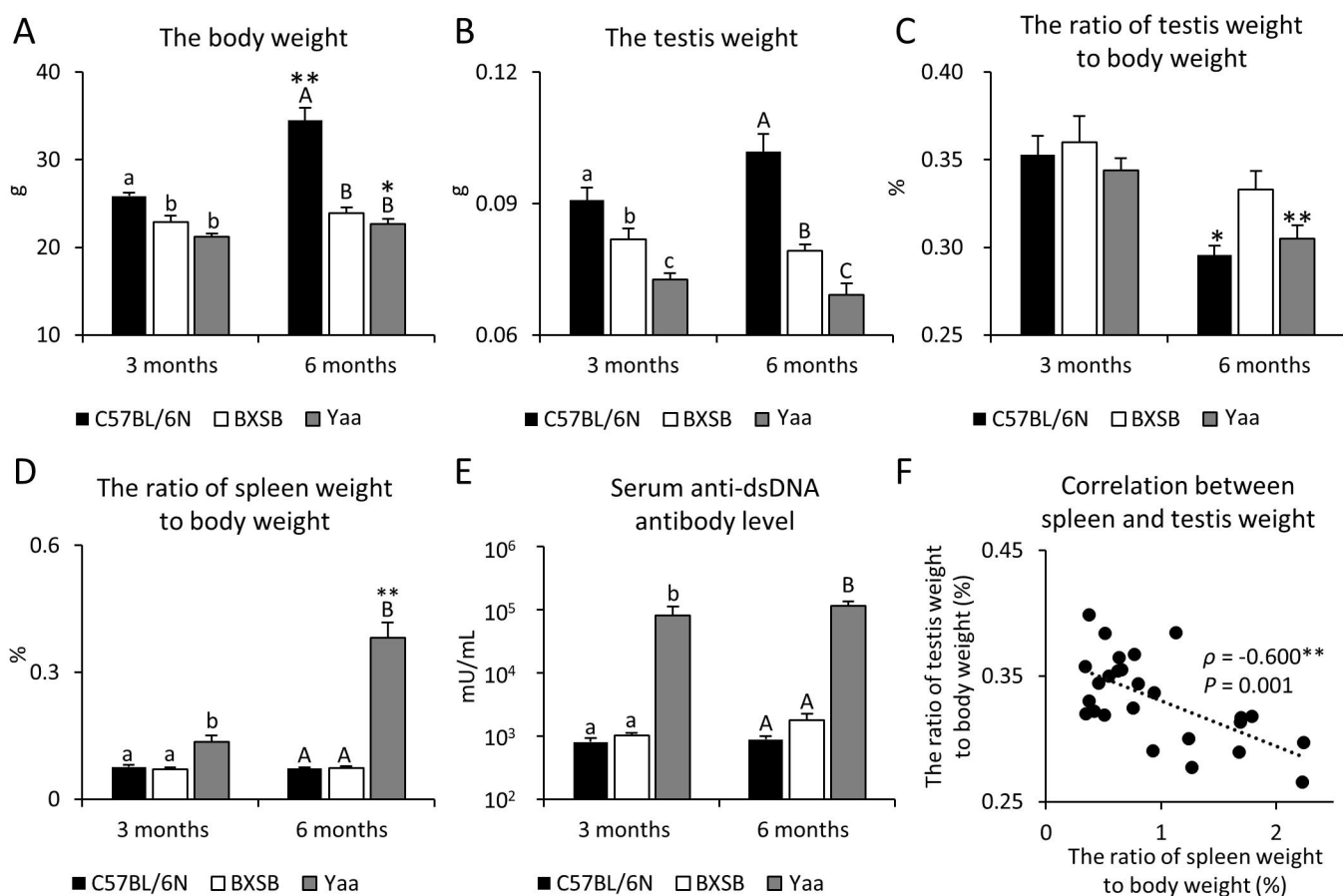


Figure 2-1. Indices of autoimmune disease condition and male reproductive function in mice

(A) Body weight. (B) Testis weight. (C) The ratio of testis weight to body weight. (D) The ratio of spleen weight to body weight. (E) The concentration of anti-dsDNA antibody in the serum. Each bar represents mean $\pm$ SE ($n \geq 5$). Significant differences among strains are shown by the letters above each bar. A lowercase letter represents the difference in 3-month-old mice. An uppercase letter represents the difference in 6-month-old mice. $P < 0.05$ (Kruskal–Wallis test followed by Scheffe’s method). Significant differences between different ages in the same strain are indicated with an asterisk. *: $P < 0.05$, **: $P < 0.01$ (Mann–Whitney U -test). (F) Correlation between the ratio of testis weight to body weight and that of spleen in Yaa. ρ : Spearman’s rank correlation coefficient ($n = 26$). $P = 0.001$.

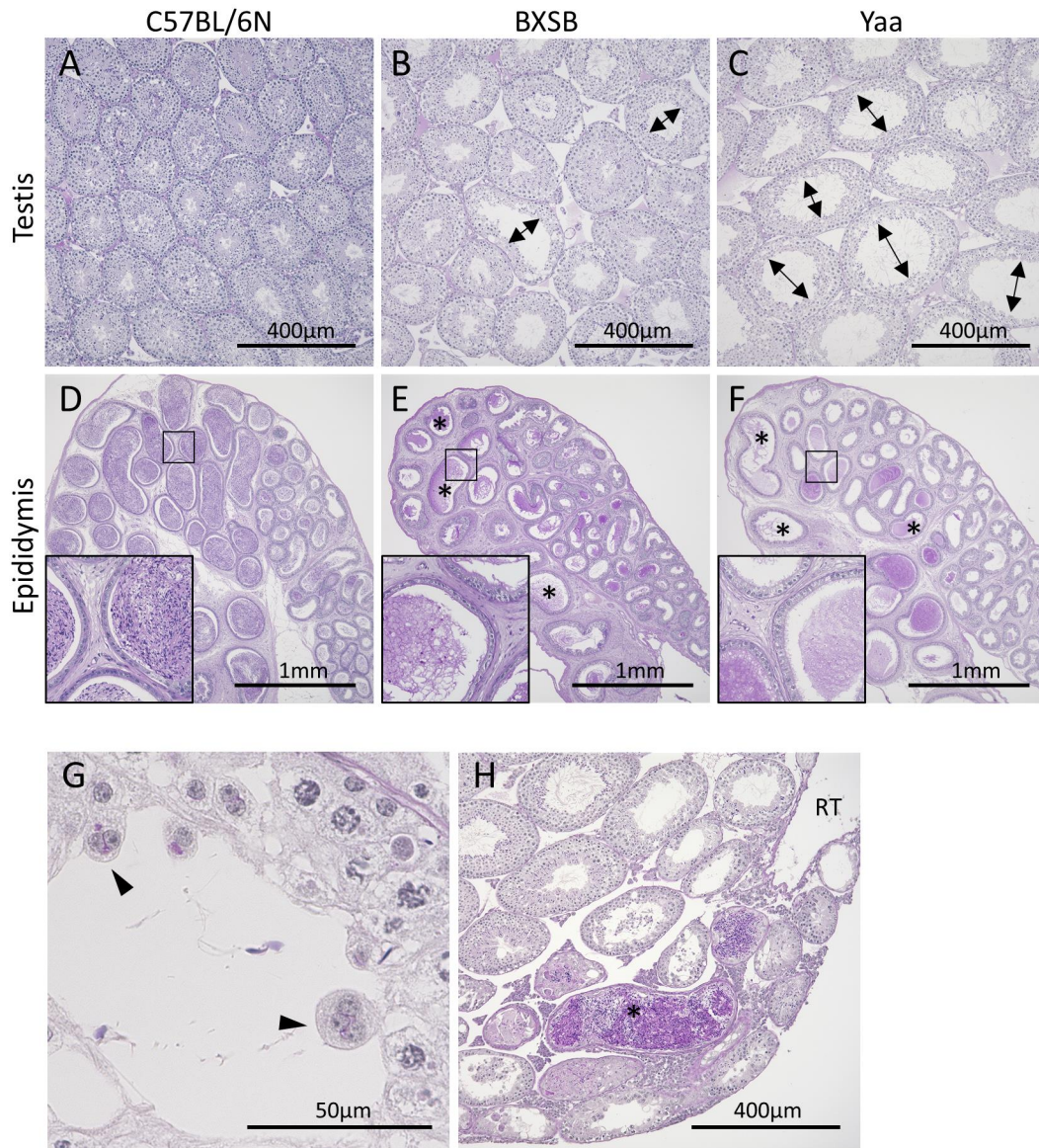


Figure 2-2. Comparison of histopathological features in mouse testes and epididymis
 (A-F) Representative images of the testes and epididymides of C57BL/6N (A and D), BXSB (B and E), and Yaa (C and F) at 3 months of age. Bidirectional arrows represent the lumen of the seminiferous tubules. Asterisks represent the loss of sperm in the lumen of the tail of the epididymis. (G-H) Representative images of histopathology in Yaa testis at 6 (G) and 3 months (H). Arrowheads represent multinucleated cells. The asterisk shows the rete testis dilated and filled with sperm. RT: rete testis.

