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Castrated autoimmune glomerulonephritis mouse model shows attenuated glomerular sclerosis with altered parietal epithelial cell phenotype

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Short Title: Castration attenuates glomerular sclerosis in mice

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Abstract

Sex hormones help in maintaining proper immunity as well as renal homeostasis in mammals, and these multi-functional properties characterize the onset of sex-dependent diseases. To clarify the contribution of sex hormones to autoimmune disease-related renal pathogenesis, BXSB/MpJ-*Yaa* was investigated as a murine autoimmune glomerulonephritis model. BXSB/MpJ-*Yaa* and its wild-type, BXSB/MpJ-*Yaa*$^{+}$ 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/MpJ-*Yaa*$^{+}$. At 3 months, both sham-operated and castrated BXSB/MpJ-*Yaa* manifested splenomegaly, autoantibody production, and glomerulonephritis, and castrated BXSB/MpJ-*Yaa* tended to show heavier spleen weights than the sham-operated group. At 6 months, both the treated BXSB/MpJ-*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 BXSB/MpJ-*Yaa* was significantly milder than in sham-operated mice at 4 months, but those of both the treated BXSB/MpJ-*Yaa* were comparable at 6 months. The examined renal histopathological indices in parietal epithelial cells (PECs) were remarkably altered by castration. Briefly, castration decreased the height of PECs and total PEC number in BXSB/MpJ-*Yaa* at 6 months. For immunostaining, PECs facing the injured glomeruli of BXSB/MpJ-*Yaa* expressed CD44, an activated PEC marker, and CD44-positive PECs showed nuclear localization of the androgen receptor and proliferation marker

Ki67. CD44- or Ki67-positive PECs were significantly fewer in castrated group than in sham-operated BXSB/MpJ-*Yaa* at 6 months. Further, quantitative indices for CD44-positive PEC number and frequency in renal corpuscles positively correlated with glomerular sclerotic severity in BXSB/MpJ-*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 BXSB/MpJ-*Yaa*, as PEC activation resulted in glomerular sclerosis.

Keywords

Autoimmune disease, Kidney disease, Glomerulonephritis, Glomerular sclerosis, Androgen

Impact Statement

The prevalence of kidney disease is increasing globally. The loss of kidney function is irreversible, and dialysis and kidney transplantation are the primary treatments; however, they are complex and expensive. Importantly, most patients undergoing dialysis are men. Androgens have been suggested as a risk factor, but how androgens contribute to the pathogenesis of kidney disease remains unclear. Here, we histologically investigated the renal pathology of a glomerulonephritis model and found milder glomerular sclerotic lesions in castrated mice, suggesting an exacerbating effect of androgens on glomerular sclerosis development. Furthermore, we report a clear expression of the androgen receptor in parietal epithelial cells (PECs), but not in glomerular cells, showing altered number and

property owing to castration. Taken together, our results provide new evidence that androgens contribute to the pathogenesis of kidney disease by mediating the PEC phenotype; thus, it is crucial to understand the mechanism of male-dominant symptoms.

Introduction

In mammals, sex hormones play an essential role in maintaining proper reproductive function. In addition, sex hormones have been regarded as modulators of immune function, resulting in clear sex-related disparities in immune system diseases. Systemic lupus erythematosus (SLE), a refractory autoimmune disease showing skin rash, joint pain, and anti-dsDNA autoantibody, represents significant sex-related disparities. 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 females.[1] These data suggest that estrogens are an exacerbating factor in the etiology of SLE.

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 glomerulus.[2] Importantly, 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.[3,4] 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 by sex hormones in terms of development,

81 homeostasis, and disease conditions. Female rats have been reported to have smaller kidneys than
82 males, and castrated mice also have a decreased kidney size.[5] Histologically, the PECs and proximal
83 tubular epithelial cells in mice have been found to be larger in males than in females,[6] suggesting the
84 involvement of androgens in kidney function. Androgen receptor (AR) expression has been confirmed
85 in proximal tubular epithelial cells in mice and rats.[7] In addition, AR mRNA expression was reported
86 in mouse glomeruli, including podocytes, using PCR analysis, and AR proteins in cultured mesangial
87 cells,[7–9] suggesting the contribution of androgens to glomerular homeostasis as well as the
88 pathogenesis of kidney diseases. Clinical studies have reported that several kidney diseases, including
89 glomerulonephritis, glomerulosclerosis, membranous nephropathy, and IgA nephropathy, are more
90 prevalent and serious in males.[10–12] Experimental studies have supported androgens as a risk factor for
91 kidney diseases, as shown by the protective effect of castration in several kidney disease models
92 induced by ureteral obstruction, ischemia-reperfusion, and diabetes[13–15]; in contrast, estrogen is
93 suggested to have a protective role in kidney disease, as demonstrated in human and animal models.[16]
94 On the other hand, for autoimmune nephritis, we have previously reported that androgens played the
95 protective role in pathology of autoimmune glomerulonephritis in congenic mice unlike other kidney
96 disease models.[17] Furthermore, examination of autoimmune-prone NZB-derived F1 mice showed that
97 estrogen promotes autoimmune glomerulonephritis due to excessive activation of cellular immunity.[18]
98 These results suggest that autoimmune disease-related nephritis is complicatedly formed by systemic
99 autoimmune conditions and sex-related factors.

The BXSB/MpJ-*Yaa* mouse (Yaa) is a representative model of male-dominant systemic autoimmune disease, characterized by autoantibody production and splenomegaly due to the excessive proliferation of autoreactive lymphocytes.[19] Yaa develops severe membranous proliferative glomerulonephritis (MPGN), characterized by glomerular hypertrophy, thickening of GBM, and expansion of sclerotic lesions.[20] Yaa is derived from the BXSB/MpJ-*Yaa*$^{+}$ (BXSB) strain, and is presumed to develop the disease due to Y-linked autoimmune acceleration (*Yaa*) mutations, duplication of some genes on the telomere region of X chromosome to that of the Y chromosome in males.[21] Previous studies revealed that the BXSB genome is also suspected to develop autoimmune disease-prone phenotypes, as aged female BXSB manifested autoantibody production and glomerulonephritis.[22] In addition to *Yaa* mutation, we 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. 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 [23] However, detailed pathology of autoimmune glomerulonephritis in Yaa, contributed by sex hormones has not been discussed so far.

In the present study, we 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. At 6 months, castrated Yaa clearly showed

milder sclerotic lesions in glomeruli, although autoimmune indices were equivalent between treatments. 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

BXSB (without *Yaa* mutation) and Yaa mice were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). 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, and all animal experiments were approved by the Institutional Animal Care and Use Committee of Hokkaido University and the Faculty of Veterinary Medicine, Hokkaido University (approval No. 15-0079, 20-0012). Our 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, 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 the ratio to body weight 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-double-stranded DNA (dsDNA) antibody levels were measured using the Mouse Anti-dsDNA ELISA Kit (Shibayagi, Gunma, Japan), 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) and Urinary Creatinine Assay Kit (Detroit R&D, Inc.; Detroit, MI, USA), and subsequently, the urinary albumin creatinine ratio (uACR) was calculated. We 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, kidney samples were cut into 2-μm thick sections. The sections were

deparaffinized and then stained with periodic acid-Schiff-hematoxylin (PAS-H), periodic acid methenamine-silver (PAM), and Masson's trichrome (MT) stains. In histoplanimetrical analysis, histological sections were converted into digital images by scanning with NanoZoomer 2.0-RS (Hamamatsu Photonics K.K., Shizuoka, Japan). Nuclear numbers in the glomerulus and glomerular area were quantified using the NDP.view2 program (Hamamatsu Photonics K.K., Shizuoka, Japan). 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

Deparaffinized sections were treated for antigen retrieval in 20 mM Tris-HCl buffer (pH 9.0) at 110 °C for 15 min. For immunohistochemistry, sections were soaked in methanol containing 0.3% H_2O_2 for 20 min at room temperature. After washing and blocking with normal goat or donkey serum for 1 h at room temperature, sections were incubated with primary antibodies, listed in Table 1, at 4 °C for over 16 h. After washing, the sections were incubated with secondary antibodies, listed in Table 1. For immunohistochemistry, the sections were incubated with streptavidin-horseradish peroxidase (SABPO kit; Nichirei, Tokyo, Japan) for 30 min at room temperature, and then incubated with 3,3-diaminobenzidine tetrahydrochloride-0.3% H_2O_2 solution. Finally, the sections were counterstained with hematoxylin. For immunofluorescence, 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, Osaka, Japan), and were analyzed using a BX analyzer.

In histoplanimetrical 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.