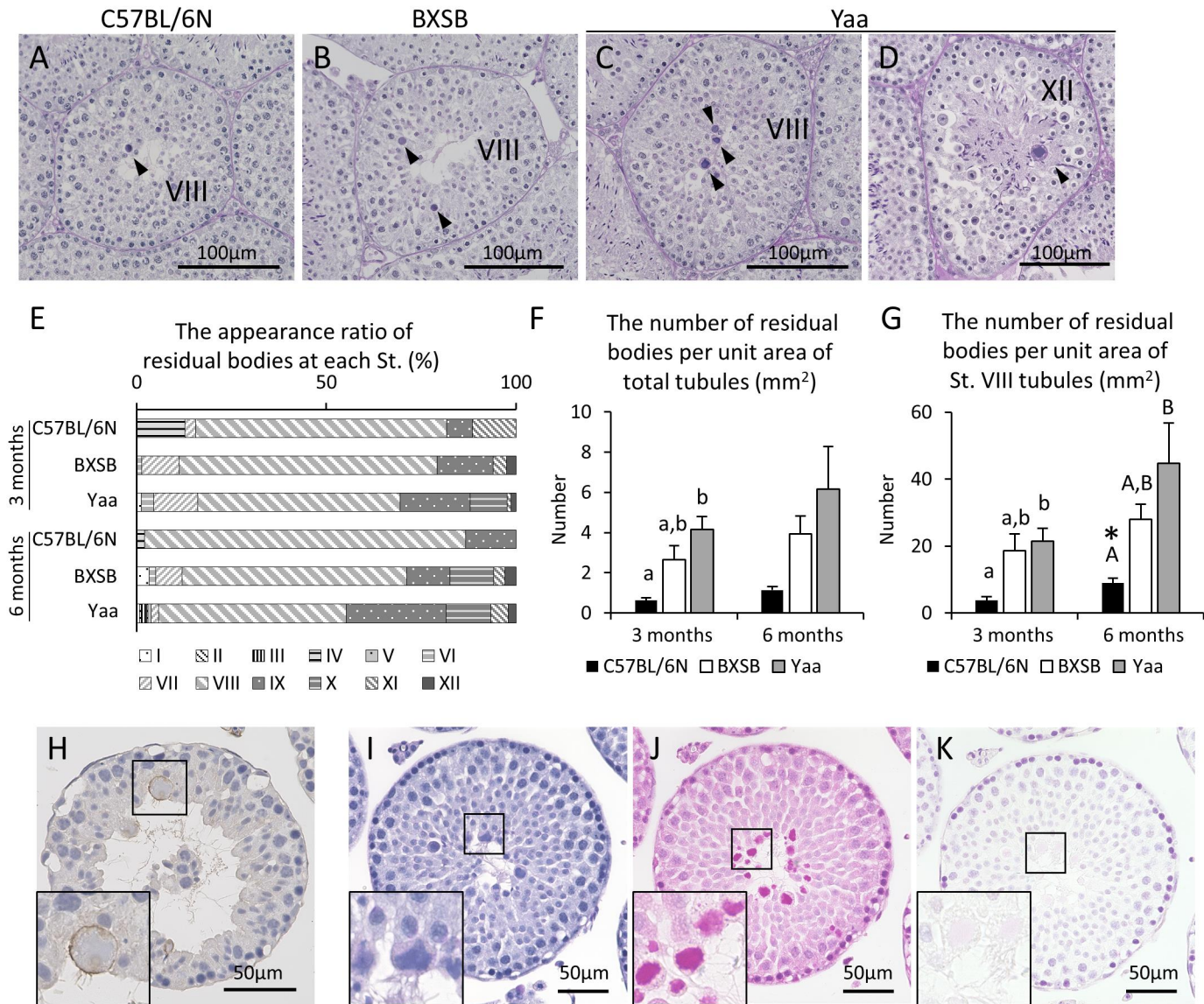


Figure 2-3. Analysis of residual bodies in mouse seminiferous tubules

(A-C) Histology of St. VIII seminiferous tubules of 3-month-old C57BL/6N (A), BXSB (B), and Yaa (C). (D) Representative image of an atypical residual body in St. XII seminiferous tubules of 3-month-old Yaa. Arrowheads represent residual bodies. All sections were fixed with Bouin's fluid and stained with PAS-H. Roman numerals indicate the stage of the seminiferous epithelial cycle. (E) The appearance ratio of residual bodies at each stage of seminiferous cycle. (F and G) The number of residual bodies per unit area of total (F) and St. VIII seminiferous tubules (G). Each bar represents mean $\pm$ SE (n = 5). Significant differences among strains are shown by the letters above each bar. $P < 0.05$ (Kruskal-Wallis test followed by Scheffe's method). A lowercase letter represents the difference in 3-month-old mice. An uppercase letter represents the difference in 6-month-old mice. Significant differences between different ages in the same strain are indicated with an asterisk. *: $P < 0.05$ (Mann-Whitney *U*-test). (H) Immunohistochemistry for tACE in cross seminiferous tubules in Yaa at 3 months of age. (I-K) Representative serial images of 3-month-old Yaa seminiferous tubule stained with PAS-H (I), methyl green-pyronin (J), and methyl green-pyronin with pre-treatment of RNase (K).

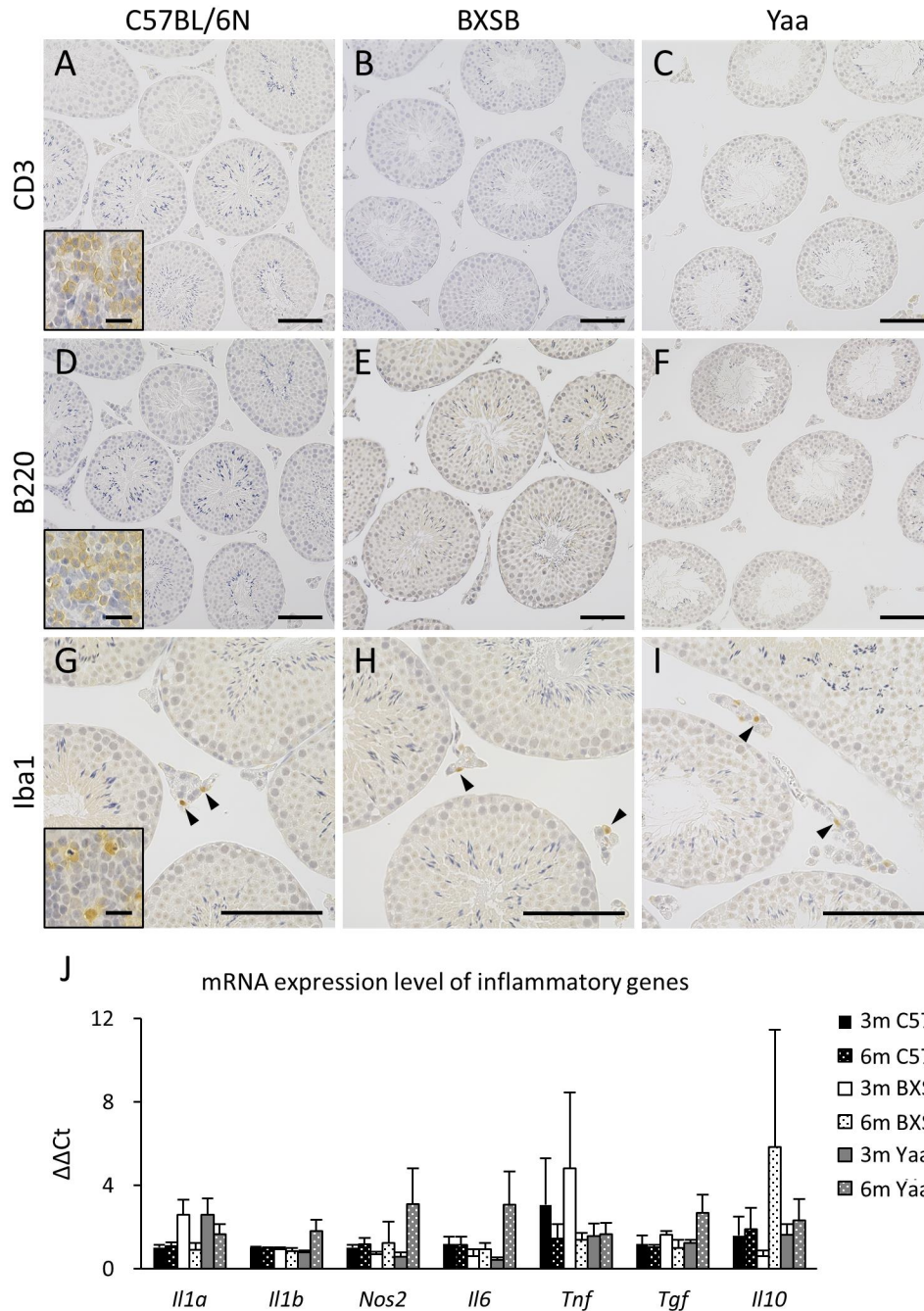


Figure 2-4. Analysis of the immunological changes in mouse testes

(A-I) Immunostained sections of mouse testes fixed with 4% paraformaldehyde. Immunohistochemistry for CD3 (A-C), B220 (D-F), and Iba1 (G-I) in cross seminiferous tubules in C57BL/6N, BXSB, and Yaa at 3 months of age. Arrowheads represent macrophages. Sections of the spleen are shown in insets. Bars = 100 µm. Bars (insets) = 10 µm.

(J) Relative mRNA expression of inflammation-related genes in C57BL/6N, BXSB, and Yaa testes at 3 and 6 months (m) of ages. The expression levels were normalized to the levels of *Actb*. Each bar represents mean $\pm$ SE (n = 5).

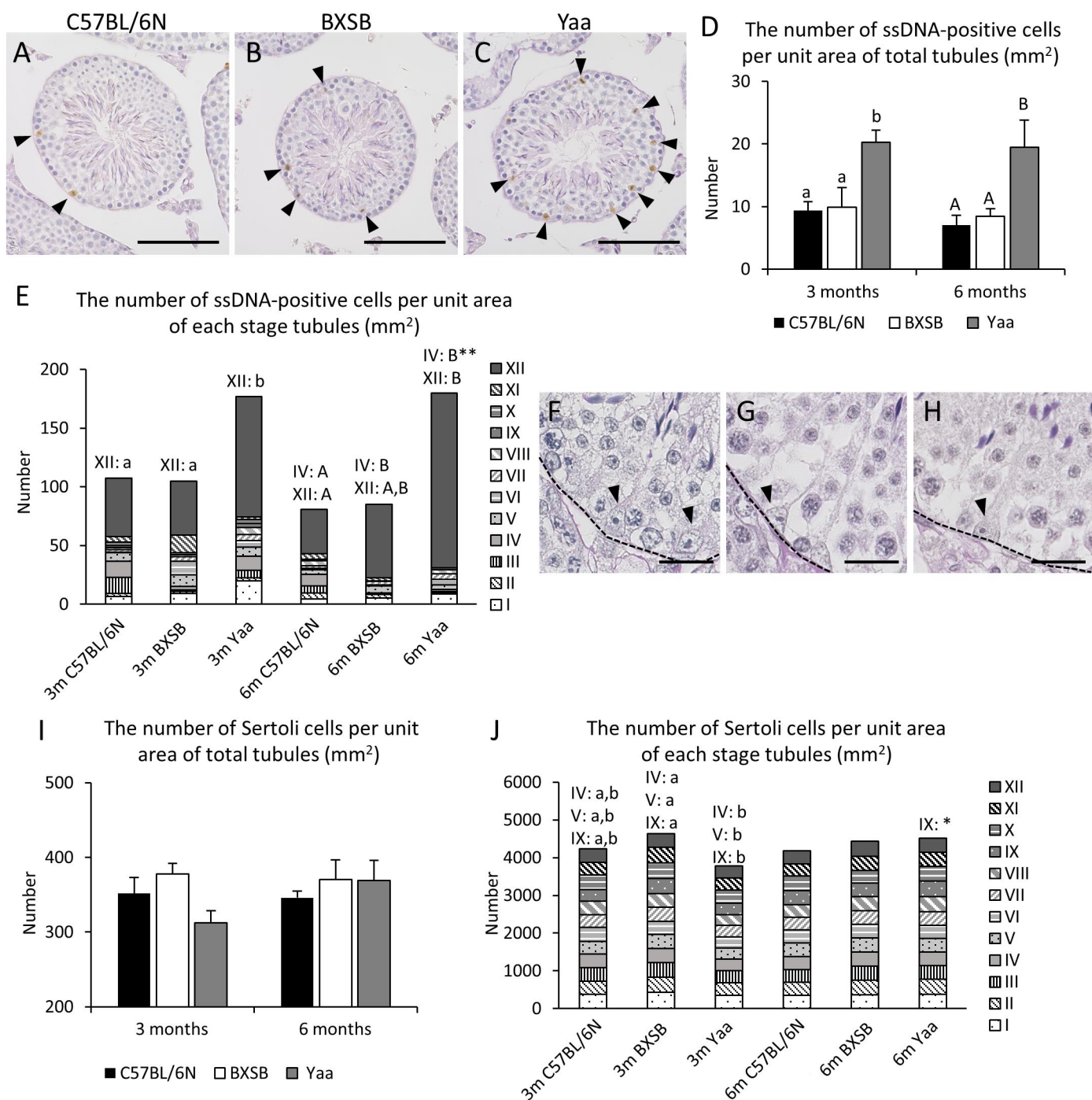


Figure 2-5. Analysis of apoptotic cells in mouse testes

(A-C) Immunostaining of ssDNA in cross seminiferous tubules at St. XII in 3-month-old C57BL/6N (A), BXSB (B), and Yaa (C). Arrowheads represent apoptotic cells. Bars = 50 μ m. (D-E) The number of ssDNA-positive cells per unit area of all examined (D) and each stage (E) seminiferous tubules in a section of mouse testis testis at 3 and 6 months (m) of ages. Each bar represents mean $\pm$ SE (n = 5). Roman numerals indicate the stage of the seminiferous epithelial cycle. Significant differences among strains are shown by the letters above each bar. $P < 0.05$ (Scheffe's method). A lowercase letter represents the difference in 3-month-old mice. An uppercase letter represents the difference in 6-month-old mice. Significant differences between different ages in the same strain are indicated with an asterisk. *: $P < 0.05$, **: $P < 0.01$ (Mann-Whitney *U*-test). (F-H) Histology of Sertoli cells in cross seminiferous tubules at St. IV-V in 3-month-old C57BL/6N (F), BXSB (G), and Yaa (H). Sections were fixed with Bouin's fluid and stained with PAS-H. Arrowheads represent Sertoli cells. Bars = 20 μ m. (I-J) The number of Sertoli cells per unit area of total (I) and each stage (J) seminiferous tubules in a section of mouse. Each bar represents mean $\pm$ SE (n = 5). Roman numerals indicate the stage of the seminiferous epithelial cycle. Significant differences among strains are indicated with letters above each bar. $P < 0.05$ (Scheffe's method). A lowercase letter represents the difference in 3-month-old mice. Significant differences between different ages in the same strain are indicated with an asterisk. *: $P < 0.05$, **: $P < 0.01$ (Mann-Whitney *U*-test).