Reverse transcription and quantitative PCR (qPCR)

Total RNA was isolated from the kidneys using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), following the manufacturer's protocol. 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 using synthesized cDNA, THUNDERBIRD

SYBR qPCR Mix (TOYOBO, Osaka, Japan), and a real-time thermal cycler (CFX Connect; BIO-RAD, California, USA), following the manufacturer's instructions. Gene expression in the testes was normalized to the expression of actin beta (*Actb*). The details of the primers are shown in Table 2.

Statistical analysis

The results are expressed as mean ± standard error (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

We first compared the physiological parameters of sham-operated and castrated mice to evaluate the effect of castration. Figure 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 1b). Castration did not induce any change in the spleen weight of BXSB mice, but decreased the body weight in castrated mice at both ages (Fig 1c and d). 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 1c), which resulted in a significant difference in spleen weight to body weight ratio (Fig 1d). On histological examination of the spleen, Yaa showed expanded white bulb area compared to BXSB, but there was no significant difference between the treatment groups (Supplemental Fig 1a). At 6 months, there was no significant difference in spleen weight, body weight ratio, or histology between castrated and sham-operated Yaa (Fig 1d, Supplemental Fig 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 1e). 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

We 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 2a). 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 2b 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 (Fig 2b). At 6 months, severe MPGN characterized by glomerular hypertrophy, thickened GBM, and expansion of sclerotic lesions was observed in both treatment groups; however, castrated mice showed milder expansion of sclerotic lesions compared with sham-operated mice (Fig 2c). 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 2d); however, these pathological changes were scarcely observed in castrated Yaa. Comparison of histoplanimetries between different treatment groups at 6 months did not alter the area of glomerulus and the number of glomerular nuclei due to castration (Fig 2e and f). On the other hand, sclerotic lesion area and its fraction of glomerulus were remarkably lower in castrated mice than in sham-operated Yaa (Fig 2g and h).

Functional changes in podocytes of castrated Yaa kidney

We 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 3a 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. Quantitative PCR analysis showed significantly higher levels of podocyte functional markers in castrated mice than in sham-operated mice (Fig 3b).

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 3c). 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 3d and e).

Analysis of androgen receptor in 6-month-old Yaa kidney

Next, we 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 4a 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, 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. 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 4b).

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

PECs have been demonstrated to contribute in the formation of glomerulosclerosis.[24] 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 lesions.[25] We first examined the expression of CD44 in 6-month-old Yaa kidneys, using immunohistochemistry. CD44-positive PECs were rarely observed in the renal corpuscle with uninjured glomeruli in both the treated Yaa groups (Fig 5a). 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. 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 properties[26]. 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 histoplanimetrical analysis, CD44-positive PEC number was significantly decreased in castrated Yaa at 6 months (Fig 5b). Furthermore, castrated mice showed a lower frequency of renal corpuscles with CD44-positive PECs (Fig 5c). Figure 5d 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 5e shows a representative immunofluorescence for CD44 and Ki67 in Yaa kidneys showing MPGN. 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. Histoplanimetrical analysis showed a significant decrease in Ki67-positive PEC number in castrated Yaa (Figure 5f). Further, the total number of PECs were significantly lower in castrated kidneys (Fig

303 5g).

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

313

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.[27] 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.[28] 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.[29] 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[22], 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[15]. The etiology has not been elucidated, but increase in transforming growth factor-β level in the glomerulus by androgens could be a potent pathogenesis of glomerular sclerotic lesions[9]. 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.

352 Formation of glomerular sclerotic lesions is commonly observed in various kidney diseases,
353 including focal segmental glomerulosclerosis, diabetes nephritis, and lupus nephritis, characterized by
354 ECM accumulations.[30] Disruption of the glomerular filtration barrier that podocytes maintain is the
355 primary pathological event, which results in the leakage of circulating proteins into primitive urine.
356 Previous studies reported that mouse podocytes and mesangial cells express AR, and androgens have
357 been suggested to induce apoptosis of cultured podocytes and promote ECM production in mesangial
358 cells *in vivo* [7–9]. However, in the current model, AR was observed significantly in the nuclei of PECs,
359 but not in those of podocytes and mesangial cells. Importantly, PEC is suggested to be involved in the
360 formation of glomerular sclerotic lesions once podocytes are injured, although the molecular
361 mechanisms are still poorly defined.[24] PECs tend to proliferate, produce ECM, and migrate to tuft to
362 seal the disrupted filtration barrier. As a result, glomeruli increase sclerotic lesions due to the
363 accumulation of ECM that PECs and mesangial cells mainly produce.
364 Recently, PECs have been divided into subpopulations classified by specific molecules,[26] and
365 CD44 has been established as a marker of activated PECs.[31] CD44 is a cell membrane protein, which
366 functions as a receptor of hyaluronic acid and ECM, and has been reported to be involved in cell
367 proliferation, migration, and malignancy in various cancers, such as prostate cancer and breast
368 cancer.[32] In the kidney, CD44-positive PECs are considered to have the ability to proliferate, migrate,
369 and produce ECM as well. Furthermore, previous studies have reported the contribution of CD44-
370 positive PECs in the formation of glomerulosclerosis and crescents.[25] Our results confirmed CD44