Chapter 3

The effect of castration on the pathogenesis of systemic autoimmune disease and glomerulonephritis

Introduction

The functional crosstalk between reproductive system and autoimmunity is bidirectional. The abnormal autoimmunity could cause the altered morpho-function of reproductive tracts, ovary and testis, as shown in chapter 1 and 2. The current study focuses on the opposite side effect – the disrupted reproductive function could affect the immunity in the model mouse. In particular, castrated mice were investigated in this chapter, as sex hormones have been regarded as strong modulators of immune function, besides reproductive function in mammals. There is a definite functional difference between female and male hormones; estrogens mainly activate immune reactions and androgens suppress^{8,66)}, which results in clear sex-related disparities in immune diseases⁴⁸⁾. Indeed SLE incidence rates in pre-menopausal women to age-matched men are 8:1-15:1; however, prevalence is lower in pre-adolescent and post-menopause females⁷⁷⁾. These data suggest that estrogens are an exacerbating factor in the etiology of SLE.

Importantly, lupus nephritis is the most frequent complication in patients with SLE. Autoimmune factors directly affect the pathogenesis of kidney disease. Besides local inflammation in the kidney due to disrupted autoimmunity, deposition of immune complexes and complement activation lead to thickening of the glomerular basement membrane (GBM), and spike-like structures; in addition, accumulation of extracellular matrix (ECM) produced by mesangial cells and parietal epithelial cells (PECs) results in sclerotic and crescentic lesions in the glomerulus¹²⁰⁾. Studies have reported that human male patients with SLE show more severe lupus nephritis than women, whereas the prevalence of lupus nephritis in women is higher than that in men due to a higher incidence of SLE^{34,89)}. This evidence suggests the involvement of sex hormones in the pathogenesis of lupus nephritis, in association with the immune system.

The kidney is an organ that can be affected directly by sex hormones in terms of development, homeostasis, and disease conditions. Female rats have been reported to have smaller kidneys than males, and castrated mice also have a decreased kidney size⁵⁷⁾. Histologically, the PECs and proximal tubular epithelial cells in mice have been found to be larger in males than in females⁴⁾, suggesting the involvement of androgens in kidney function. Androgen receptor (AR) expression has been confirmed in proximal tubular epithelial cells in mice and rats⁸⁵⁾. In addition, *Ar* mRNA expression was reported in mouse glomeruli, including podocytes, using PCR analysis, and AR proteins in cultured mesangial

cells ^{19,21,85)}, suggesting the contribution of androgens to glomerular homeostasis as well as the pathogenesis of kidney diseases. Clinical studies have reported that several kidney diseases, including glomerulonephritis, glomerulosclerosis, membranous nephropathy, and IgA nephropathy, are more prevalent and serious in males ^{9,73,93)}. Experimental studies have supported androgens as a risk factor for kidney diseases, as shown by the protective effect of castration in several kidney disease models induced by ureteral obstruction, ischemia-reperfusion, and diabetes ^{11,40,98)}; in contrast, estrogen is suggested to have a protective role in kidney disease, as demonstrated in human and animal models ⁴⁵⁾. On the other hand, androgen has been reported to play the protective role in pathology of autoimmune glomerulonephritis in congenic mice unlike other kidney disease models ³⁷⁾. Furthermore, examination of autoimmune-prone NZB-derived F1 mice showed that estrogen promotes autoimmune glomerulonephritis due to excessive activation of cellular immunity ⁸⁴⁾. These results suggest that autoimmune disease-related nephritis is complicatedly formed by systemic autoimmune conditions and sex-related factors.

Yaa develops severe membranous proliferative glomerulonephritis (MPGN), characterized by glomerular hypertrophy, thickening of GBM, and expansion of sclerotic lesions ⁴⁷⁾. In addition to the Y-linked autoimmune accelerating mutation, previous works have reported that podocyte injuries due to the immune-associated genes on chromosome 1 contribute to the etiology of glomerulonephritis in Yaa, resulting in albuminuria ⁴⁶⁾. The effect of gonadectomy on Yaa was previously reported; there was little effect on their phenotypes; nevertheless, castrated Yaa was slightly accelerated to develop the autoimmune disease ²⁰⁾. However, detailed pathology of autoimmune glomerulonephritis in Yaa, contributed by sex hormones has not been discussed so far.

In this chapter, the author histologically investigated the influence of castration on the pathogenesis of systemic autoimmune disease and glomerulonephritis in Yaa. Castrated Yaa accelerated the development of the autoimmune disorder represented by early splenomegaly at 3 months, but there was no difference in renal pathology. However, castrated Yaa clearly showed milder sclerotic lesions in glomeruli compared to sham-operated group. These results show that androgens mediate the onset of systemic autoimmune disease condition at an early stage but exacerbate glomerular sclerosis with priority over the effect of autoimmunity in the later stages of the disease in Yaa.

Materials and methods

Animals and sample preparation

Male BXSb and Yaa were purchased from Japan SLC, Inc.. Mice were castrated or sham-operated at 3-weeks-old, under combination anesthesia (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol) with buprenorphine (0.1 mg/kg). First, the middle of the scrotum was incised in both the treated groups. Testes from both sides, epididymis, and part of the deferent duct were removed in the castrated group. During sham-operation, the testes were just held and pulled lightly, and then were placed back into the scrotum. Mice were maintained according to *The Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine* (Approval No. 15-0079, 20-0012). This animal experiment program was approved by the Association for Assessment and Accreditation of Laboratory Animal Care International). Urine was continuously collected from mice 3 to 6 months of age, by pressure urination. At 3 or 6 months old, under deep anesthesia (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol), after body weights were measured, blood samples were collected by cutting the carotid artery. After euthanasia by cervical dislocation, the kidney and spleen were collected. The weights of the kidney and spleen were measured, and S/B was compared in each group.

Serological and urinary analyses

Serum and urine were used to evaluate physiological parameters. Serum testosterone levels were measured using the Testosterone ELISA Kit (IBL, Gunma, Japan), to evaluate male reproductive functions. In addition, serum anti-dsDNA antibody levels were measured using the Mouse Anti-dsDNA antibody ELISA Kit (Shibayagi), to evaluate systemic autoimmune conditions. As for renal function analysis, serum blood urea nitrogen (BUN) and serum creatinine (Cre) were measured using a Fuji Dri-Chem 7000v instrument (Fujifilm, Tokyo, Japan). Urinary albumin and creatinine levels were measured using the LBIS Mouse Albumin ELISA Kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and Urinary Creatinine Assay Kit (Detroit R&D, Inc.; Detroit, MI, USA), and subsequently, the urinary albumin creatinine ratio (uACR) was calculated. The author followed the manufacturer's instructions for performing all the assays.

Histological analysis

The collected organs were fixed in 4% paraformaldehyde for approximately 24 h. After embedding in paraffin, spleen and kidney samples were cut into 3- or 2- μ m thick sections, respectively. The sections were deparaffinized and then stained with HE, PAS-H, periodic acid methenamine-silver (PAM), and Masson's trichrome (MT) stains. In histoplanimetric analysis, histological sections were converted into digital images by scanning with NanoZoomer 2.0-RS. Nuclear numbers in the glomerulus and glomerular area were quantified using the NDP.view2 program. The sclerotic lesion area of more than 20 glomeruli was measured using sections stained with MT, using a BX analyzer (Keyence, Osaka, Japan), and the sclerotic lesion fraction was calculated as the percentage of the sclerotic area to the glomerular area.

Immunostaining

Immunohistochemistry were performed as done in chapter 1 and 2. For antigen retrieval, 20 mM Tris-HCl buffer (pH 9.0) was used. After washing and blocking, sections were incubated with primary antibodies. For immunofluorescence, after antigen retrieval and blocking with normal donkey serum for 1 h at room temperature, sections were incubated with primary antibodies, listed in Table 3-1, at 4 °C for over 16 h. After washing, the sections were incubated with secondary antibodies, and then sections were incubated with Hoechst 33342 (1:500; Dojindo, Kumamoto, Japan) for nuclear staining at room temperature for 15 min. All the immunofluorescence sections were observed under an All-in-One Fluorescence Microscope BZ-X710 (Keyence), and were analyzed using a BX analyzer. The detailed information of used reagents is described in Table 3-1.

In histoplanimetric analysis, immunostained sections for CD44 were converted into digital images by scanning with NanoZoomer 2.0-RS. With more than 50 renal corpuscles, the total number and CD44-positive PECs were counted, and the circumference length of each Bowman's capsule was measured with the NDP.view2 program. Then, the numbers of total and CD44-positive PECs per unit length of Bowman's capsules were calculated. In addition, the number of renal corpuscles, including the CD44-positive PECs as well as the total number of renal corpuscles in a section were calculated, followed by the evaluation of CD44-positive renal corpuscle ratio. Further, in immunofluorescent sections, the number of antigens identified using monoclonal antibody Ki67 (Mki67, also known as

Ki67)-positive PECs was counted by observing under a BZ-X710 microscope, in more than 100 renal corpuscles, and the number of Ki67-positive PECs in a renal corpuscle was calculated.

RT and qPCR

qPCR was performed as done in chapter 2. Total RNA was isolated from the kidneys using TRIzol Reagent (Thermo Fisher Scientific). Synthesized cDNA was used for the analysis of gene expression levels using synthesized cDNA, THUNDERBIRD SYBR qPCR Mix (TOYOBO), and a real-time thermal cycler (CFX Connect; BIO-RAD). Gene expression in the kidneys was normalized to the expression of *Actb* and the details of the primers are described in Table 3-2.

Statistical analysis

The results are expressed as mean $\pm$ SE, and were analyzed using non-parametric statistical methods. Data between different treatments in the same strain, different strains with the same treatment, and different ages in the same strain with the same treatment were compared using the Mann-Whitney *U*-test. Spearman's correlation coefficient was used to analyze the correlation between two parameters. All the values were considered statistically significant at $P < 0.05$.

Results

Physiological change in castrated mice

The author first compared the physiological parameters of sham-operated and castrated mice to evaluate the effect of castration. Figure 3-1A shows the blood testosterone level of each group. All castrated mice at both ages showed significant decrease in their testosterone levels compared with those of sham-operated mice. Body weights of all castrated mice were also lower than those of sham-operated mice of the same strain at 3 and 6 months (Fig. 3-1B). Serum anti-dsDNA antibody levels were higher in Yaa than in BXSB of all the examined groups, but no difference was observed between castrated and sham-operated mice of both strains at 3 and 6 months (Fig. 3-1C). Castration did not induce any change in the spleen weight of BXSB mice, but increased S/B in castrated mice at both ages (Fig 3-1D and E). On the other hand, castrated Yaa at 3 months showed a tendency of increased spleen weight compared to the sham-operated group ($P=0.083$, Fig. 3-1D), which resulted in a significant difference in S/B (Fig. 3-1E). In histological examination of the spleen, Yaa showed expanded white bulb area compared to BXSB, but there was no significant difference between the treatment groups (Fig. 3-2A). At 6 months, there was no significant difference in spleen weight, S/B, or histology between castrated and sham-operated Yaa (Fig. 3-1D, E and 2B). These data suggest that castration evidently did not have any effect on the immune system but might affect the onset of autoimmune disease of Yaa.

Histological changes in the kidney of castrated mice

The author next examined the histology of the kidneys in mice to investigate the effect of castration on renal pathogenesis. In BXSB, sham-operated mice did not show glomerular pathology at 3 and 6 months, and there were no significant histological changes in the glomerulus due to castration (Fig. 3-3A). On the other hand, castrated BXSB showed a flattened morphology of PEC, while the sham-operated BXSB rendered a more cuboidal shape at both the ages. Figure 3-3B shows representative glomerular histology of 3-month-old Yaa. Both the treated mice showed MPGN with glomerular hypertrophy and expansion of mesangial lesions, but there were no treatment-related histological changes in the renal corpuscle except for flat PEC, as observed in castrated BXSB. At 6 months, severe MPGN characterized by glomerular hypertrophy, thickened GBM, and expansion of sclerotic lesions was observed in both treatment groups (Fig. 3-3C–H); however, castrated mice showed milder

expansion of sclerotic lesions compared with sham-operated mice (Fig. 3-3F–H). Furthermore, some sham-operated Yaa showed more severe MPGN, characterized by global sclerotic lesions and crescent formed by stratified PECs and ECM attached to the capillary tuft (Fig. 3-3I and J); however, these pathological changes were scarcely observed in castrated Yaa. Comparison of histoplanimetry between different treatment groups at 6 months did not alter the area of glomerulus and the number of glomerular nuclei due to castration (Fig. 3-3K and L). On the other hand, sclerotic lesion area and its fraction of glomerulus were remarkably lower in castrated mice than in sham-operated Yaa (Fig. 3-3M and N).

Functional changes in podocytes of castrated Yaa kidney

The author further investigated the morpho-function of podocytes of 6-month-old Yaa, since formation of sclerotic lesions is considered to be the result of loss of podocyte function. Figure 3-4A to F shows immunofluorescence of podocyte slit diaphragm molecules (nephrin and podocin) and podocyte cytoskeleton molecule (synaptopodin) in Yaa glomerulus at 6 months. In both the treated Yaa, expression of all the molecules was faint at the center of the glomerulus. On comparing the treatments, partial granular patterns were observed at the glomerular edge in the sham-operated group, but a linear positive reaction was relatively maintained in the castrated Yaa for all the markers. qPCR analysis showed significantly higher levels of podocyte functional markers in castrated mice than in sham-operated mice (Fig. 3-4G).

Loss of podocyte slit diaphragm molecules results in albumin leakage into the urine. uACR was compared between sham-operated and castrated Yaa from 3 to 6 months (Fig. 3-4H). At 3 months, uACR levels were equivalent between the two treatment groups. On the other hand, castrated mice demonstrated significantly decreased levels of uACR at 4 months compared to sham-operated mice, and this tendency continued till 5 months, but without statistical significance. Eventually, uACR was comparable between the two groups at 6 months. Serum BUN and Cre in castrated Yaa were observed to be lower than those in sham-operated mice, but without statistical significance (Fig. 3-4I and J).

Analysis of AR in 6-month-old Yaa kidney

Next, the author examined AR in Yaa kidney to determine the role of androgen in the pathogenesis of glomerulonephritis in Yaa. In 6-month-old Yaa, kidney weight was significantly decreased by

castration (sham-operated (g): 0.168 ± 0.010 , castrated (g): 0.115 ± 0.006). Figure 3-5A to F shows immunohistochemistry for AR in 6-month-old Yaa kidney. In sham-operated kidneys, a clear positive reaction was detected in the nuclei of proximal tubules in the cortex and outer stripe of the outer medulla (Fig. 3-5A, B, D, and E), and their cytoplasm was slightly positive. As for the cells in the renal corpuscle, immunopositive reaction was clearly detected in the nuclei of PECs in sham-operated Yaa, but not in glomerular cells (Fig. 3-5C and F). Castrated mice showed similar localization of AR in the kidney, but positive reactions in the nuclei of both tubulointerstitium and PECs were relatively weak as compared with those in the sham-operated Yaa. However, on comparing the mRNA levels of *Ar* in Yaa kidney, no difference was observed between sham-operated and castrated mice (Fig. 3-5G).

Analysis of PEC phenotype in 6-month-old Yaa kidney

PECs have been demonstrated to contribute in the formation of glomerulosclerosis⁶⁹⁾. In particular, activated PECs, characterized by CD44 expression on their cell membrane, are considered to have the potential to migrate, proliferate, and produce ECM, and play a role in the pathogenesis of glomerular sclerotic lesions²²⁾. The author first examined the expression of CD44 in 6-month-old Yaa kidneys, using immunohistochemistry (Fig. 3A–D). CD44-positive PECs were rarely observed in the renal corpuscle with uninjured glomeruli in both the treated Yaa groups (Fig. 3-6A and C). On the other hand, CD44-positive reaction was clearly observed in the PECs of renal corpuscles with injured glomeruli showing hypertrophy and expansion of sclerotic lesions (Fig. 3-6B and D). Particularly, PECs located in the middle (equator) part of Bowman's capsule, representing middle, between flat and cuboidal, height, frequently showed CD44-positive reactions compared to other parts of the PECs in both the groups. These PECs have been classified as intermediate PECs having proliferation and migration properties⁵³⁾. Sham-operated Yaa clearly showed CD44 expression on their cell membrane, particularly on the surface facing the capsular lumen, but in castrated mice, cell membrane reaction was unclear due to flat morphology of PECs. In histoplanimetric analysis, CD44-positive PEC number was significantly decreased in castrated Yaa at 6 months (Fig. 3-6E). Furthermore, castrated mice showed a lower frequency of renal corpuscles with CD44-positive PECs (Fig. 3-6F). Figure 3-6G shows immunofluorescence of CD44 and AR in sham-operated Yaa at 6 months, having glomeruli with sclerotic lesions. Many PECs were positive for CD44, and stratified PECs clearly showed positive

reactions for both CD44 and AR. For the analysis of proliferating cells, Ki67 was used with immunostaining. Figure 3-6H and I show a representative immunofluorescence for CD44 and Ki67 in kidneys showing MPGN of sham-operated and castrated Yaa. Ki67-positive cells were observed in the glomerulus and PECs of both the treated Yaa groups at 6 months. In sham-operated Yaa, in particular, Ki67-positive reactions were observed in the stratified PECs that showed a positive reaction for CD44, whereas stratified PECs were rarely observed in castrated Yaa (Fig. 3-6H). Histoplanimetric analysis showed a significant decrease of Ki67-positive PEC number in castrated Yaa (Fig. 3-6J). Further, the total number of PECs were significantly lower in castrated kidneys (Fig. 3-6K).

Table 3-3 shows correlation between sclerotic lesion and examined parameters in 6-month-old Yaa of both the treatment groups. The area of sclerotic lesion showed correlation with spleen weight, but sclerotic fraction and uACR showed no correlation with autoimmune disease indices. In addition, CD44 parameters showed weak positive correlation with spleen weight, but not with anti-dsDNA antibody level. Meanwhile, both area and fraction of sclerotic lesions were significantly and positively correlated with PEC parameters, CD44-positive PEC number, CD44-positive renal corpuscle ratio, and total PEC number. The sclerotic area, in particular, showed positive correlation with CD44-positive PEC parameters. Further, uACR was also clearly associated with CD44 expression in PECs, and CD44 parameters showed positive correlation with PEC number.

Discussion

In both healthy and autoimmune disease groups, castration decreased serum testosterone levels and body weight 3 months onwards. This would be because androgens contribute to increasing the weights of bones and muscles ¹¹²⁾. In the comparison of autoimmune indices, castration seemed to accelerate the onset of autoimmune disease, but did not have any effect on the late pathological stage of Yaa. Splenomegaly is generally observed when the immune system is disrupted in certain conditions, such as autoimmune diseases and infections ⁵⁸⁾. Importantly, gonadectomy could be a trigger for autoimmune disorders. In particular, the lack of androgens caused due to castration could lead to autoimmune disorders; this has been reported in a man who underwent testicular removal followed by estrogen administration therapy, and developed SLE ⁸¹⁾. The lack of immunosuppressive function of androgen could result in excessive activation of immune cell proliferation in Yaa as well. However, there was no influence of castration on BXSB immunological condition during the examination period, and there was no treatment-related difference in autoantibody levels in both the BXSB and the Yaa. Thus, male hormones could be important in suppressing autoimmunity, particularly, cellular immunity, but their absence would not affect the immune system if it is functioning normally in this experimental model.

Yaa represents lupus nephritis-like glomerulonephritis with the progression of systemic autoimmune disease. At 3 months, Yaa showed MPGN, but there was no treatment-related difference in renal pathology except for PEC flattening, suggesting that androgen would not be involved in the onset of Yaa glomerulonephritis, although castration significantly decreased kidney weight. Importantly, female BXSB also developed splenomegaly and MPGN 10 months onwards ⁴⁶⁾, while no renal lesion was observed in male BXSB without *Yaa* mutation at the same age. The present study adds evidence that androgens do not directly affect BXSB renal pathologies. These findings indicate that predispositions of the BXSB genome, including the development of MPGN, can be more sensitively affected by estrogen than androgens, even if there is a subsequent effect of androgens.

More severe glomerular pathology characterized by the expansion of sclerotic lesions in the glomerulus in 6-month-old Yaa would be associated with autoimmune disease progression, as shown by a positive correlation with spleen weight. On the other hand, castrated Yaa clearly showed milder sclerotic lesions compared with the sham-operated group, whereas there were no treatment differences in nuclear number and glomerular area. These results strongly suggest that formation of sclerotic lesions

would be specifically affected by androgens in Yaa, even though androgen was suggested to have a protective effect in other autoimmune nephritis models. Several studies have reported that androgens promote the formation of sclerotic lesions in the glomerulosclerosis disease model ⁴⁰⁾. The etiology has not been elucidated, but increase in TGF β level in the glomerulus by androgens could be a potent pathogenesis of glomerular sclerotic lesions in mice ²¹⁾. Based on these results, autoimmune nephritis in Yaa would be complicatedly formed by genetic factors, systemic autoimmunity, and sex hormones, but the effect of androgens would be superior to immunosuppression.

Formation of glomerular sclerotic lesions is commonly observed in various kidney diseases, including focal segmental glomerulosclerosis, diabetes nephritis, and lupus nephritis, characterized by ECM accumulations ¹⁸⁾. Disruption of the glomerular filtration barrier that podocytes maintain is the primary pathological event, which results in the leakage of circulating proteins into primitive urine. Previous studies reported that mouse podocytes and mesangial cells express AR, and androgens have been suggested to induce apoptosis of cultured podocytes and promote ECM production in mesangial cells *in vivo* ^{19,21,85)}. However, in the current model, AR was observed significantly in the nuclei of PECs, but not in those of podocytes and mesangial cells. Importantly, PEC is suggested to be involved in the formation of glomerular sclerotic lesions once podocytes are injured, although the molecular mechanisms are still poorly defined ⁶⁹⁾. PECs tend to proliferate, produce ECM, and migrate to tuft to seal the disrupted filtration barrier. As a result, glomeruli increase sclerotic lesions due to the accumulation of ECM that PECs and mesangial cells mainly produce.

Recently, PECs have been divided into subpopulations classified by specific molecules ⁵³⁾, and CD44 has been established as a marker of activated PECs ²³⁾. CD44 is a cell membrane protein, which functions as a receptor of hyaluronic acid and ECM, and has been reported to be involved in cell proliferation, migration, and malignancy in various cancers, such as prostate cancer and breast cancer ⁹⁰⁾. In the kidney, CD44-positive PECs are considered to have the ability to proliferate, migrate, and produce ECM as well. Furthermore, previous studies have reported the contribution of CD44-positive PECs in the formation of glomerulosclerosis and crescents ²²⁾. The present results confirmed CD44 expression on PECs facing injured glomeruli, but not in renal corpuscles with uninjured glomeruli, and a positive correlation with sclerotic indices indicated CD44 function in the formation of sclerotic lesions in Yaa. Additionally, the decreased number of CD44-positive PECs and the positive ratio of CD44 in the

renal corpuscles of 6-month-old castrated Yaa highlighted the effect of androgens on CD44 expression. The decreased number of Ki67-positive PECs suggests that the proliferation of PEC is suppressed in castrated Yaa, which might result in the decrease of total number of PECs. Thus, the author infers that morpho-functional changes in PECs under androgen-poor conditions play a key role in the attenuation of glomerular sclerosis in Yaa.

AR is stably expressed in the cytoplasm of widespread cells and tissues. After androgens bind to AR, androgen/AR (A/AR) complexes translocate to the nucleus and function as transcription factors ¹⁶⁾. The involvement of A/AR signaling in CD44 expression has been suggested in some epithelial tumors. Prostate cancer develops androgen-dependence, and *in vitro* analysis showed that AR expression mediates CD44 expression in prostate cancer cells ^{29,99)}. More importantly, androgen-mediated CD44 expression is reported to lead epithelial-mesenchymal transition (EMT) in cancer cells, which allows cells to proliferate and produce ECM excessively ⁶³⁾. In renal pathology, PECs are observed to undergo EMT, resulting in the proliferation and production of ECM, thereby forming crescent and glomerular sclerotic lesion ²⁷⁾. In this study, the author revealed that A/AR might mediate the PEC phenotype, in particular, expressing CD44 and undergoing EMT in Yaa glomerulonephritis. Moreover, decreased immunopositive reaction of AR in the PEC nucleus in castrated mice compared with sham-operated PEC, despite equivalent mRNA expression levels of *Ar* in the whole kidney, supported the decreased activity of A/AR signaling pathway, owing to castration.