371 expression on PECs facing injured glomeruli, but not in renal corpuscles with uninjured glomeruli,
372 and a positive correlation with sclerotic indices indicated CD44 function in the formation of sclerotic
373 lesions in Yaa. Additionally, the decreased number of CD44-positive PECs and the positive ratio of
374 CD44 in the renal corpuscles of 6-month-old castrated Yaa highlighted the effect of androgens on
375 CD44 expression. The decreased number of Ki67-positive PECs suggests that the proliferation of
376 PEC is suppressed in castrated Yaa, which might result in the decrease of total number of PECs. Thus,
377 we infer that morpho-functional changes in PECs under androgen-poor conditions play a key role in
378 the attenuation of glomerular sclerosis in Yaa.

379 AR is stably expressed in the cytoplasm of widespread cells and tissues. After androgens bind to
380 AR, androgen/AR (A/AR) complexes translocate to the nucleus and function as transcription factors.[33]
381 The involvement of A/AR signaling in CD44 expression has been suggested in some epithelial
382 tumors. Prostate cancer develops androgen-dependence, and *in vitro* analysis showed that AR
383 expression mediates CD44 expression in prostate cancer cells.[34,35] More importantly, androgen-
384 mediated CD44 expression is reported to lead epithelial-mesenchymal transformation (EMT) in
385 cancer cells, which allows cells to proliferate and produce ECM excessively.[36] In renal pathology,
386 PECs are observed to undergo EMT, resulting in the proliferation and production of ECM, thereby
387 forming crescent and glomerular sclerotic lesion.[37] In this study, we revealed that A/AR might mediate
388 the PEC phenotype, in particular, expressing CD44 and undergoing EMT in Yaa glomerulonephritis.
389 Moreover, decreased immunopositive reaction of AR in the PEC nucleus in castrated mice compared

390 with sham-operated PEC, despite equivalent mRNA expression levels of *Ar* in the whole kidney,
391 supported the decreased activity of A/AR signaling pathway, owing to castration.

392 Castrated Yaa showed decreased levels of uACR compared to the sham group at 4 months, when
393 MPGN progressed severely in Yaa. This suggests that castration prevents progression of renal lesions
394 in Yaa. There is a less possibility of direct contribution of androgens to glomerular injury because
395 there is no expression of AR in the glomerulus. It is rather reasonable that androgens had already
396 affected PECs at 4 months to promote the formation of sclerotic lesions. Furthermore, androgen is
397 thought to increase albuminuria in some strains of mice and rats, as male animals show higher urinary
398 protein levels than females.[38,39] Albuminuria from glomerulus is reported to induce CD44 expression
399 in PECs in a glomerulonephritis mouse model.[40] Positive correlation between CD44-related indices
400 and uACR level in 6-month-old Yaa indicates their close association with Yaa as well. Higher uACR
401 levels in sham-operated Yaa than in the castrated group at 4 and 5 months suggest that androgens
402 induce the leakage of albumin, which could enhance CD44 expression in PECs. As a result, formation
403 of glomerular sclerosis lesions synergistically exacerbated renal pathology in Yaa. However, at the
404 late disease stage, castrated Yaa showed equivalent levels of uACR with sham-operated Yaa. It could
405 be reasoned that castrated Yaa developed more severe autoimmune diseases and MPGN than in early
406 stages, and the formation of global sclerotic lesions in sham-operated Yaa lead to glomerular
407 dysfunction, resulting in decreased uACR level.