Castrated Yaa showed decreased levels of uACR compared to the sham group at 4 months, when MPGN progressed severely in Yaa. This suggests that castration prevents progression of renal lesions in Yaa. There is a less possibility of direct contribution of androgens to glomerular injury because there is no expression of AR in the glomerulus. It is rather reasonable that androgens had already affected PECs at 4 months to promote the formation of sclerotic lesions. Furthermore, androgen is thought to increase albuminuria in some strains of mice and rats, as male animals show higher urinary protein levels than females ^{60,83)}. Albuminuria from glomerulus is reported to induce CD44 expression in PECs in a glomerulonephritis model mouse.¹²¹⁾ Positive correlation between CD44-related indices and uACR level in 6-month-old Yaa indicates their close association with Yaa as well. Higher uACR levels in sham-operated Yaa than in the castrated group at 4 and 5 months suggest that androgens induce the leakage of albumin, which could enhance CD44 expression in PECs. As a result, formation of glomerular sclerosis

lesions synergistically exacerbated renal pathology in Yaa. However, at the late disease stage, castrated Yaa showed equivalent levels of uACR with sham-operated Yaa. It could be reasoned that castrated Yaa developed more severe autoimmune diseases and MPGN than in early stages, and the formation of global sclerotic lesions in sham-operated Yaa lead to glomerular dysfunction, resulting in decreased uACR level.

In conclusion of this chapter, castration caused accelerated onset of autoimmune diseases, but attenuated sclerotic lesions in Yaa, a male-dominant autoimmune disease model mouse. In the pathogenesis of autoimmune disease, androgen is shown to delay the onset of disease, but could not ultimately reduce disease severity. In contrast, renal pathology is definitely exacerbated with the expansion of sclerotic lesions in the presence of androgen. These results suggest the importance of the effect of androgen on the formation of glomerular sclerosis over the immunosuppressive effect in this autoimmune glomerulonephritis model. Further, during the pathogenesis of sclerotic lesion in Yaa, A/AR signaling pathway would be involved in the activation of PECs, leading to proliferating and producing ECM, characterized by the expression of CD44. These findings reveal the role of male sex hormones in immunity and kidney homeostasis, and provide novel insights into the pathological crosstalk of male reproduction and autoimmune-related disease.

Summary

In the relationship of reproductive and immune function, sex hormones play critical roles in maintaining proper immunity. Imbalance of sex hormones causes organ-specific immune disease condition including kidney disease as well as systemic autoimmune disease. In this chapter, castrated Yaa was investigated as a murine autoimmune glomerulonephritis model, to clarify the contribution of sex hormones to autoimmune disease-related renal pathogenesis. Yaa and its wild-type, BXSB were castrated or sham-operated at 3 weeks and examined until 6 months of age. Both castrated strains showed significantly lower serum testosterone levels and body weights than sham-operated mice. Castration did not change the disease phenotypes in BXSB. At 3 months, both sham-operated and castrated Yaa manifested splenomegaly, autoantibody production, and glomerulonephritis, and castrated Yaa tended to show heavier spleen weights than the sham-operated group. At 6 months, both the treated Yaa showed equivalent autoimmune disease conditions; however, castrated mice clearly showed milder glomerular sclerotic lesions than the sham-operated groups. Urinary albumin excretion in castrated Yaa was significantly milder than in sham-operated mice at 4 months, but those of both the treated Yaa were comparable at 6 months. The examined renal histopathological indices in PECs were remarkably altered by castration. Briefly, castration decreased the height of PECs and total PEC number in Yaa at 6 months. For immunostaining, PECs facing the injured glomeruli of Yaa expressed CD44, an activated PEC marker, and CD44-positive PECs showed nuclear localization of AR and proliferation marker Ki67. CD44- or Ki67-positive PECs were significantly fewer in castrated group than in sham-operated Yaa at 6 months. Further, quantitative indices for CD44-positive PEC number and frequency in renal corpuscles positively correlated with glomerular sclerotic severity in Yaa. In conclusion, androgen seemed to have an effect on both systemic immunity and renal morpho-function; however, the effect on the latter could be more clearly observed in Yaa, as PEC activation resulted in glomerular sclerosis. Thus, results in this final chapter provided novel evidences about the role of reproductive function in the pathogenesis of autoimmune kidney disease in the crosstalk between reproduction and immunity.

Tables and figures

Table 3-1. Antibodies

Primary Antibody	Source	Detection (Application)	Dilution	Blocking	Antigen retrieval	Secondary Antibody (IHC)	Secondary Antibody (IF)
Rabbit anti-nephrin	IBL	Podocyte slit diaphragm (IF)	1:400	5% NDS	10 mM CB (pH 6.0) 110°C, 15 min	-	Alexa Fluor 488-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rabbit anti-podocin	IBL	Podocyte slit diaphragm (IF)	1:800	5% NDS	10 mM CB (pH 6.0) 110°C, 15 min	-	Alexa Fluor 488-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Mouse anti-synaptopodin	Fitzgerald, (North Acton, MA, USA)	Podocyte cytoskeleton (IF)	1:100	5% NDS	10 mM CB (pH 6.0) 110°C, 15 min	-	Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rabbit anti-androgen receptor	Abcam (Cambridge, UK)	Androgen receptor (IHC, IF)	1:500 (IHC) 1:200 (IF)	10% NGS (IHC) 5% NDS (IF)	20mM Tris-HCl (pH 9.0) 121°C, 10 min	Biotin-conjugated goat anti-rabbit IgG antibody (SABPO(R) Kit, Nichirei)	Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rat anti-CD44	BD Biosciences (San Jose, CA, USA)	Activated parietal epithelial cell (IHC, IF)	1:400 (IHC) 1:800 (IF)	10% NGS (IHC) 5% NDS (IF)	20mM Tris-HCl (pH 9.0) 110°C, 15 min	Goat anti-rat IgG antibody (Santa Cruz)	Alexa Fluor 488-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rabbit anti-Ki67	Abcam	Proliferating cell (IF)	1:800	5% NDS	20mM Tris-HCl (pH 9.0) 110°C, 15 min	-	Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)

CB: citrate buffer. NGS: normal goat serum. NDS: normal donkey serum. IHC: immunohistochemistry. IF: immunofluorescence.

Table 3-2. Primers

Gene name (accession no.)	Official symbol	Primer sequence (5'-3')	Product size (bp)
Nephrosis 1, nephrin (NM_019459)	<i>Nphs1</i>	F: ACCTGTATGACGAGGTGGAGAG R: TCGTGAAGAGTCTCACACCCAG	218
Nephrosis 2, podocin (NM_130456)	<i>Nphs2</i>	F: AAGGTTGATCTCCGTCTCTCCAG R: TTCCATGCGGTAGTAGCAGAC	105
Synaptopodin (NM_177340.2)	<i>Synpo</i>	F: CATCGGACCTTCTTCCTGTG R: TCGGAGTCTGTGGGTGAG	90

Table 3-3. Correlation analysis among examined parameters in 6-month-old Yaa

Autoimmune disease			Activation of PECs		
<i>Parameters</i>		Serum dsDNA antibody level	Spleen weight	CD44-positive renal corpuscle ratio	Total PEC number
Sclerotic lesion	Sclerotic lesion area	ρ	0.003	0.515*	
		P	0.991	0.029	
lesion	Sclerotic lesion fraction	ρ	-0.124	0.302	
		P	0.649	0.223	
Glomerular function	uACR	ρ	0.297	0.612	
		P	0.405	0.060	
Activation of PECs	CD44-positive PEC number	ρ	0.196	0.417	
		P	0.468	0.085	
of PECs	CD44-positive renal corpuscle ratio	ρ	0.210	0.475*	
		P	0.434	0.047	

* $P < 0.05$, ** $P < 0.01$. ρ : Spearman's rank correlation coefficient, $n = 18$. uACR: urinary albumin creatinine ratio, PEC: parietal epithelial cell

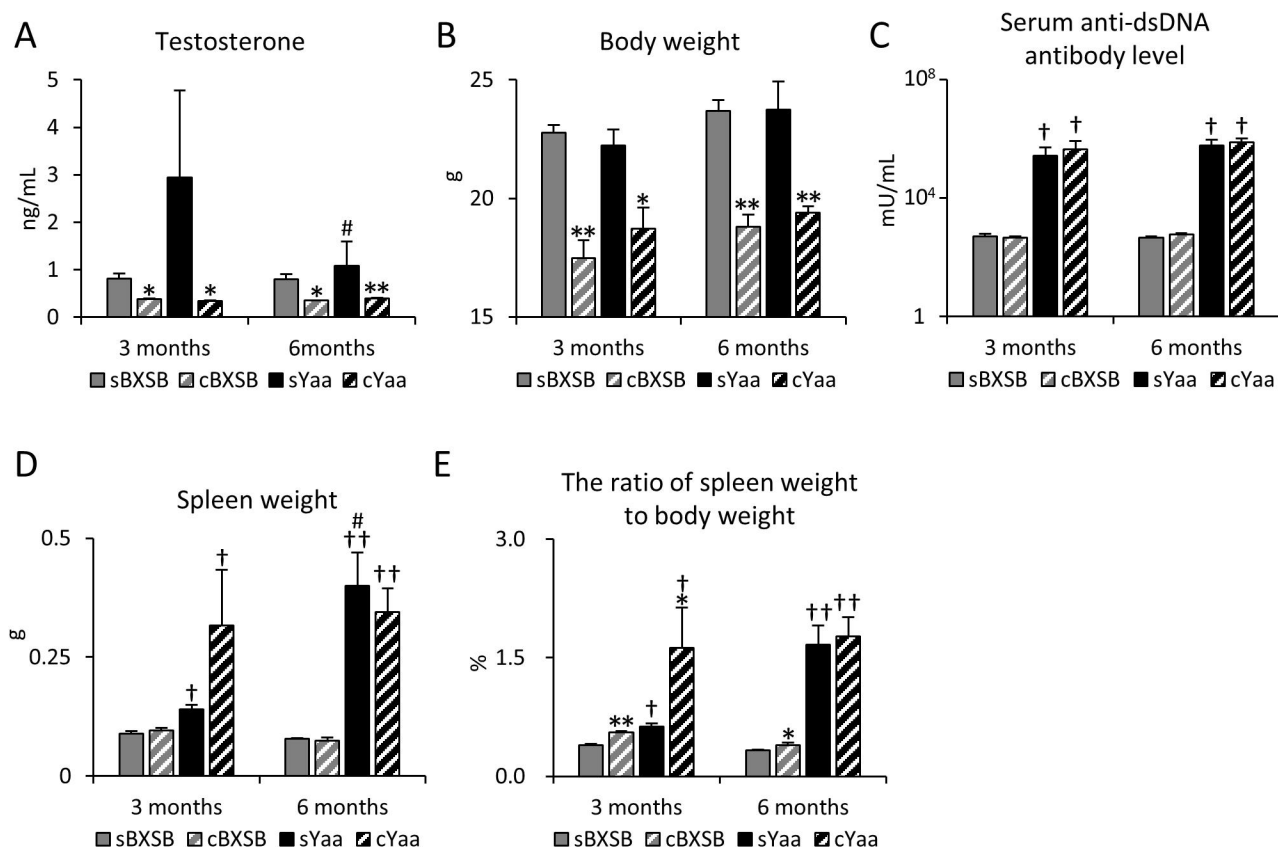


Figure 3-1. Indices of male reproductive function and autoimmune disease condition in mice

(A) The serum level of testosterone. (B) Body weight. (C) The serum level of anti-dsDNA antibody. (D) Spleen weight. (E) The ratio of spleen weight to body weight. s: sham-operated; c: castrated. Significant differences in castrated groups against sham-operated group in the same strain at the same age are indicated with an asterisk. *: $P < 0.05$, **: $P < 0.01$ (Mann-Whitney U -test). Significant differences in Yaa against BXSB with the same treatment at the same age are indicated with a dagger. †: $P < 0.05$, ††: $P < 0.01$ (Mann-Whitney U -test). Significant differences in 6-month-old groups against 3-month-old groups in the same strain with the same treatment are indicated with a hash tag. #: $P < 0.05$ (Mann-Whitney U -test).

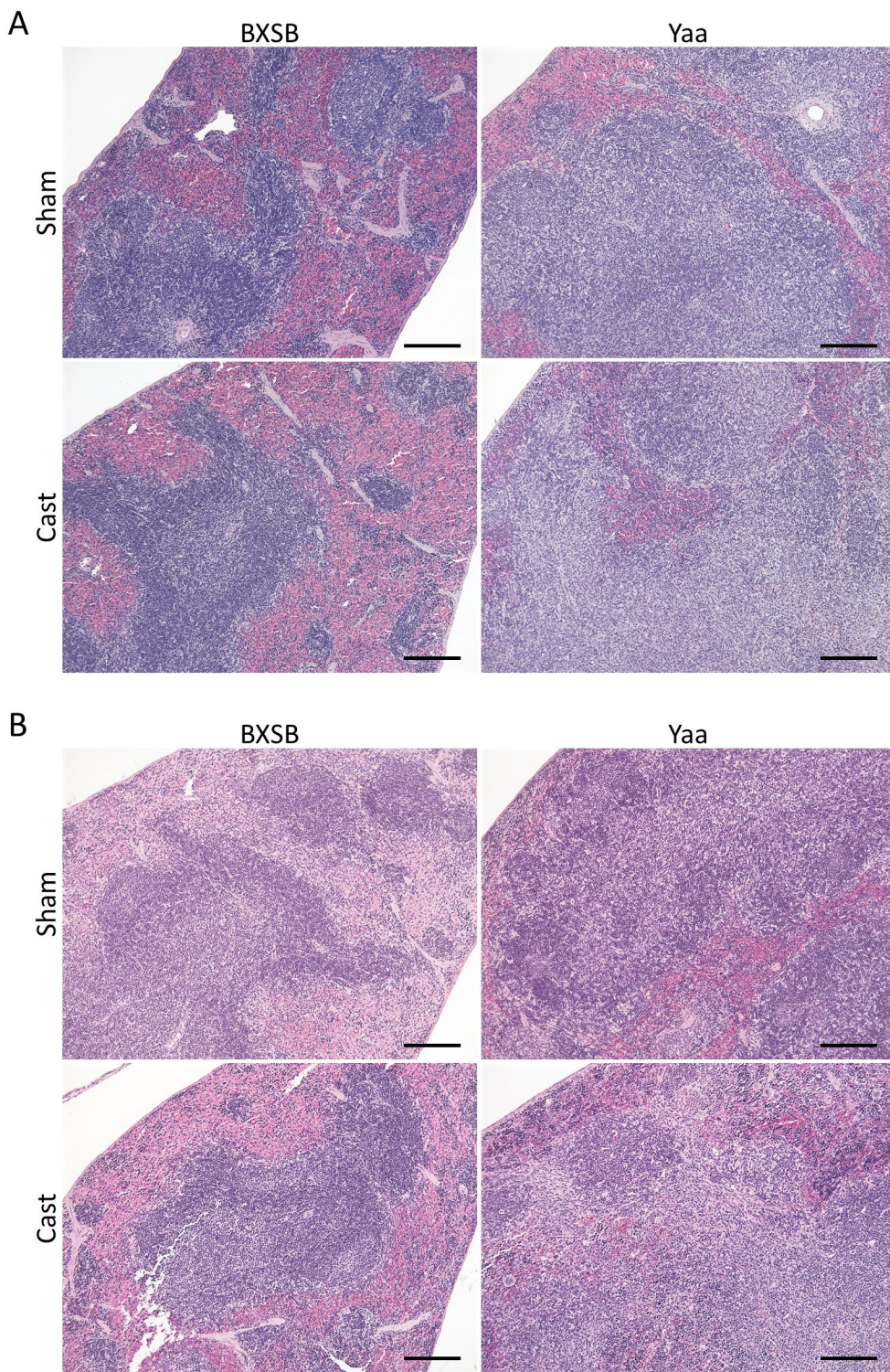


Figure 3-2. Histopathology of spleen in mice

(A) Histology of the spleens in sham-operated and castrated BXSB and Yaa at 3 months. Yaa shows expanded white bulb area compared to BXSB of the same treated group, but there is no significant difference between sham-operated and castrated groups in both strains. (B) Histology of spleens in sham-operated and castrated BXSB and Yaa at 6 months. Both the strains show similar tendencies at 3 months. There is no significant difference for treatment in either strain. All sections were stained with HE. Bars = 200 μm.

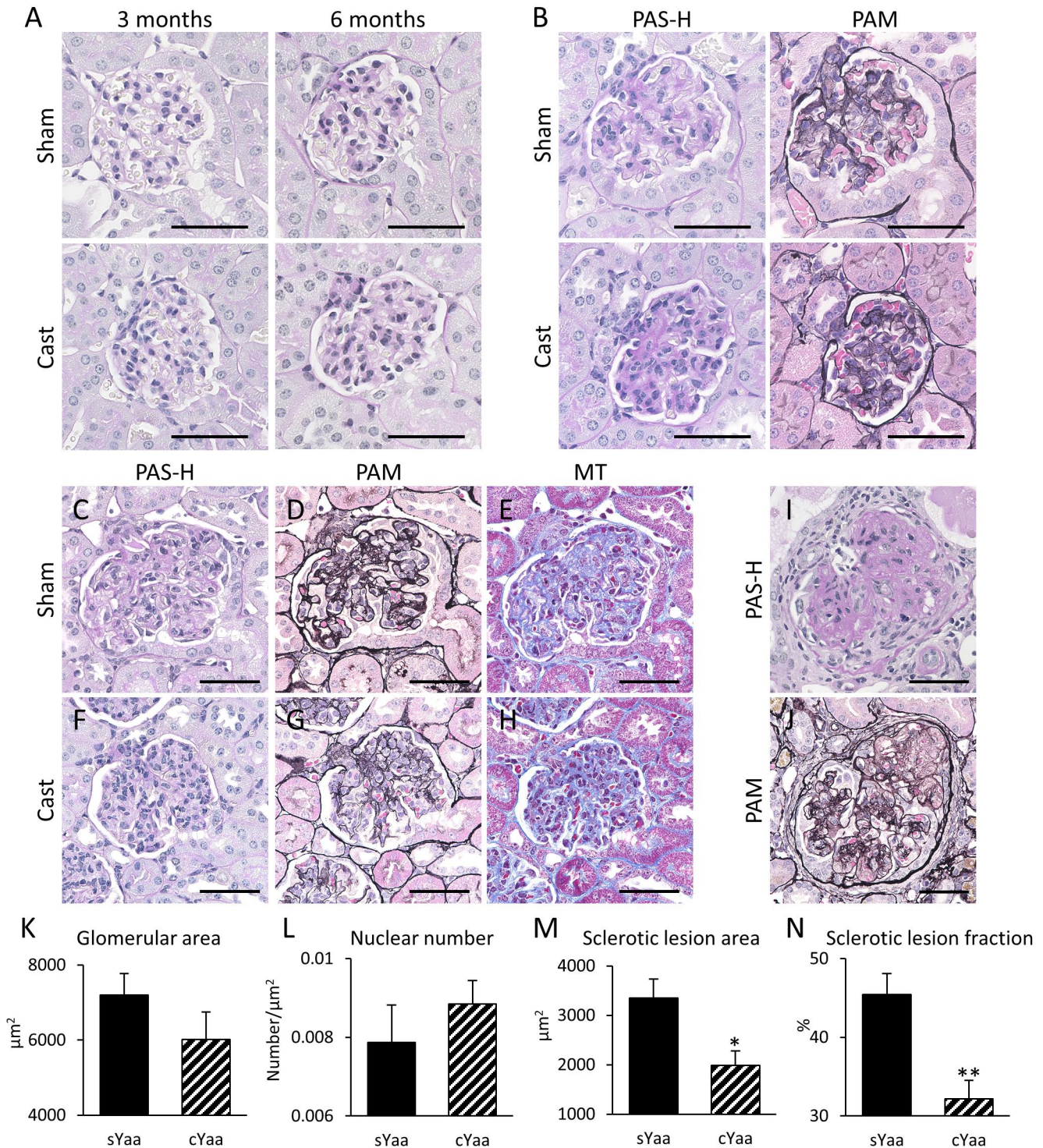


Figure 3-3. Histopathological analysis of glomeruli in mice

(A) Histology of glomeruli in sham-operated and castrated BXSBS at 3 and 6 months. Sections were stained with PAS-H. (B) Histology of glomeruli in sham-operated and castrated Yaa at 3 months. Glomerular hypertrophy, increase in mesangial matrix lesion, and wrinkling of the glomerular basement membrane are observed, but there are no treatment-related differences. (C-H) Histology of glomeruli in sham-operated and castrated Yaa at 6 months. Sections were stained with PAS-H (C and F), PAM (D and G), and MT (E and H). Expansion of sclerotic lesion is more significant in sham-operated Yaa than in castrated Yaa. (I and J) Representative histology of glomeruli with global sclerotic lesions stained with PAS-H (I) and PAM (J) in sham-operated Yaa at 6 months. Increased parietal epithelial cells and accumulation of extracellular matrix are significant, resulting in the adhesion of glomerular tuft and Bowman's capsule. Bars = 50 μm . (K) The area of glomeruli in sham-operated Yaa (sYaa) and castrated Yaa (cYaa) at 6 months. (L) The number of nuclei per unit area of a glomerulus in sYaa and cYaa at 6 months. (M) The area of sclerotic lesion in glomeruli in sYaa and cYaa at 6 months. (N) The fraction of sclerotic lesions in sYaa and cYaa at 6 months. Each bar represents mean $\pm$ SE (n = 8-10). Significant differences in castrated groups compared to the sham-operated group are indicated with an asterisk. *: $P < 0.05$, **: $P < 0.01$ (Mann-Whitney U -test).