408 In conclusion, castration caused accelerated onset of autoimmune diseases, but attenuated

sclerotic lesions in Yaa, a male-dominant autoimmune disease mouse model. In the pathogenesis of autoimmune disease, androgen suppresses excessive autoimmune reactions expressed earlier during the onset of disease in castrated Yaa. In contrast, renal pathology is definitely exacerbated with the expansion of sclerotic lesions in the presence of androgen. Our 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, PECs are suggested to be mediated by A/AR signaling in the pathogenesis of glomerular sclerosis in Yaa, which plays a critical role in this pathogenesis by proliferating and producing ECM. These findings provide novel insights into the sex-related disparity of renal diseases in zoobiquitous medicine.

Authors' Contributions

Y.O., O.I., T.N., and Y.K. designed the work; Y.O., O.I., M.A., and T.N. performed experiments and analyzed the data; Y.O., O.I., and Y.K. drafted and revised the manuscript. All the authors were involved in the writing of the paper and approval of the final manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Figure legends

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

(a) The serum level of testosterone. (b) Body weight. (c) Spleen weight. (d) The ratio of spleen weight to body weight. (e) The serum level of anti-double stranded DNA antibody. (f) Kidney weight. Each bar represents mean ± SE (n = 4-10). s: sham-operated; c: castrated. *: Significant differences in castrated groups against sham-operated group in the same strain at the same age (*: $P < 0.05$, **: $P < 0.01$, Mann-Whitney *U*-test). †: Significant differences in Yaa against BXSB with the same treatment at the same age († $P < 0.05$, †† $P < 0.01$, Mann-Whitney *U*-test). #: Significant differences in 6-month-old groups against 3-month-old groups in the same strain with the same treatment (#: $P < 0.05$, ##: $P < 0.01$, Mann-Whitney *U*-test).

Figure 2. Histopathological analysis of glomeruli in mice

(a) Histology of glomeruli in sham-operated and castrated BXSB at 3 and 6 months. Sections are stained with periodic acid-Schiff -hematoxylin (PAS-H). (b and c) Histology of glomeruli in sham-operated and castrated Yaa at 3 months (b) and 6 months (c). Sections are stained with PAS-H, periodic acid methenamine-silver (PAM), and Masson's trichrome (MT). At 3 months, glomerular hypertrophy, increase in mesangial matrix lesion, and wrinkling of the glomerular basement membrane were observed, but there were no treatment-related differences. These histological changes are clearer at 6 months compared with 3 months in both the treatment groups. Expansion of sclerotic

lesion is more significant in sham-operated Yaa than in castrated Yaa. (d) Representative histology of glomeruli with global sclerotic lesions stained with PAS-H and PAM 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. (e) The area of glomeruli in sham-operated Yaa (sYaa) and castrated Yaa (cYaa) at 6 months. (f) The number of nuclei per unit area of a glomerulus in sYaa and cYaa at 6 months. (g) The area of sclerotic lesion in glomeruli in sYaa and cYaa at 6 months. (h) The fraction of sclerotic lesions in sYaa and cYaa at 6 months. Each bar represents mean ± 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. Analysis of podocyte function in 6-month-old Yaa

(a) Immunofluorescence of podocyte function molecules (Podocin, Nephrin, Synaptopodin) 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 μm. (b) 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*. (c) uACR in sYaa and cYaa from 3 to 6 months. (d and e) The serum levels of serum blood urea nitrogen (BUN, d) and serum

creatinine (Cre, e) in sYaa and cYaa at 6 months. Each bar represents mean ± 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 4. Analysis of androgen receptor in 6-month-old Yaa

(a) Immunohistochemistry of androgen receptor (AR) in outer medulla, cortex, and glomerulus of Yaa kidneys at 6 months. Positive reaction is detected in nucleus of proximal tubules in medulla and cortex, and parietal endothelial cells (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 μm. (b) 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 ± SE (n = 8-10).

Figure 5. Histopathological analysis of parietal epithelial cells in 6-month-old Yaa

(a) Immunohistochemistry of CD44 in Yaa kidneys at 6 months. Arrowheads represent CD44-positive parietal epithelial cells (PECs). In renal corpuscles with uninjured glomeruli, CD44-positive PECs are not observed in both the treatment groups. PECs in renal corpuscles with injured glomeruli show positive reaction on their cell membrane. Treatment-related change of CD44 localization is not

observed. Bars = 50 μm. (b) 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. (c) The ratio of renal capsules with CD44-positive PECs in sYaa and cYaa at 6 months. (d) Immunofluorescence of androgen receptor (red, AR) 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 μm. (e) Immunofluorescence of Ki67 (red) and CD44 (green) in 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 μm. (f) The number of Ki67-positive PECs in a renal capsule in sYaa and cYaa at 6 months. (g) The total number of PECs per unit length of Bowman's capsule in sYaa and cYaa at 6 months. Each bar represents mean ± 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).