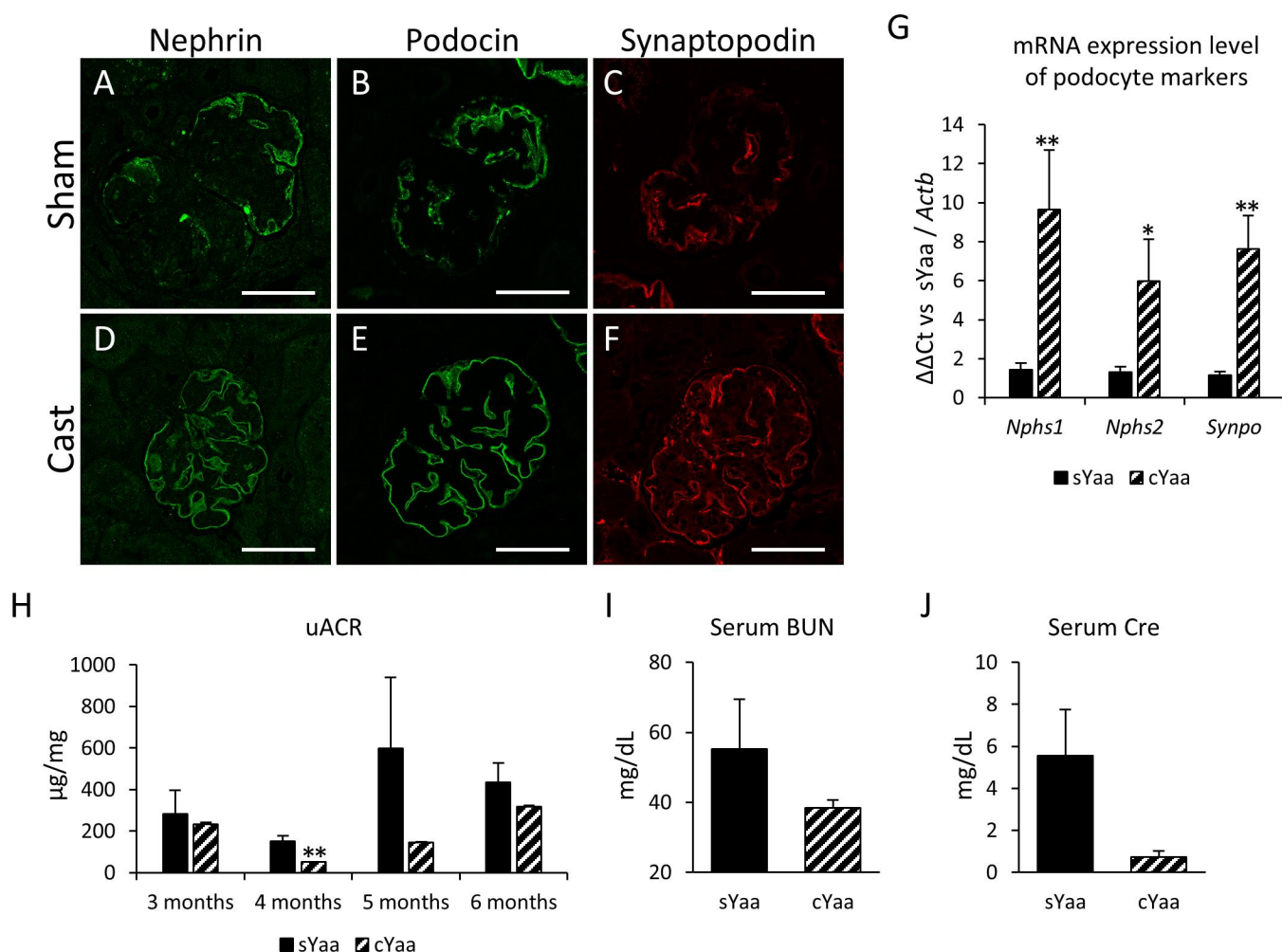


Figure 3-4. Analysis of podocyte function in 6-month-old Yaa

(A-F) Immunofluorescence of podocyte function molecules (nephrin (A and D), podocin (B and E), and synaptopodin (C and F)) in Yaa at 6 months. Nephrin-, podocin- and synaptopodin-immunopositive areas are faint at the center of the glomerulus of Yaa in both the treatment groups, but linear positive reactions at the peripheral area of glomeruli are partially eliminated in only sham-operated groups. Bars = 50 μ m. (G) Relative mRNA expression of podocyte function molecules in kidneys of sham-operated Yaa (sYaa) and castrated Yaa (cYaa). The expression levels were normalized to the levels of *Actb*. (H) uACR in sYaa and cYaa from 3 to 6 months. (I and J) The serum levels of BUN (I) and Cre (J) in sYaa and cYaa at 6 months. Each bar represents mean $\pm$ SE (n = 8-10). Significant differences in castrated groups compared to the sham-operated group are indicated with an asterisk. *: $P < 0.05$, **: $P < 0.01$ (Mann-Whitney *U*-test).

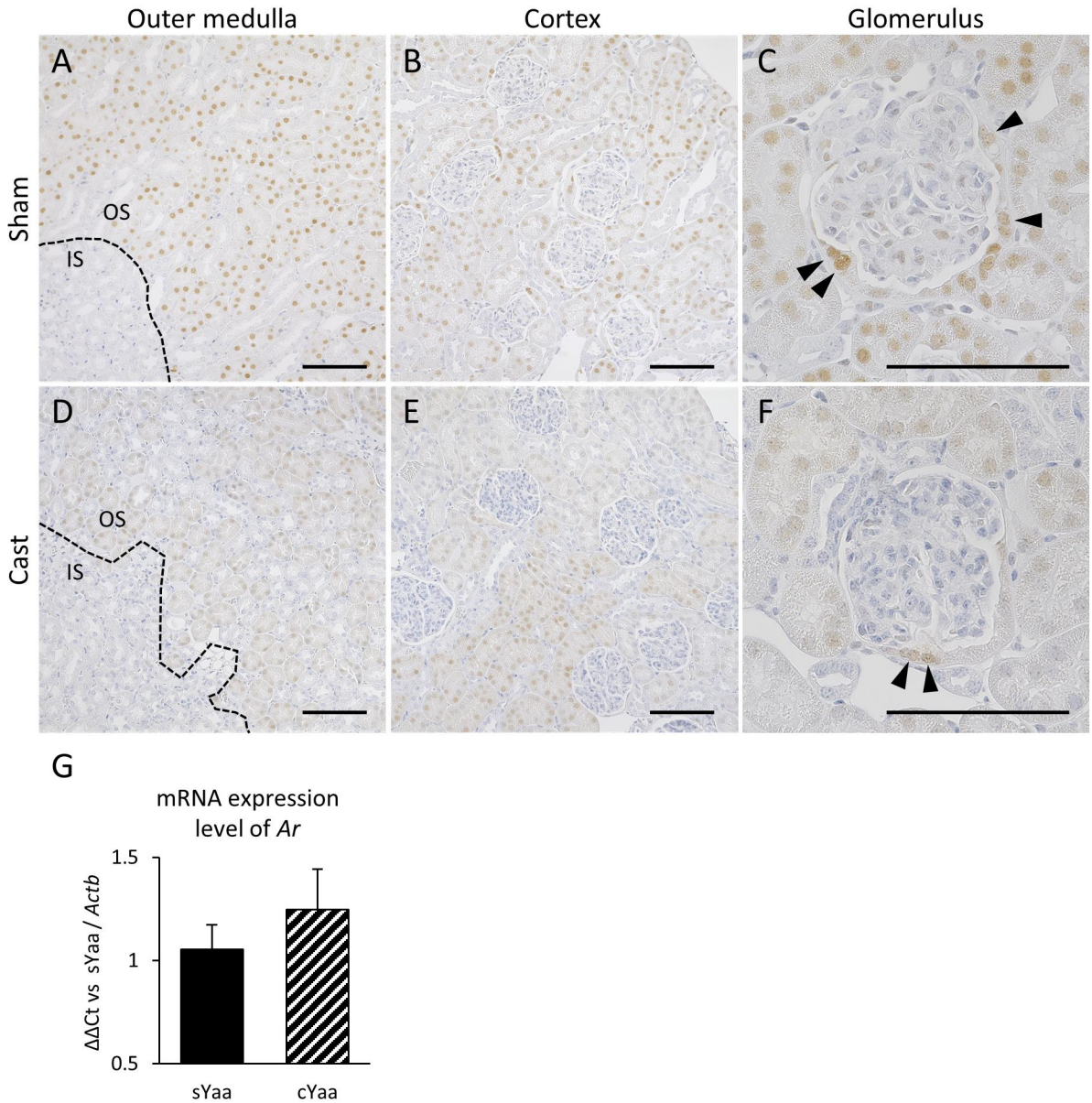


Figure 3-5. Analysis of androgen receptor in 6-month-old Yaa

(A-F) Immunohistochemistry of AR in outer medulla (A and D), cortex (B and E), and glomerulus (C and F) of Yaa kidneys at 6 months. Positive reaction is detected in nucleus of proximal tubules in medulla and cortex, and PECs. Dotted line represents the boundary between outer stripe (OS) and inner stripe (IS) in outer medulla. Arrowheads represent AR-positive PECs. Both the treatment groups show same localization, but reaction strengths in PECs tend to be lower in castrated Yaa. Bars = 100 μ m. (G) Relative mRNA expression of *Ar* in kidneys of sham-operated Yaa (sYaa) and castrated Yaa (cYaa). The expression levels were normalized to the levels of *Actb*. Each bar represents mean $\pm$ SE (n = 8-10).

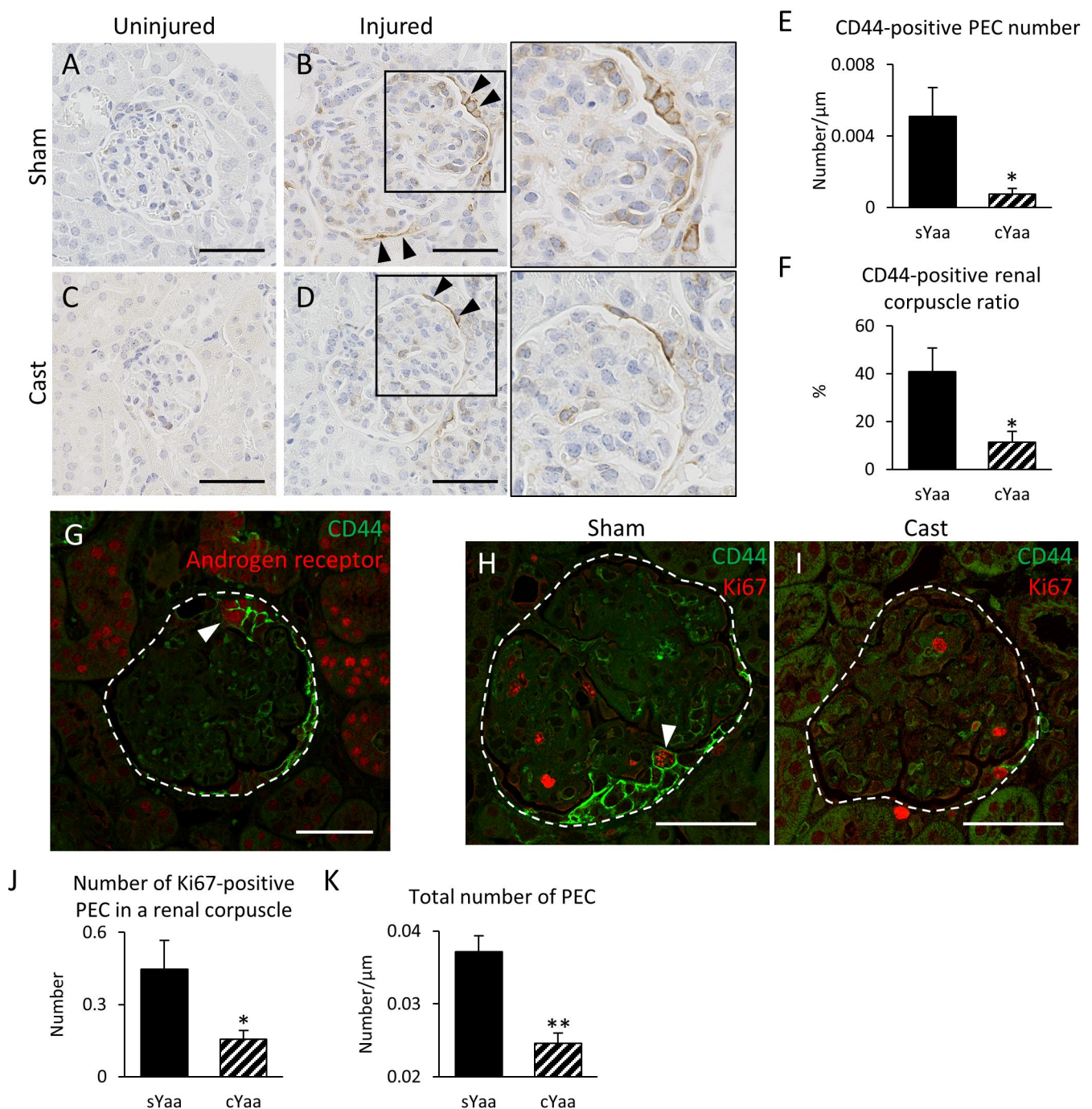


Figure 3-6. Histopathological analysis of parietal epithelial cells in 6-month-old Yaa

(A-D) Immunohistochemistry of CD44 in Yaa kidneys at 6 months. Arrowheads represent CD44-positive PECs. In renal corpuscles with uninjured glomeruli, CD44-positive PECs are not observed in both the treatment groups (A and C). PECs in renal corpuscles with injured glomeruli show positive reaction on their cell membrane (B and D). Treatment-related change of CD44 localization is not observed. Bars = 50 μ m. (E) The number of CD44-positive PECs per unit length of Bowman's capsule in sham-operated Yaa (sYaa) and castrated Yaa (cYaa) at 6 months. (F) The ratio of renal capsules with CD44-positive PECs in sYaa and cYaa at 6 months. (G) Immunofluorescence of AR (red) and CD44 (green) in sYaa at 6 months. Dotted line represents renal corpuscles. Arrowheads represent both markers-positive PECs. Some of the CD44-positive PECs stratify, and some of their nuclei are positive for AR. Bar = 50 μ m. (H and I) Immunofluorescence of Ki67 (red) and CD44 (green) in sham-operated (H) and castrated (I) Yaa at 6 months. Dotted line represents renal corpuscles. Arrowhead represents both markers-positive PECs. Some CD44-positive PECs stratify, and some of their nuclei are positive for Ki67 in sham-operated Yaa. Bars = 50 μ m. (J) The number of Ki67-positive PECs in a renal capsule in sYaa and cYaa at 6 months. (K) The total number of PECs per unit length of Bowman's capsule in sYaa and cYaa at 6 months. Each bar represents mean $\pm$ SE (n = 8-10). Significant differences in castrated groups compared to the sham-operated group are indicated with an asterisk. *: $P < 0.05$, **: $P < 0.01$ (Mann-Whitney U -test).

Conclusion

In mammals, reproductive system and immune function are closely related. Both functions play important roles in maintaining the homeostasis of another function. Proper immune reactions are essential to mediate regular oogenesis. On the contrary, male germ cells are highly segregated from systemic immune system depending on immunosuppressive environment formed by Sertoli cells and resident immune cells. Reversely, immune cells express sex hormone receptors – estrogens activate immune system whereas androgens suppress. Furthermore, many genes on sex chromosome are responsible for immune system in mammals.

Due to this close functional crosstalk between reproduction and immunity, disorder of either function could cause disease of the other system. Briefly, 30% of patients with POF have an autoimmune disease such as SLE and autoimmune thyroiditis. On the other hand, SLE incidence rates in pre-menopausal women to age-matched men are 8:1-15:1, and its prevalence is lower in pre-adolescent and post-menopause females, suggesting the pathological contribution of estrogens. As for male reproductive function, ASA is frequently detected in patients with autoimmune diseases, and disturbed immunosuppressive environment caused by chemical injection, infection, and trauma easily lead to failure of spermatogenesis in human and experimental animals. Further, SLE-related kidney disease and dermatitis become more severe in male patients than in females.

The imbalance of reproduction-immunity crosstalk is a common issue among human and animals. Altered immune condition is provoked by vaccination and infection, and infertility is a serious common problem leading to declining birthrate in our society and extinction of species in animals that could threaten the preservation of biodiversity. In veterinary field, however, more than half of domestic dogs and cats are gonadectomized from the viewpoint of animal welfare, and many farm animals are forced to be continuously pregnant, and lactate in order to satisfy food supply for humans. Importantly, gonadectomized dogs were reported to increase the incidence of immune-related diseases such as Addison's disease and thrombocytopenia. These evidences from both sides of reproductive and immune function in humans and animals imply the bidirectional effects on the pathogenesis of diseases, and highlight the necessity for further investigation. In this thesis, the author examined phenotypes in model mouse of autoimmune disease and its castrated individuals to discuss the pathological mechanism of diseases caused by altered functional crosstalk between reproduction and immunity in mammals.

The author firstly investigated the morpho-functional phenotypes of reproductive tract in female and male autoimmune disease model mice to reveal the pathological effects of excessive autoimmunity on the reproductive system in chapter 1 and 2. In chapter 3, the author examined the phenotype of castrated disease model mouse to discuss the function of androgens in the pathogenesis of autoimmune disease and glomerulonephritis.

Firstly, in the analysis of female model, mice showed the significant increase of infiltrating immune cells into ovaries with the smaller number of follicles, particularly primordial follicles, with the progression of systemic autoimmune disease. Consistent with these histological changes, estrus cycles were disrupted characterized by prolonged diestrus and shortened estrus, suggesting the abnormality of female reproductive system. On the other hand, in male mice, there was no immunological change in testes, although mice manifested severe systemic autoimmune disease. However, the number of germ cells undergoing apoptosis at the meiotic stage, in particular at St. XII, was significantly increased in autoimmune disease model. Further, the increase of residual bodies and the decrease of Sertoli cells were histologically shown with the development of autoimmune disease, suggesting altered phenotype of Sertoli cells. Thus, these results suggest that autoimmune disorder might affect both female and male reproductive function, but the mechanism is partially different. Female reproductive function could be disrupted by the direct interaction between ovary composing cells and immune cells, whereas male reproductive function would be affected by altered immunosuppressive environment without histological inflammation in mouse testes.

The analysis of castrated mice revealed that the lack of androgens caused slightly accelerated the onset of autoimmune disease characterized by splenomegaly, whereas the progression was not different onwards. However, castrated mice remarkably decreased glomerular sclerotic lesions at the late disease stage, although the initial renal pathology was equivalent with sham-operated mice. Importantly, in renal corpuscle of mice, protein of AR was observed not in glomerular cells but in nuclei of PECs using immunohistochemistry. Further, numbers of PECs in total and of activated PEC characterized by CD44 expression were significantly reduced in castrated mice, suggesting the effect of androgens on PECs to mediate their activation. Here, these results provide the evidence that PEC activation is crucial to form glomerular sclerotic lesions, and androgens would exacerbate this pathway.

The present study clearly showed that disorder of either function could result in the disruption of

another function in the pathological crosstalk between reproduction and immunity. Particularly, responsible cells leading to histological changes of organs during its pathogenesis were determined in this study. On the other hand, besides the imbalance of reproduction-immunity crosstalk, the current results suggest that genetic background of examined mice affects the phenotypes of both autoimmune and urogenital diseases. Indeed, in veterinary medicine, species- and breed-specific genetic polymorphism have been reported to be a cause of disease. Thus, further investigation of animal reproduction-immunity crosstalk is needed, and accumulation of evidences would help health of all animals including humans from the zoobiquitous viewpoint. In summary, this study provides novel basic evidences on the crosstalk between reproduction and immunity, and would contribute to elucidation of the pathogenesis of immune and reproductive diseases in mammals.

Conclusion in Japanese

自己免疫疾患モデルマウスの尿生殖器異常を導く生殖－免疫病態連関

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哺乳類の生殖と免疫機能は密に連関し、両者の機能を相互に制御する。卵巢内には免疫細胞が常在し、卵胞の成熟や排卵、黄体形成とその退行等、正常な雌性生殖機能の維持を担う。対して精巣では、セルトリ細胞等の精巣構成細胞と常在免疫細胞が特異的な免疫抑制環境“免疫学的特権領域”を形成し、精細胞は全身免疫から保護される。また、種々の免疫細胞は性ホルモン受容体を発現し、主にエストロゲンは免疫活性に、アンドロゲンはその抑制に寄与する。また、性染色体には多くの免疫関連遺伝子が座位する。

このような生殖と免疫機能の相互制御機構“生殖－免疫連関”において、一方の機能変化は他方の病態を誘導する。女性では、40歳未満で生殖機能を失う早発閉経患者の約30%が全身性エリテマトーデス（SLE）や自己免疫性甲状腺炎（橋本病）等の自己免疫異常を呈する。また、自己免疫疾患の病態形成には性ホルモンが強く影響し、女性のSLE罹患率は閉経前で男性よりも8-15倍高く、性成熟前や閉経後で低下する。男性の自己免疫疾患罹患率も高率に抗精子抗体を有し、ヒトと実験動物における化学物質や感染、外傷等による精巣内免疫抑制環境の破壊は、精上皮細胞死を特徴とする造精障害を導く。一方、男性SLE患者の腎炎や皮膚炎は女性患者よりも重篤化する。

このような生殖－免疫連関の不均衡は現代のヒトと動物の共通事象である。ワクチン接種や感染症罹患は全身免疫状態を変化させ、不妊症を代表とする生殖機能障害は、ヒトでは少子化社会、動物では種の絶滅を導き、生物多様性の維持を脅かし得る種横断的な課題となる。一方、動物福祉学的観点から、日本では半数以上の飼養犬猫が不妊化され、生産獣医療分野では人社会への安定した食糧供給のため、動物の人為的な妊娠・泌乳状態を継続的に作出する。重要なことに、性腺を摘出したイヌでは甲状腺機能低下症や血小板減少症等の免疫関連疾患罹患率が増加する。これらの証拠は、生殖－免疫連関の変化に起因する双方向性の病態形成を示唆するが、その機序は

不明である。そこで本研究は、生殖および免疫機能病態モデルの表現型を詳細に解析し、当該連関における相互制御機構とその変化に起因する病態形成メカニズムの解明を目的とした。

第一章では、自己免疫疾患モデル MRL/MpJ-*Fas*^{lpr/lpr} マウスの雌性生殖機能を経時的に観察し、自己免疫異常と卵巣形態機能の関連について考察した。第二章では、雄の自己免疫疾患モデル BXSB/MpJ-*Yaa* マウスの精巣について、形態的および分子生物学的に解析を行い、自己免疫異常が精巣特異的な免疫環境に与える影響とそのメカニズムについて考察した。第三章では、精巣摘出を施した BXSB/MpJ-*Yaa* の表現型および病態形成機序について探索し、アンドロゲンが自己免疫疾患および関連病態形成に与える影響を考察した。

はじめに、雌 MRL/MpJ-*Fas*^{lpr/lpr} の病態解析において、自己免疫疾患発症に伴い、卵巣への顕著な免疫細胞浸潤と、原始卵胞を含む卵胞数および黄体数の有意な減少がみられた。性周期について、MRL/MpJ-*Fas*^{lpr/lpr} は発情期の短縮と発情休止期の延長を特徴とする周期異常を対称群よりも早期に示し、雌性生殖機能の異常が示唆された。一方、雄 BXSB/MpJ-*Yaa* の解析において、全身自己免疫異常を呈する一方、精巣内の免疫細胞数や炎症関連分子の発現量は変化しなかった。しかし、組織学的解析において、精母細胞アポトーシス数は健常群よりも有意に増加し、その増加は精上皮ステージ XII で顕著だった。精細管内では residual body の増加とセルトリ細胞の減少がみられ、全身性自己免疫異常に伴うセルトリ細胞の機能形質変化が示唆された。以上より、マウス生殖－免疫連関において、全身性自己免疫疾患は雌性および雄性生殖機能を障害することを見出した。一方、その病態形成機序は雌雄で異なり、雌では免疫細胞等の炎症因子が卵巣構成細胞に直接的に作用するのに対し、雄では全身免疫の変化が、炎症機構を介さず精巣の免疫学的環境を変化させ、生殖機能の異常をもたらす可能性が考えられた。

他方、精巣を摘出した BXSB/MpJ-*Yaa* の自己免疫疾患発症は擬手術群よりも早期化傾向であったが、病態後期の重篤度は変化しなかった。一方、腎病理解析において、精巣摘出群の病態後期における糸球体硬化病変は、擬手術群よりも有意に軽減した。重要なことに、免疫染色において、アンドロゲン受容体は糸球体構成細胞にはみられず、腎小体内では糸球体包外壁の上皮細胞(PEC)の核内に局在し、さらに総 PEC 数および活性化 PEC 数は精巣摘出群で有意に減少した。上記の結果より、アンドロゲンは BXSB/MpJ-*Yaa* の糸球体硬化病変を増悪させ、その病理形成には PEC が関与すると考えられた。

結論として、本研究はマウスの生殖－免疫連関の変化が尿生殖器に病態を導くことを示した。特に病理組織学的手法により、各臓器の形態機能変化をもたらす実動細胞を特定し、生殖－免疫

連関を基盤とする病態形成機構において新たな知見を見出した。一方、解析した疾患モデルマウスの表現型形成には生殖－免疫連関の不均衡に加え、遺伝的素因の関与が疑われた。獣医療においても、動物種、血統または品種特異的な遺伝的多型は疾患発症の一因となり、生殖－免疫連関異常との関連性評価は意義深い。汎動物学的観点より、動物から得る医科学的知見は動物のみならずヒト疾患の診断・治療に応用でき、獣医学領域における基礎研究の発展は動物とヒトの健全性達成に欠かせない。以上、本研究は哺乳類の生殖－免疫連関における新たな基礎知見の提供を通じ、動物とヒトの免疫異常、生殖機能障害およびそれらの病態連関の解明に寄与するものである。

Acknowledgement

I would like to extend thanks and my appreciation to those who have encouraged me during years of graduate studies. First and foremost, I would like to express my gratitude towards my supervisor, Professor Yasuhiro Kon for his great support, advice, and guidance throughout my all university days to complete this thesis project. I would appreciate Associate Professor Osamu Ichii teaching me thousands of things to progress scientific works, and always supporting me. I thank Professor Mitsuyoshi Takiguchi and Professor Seiji Katagiri for time taken to provide advice, critical comments and assistance in completing the thesis. I would also like to thank fellows in Laboratory of Anatomy including Mrs. Masae Ishikawa and my friends in our faculty for their encouragement, support, and great times. Finally, I sincerely express my deepest gratitude and sorrow to all animals supporting this study.

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