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Table 1. Antibodies

Primary Antibody	Source	Detection (Application)	Dilution	Blocking	Antigen retrieval	Secondary Antibody (IHC)	Secondary Antibody (IF)
Rabbit anti-nephrin	IBL (Gunma, Japan)	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 (Gunma, Japan)	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, (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 (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 (CA, USA)	Alexa Fluor 488-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rabbit anti-Ki67	Abcam (Cambridge, UK)	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.

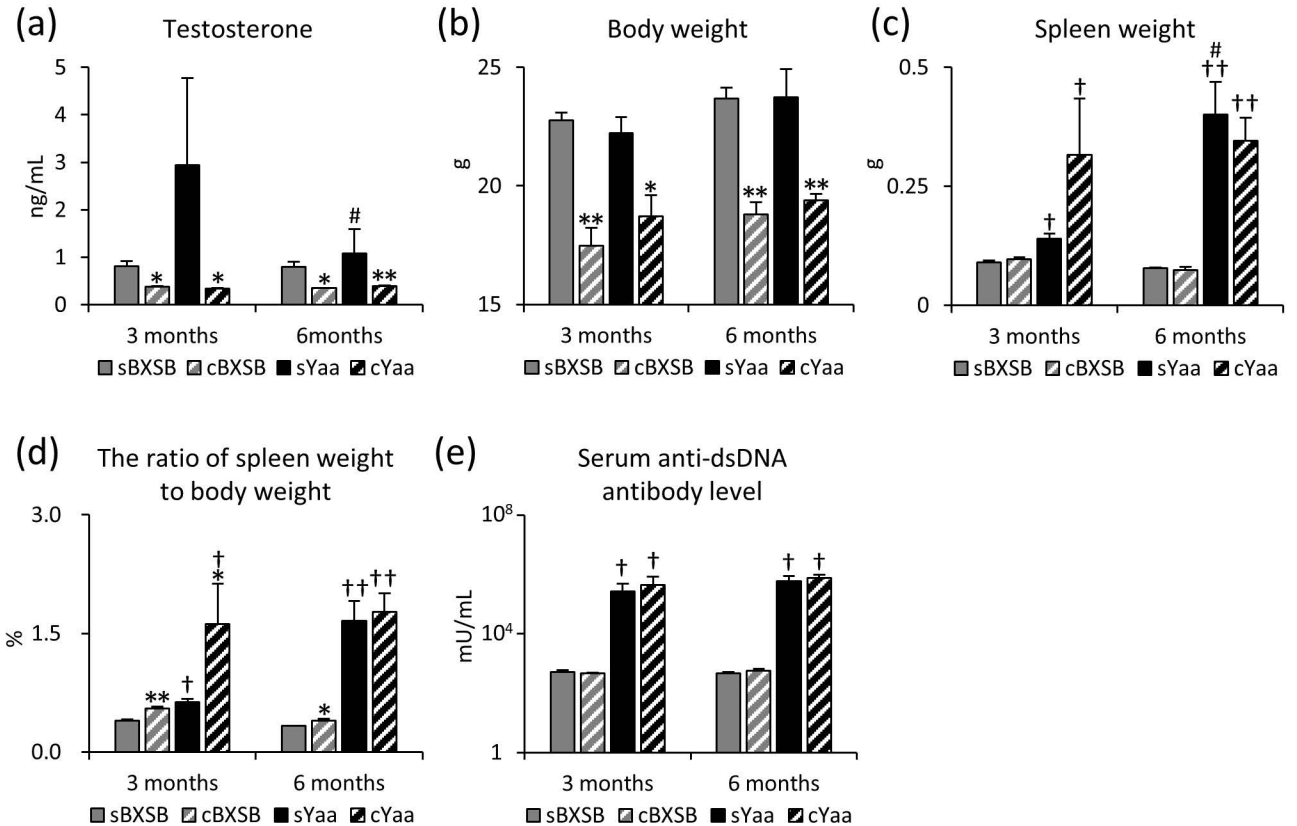
Table 2. Primers

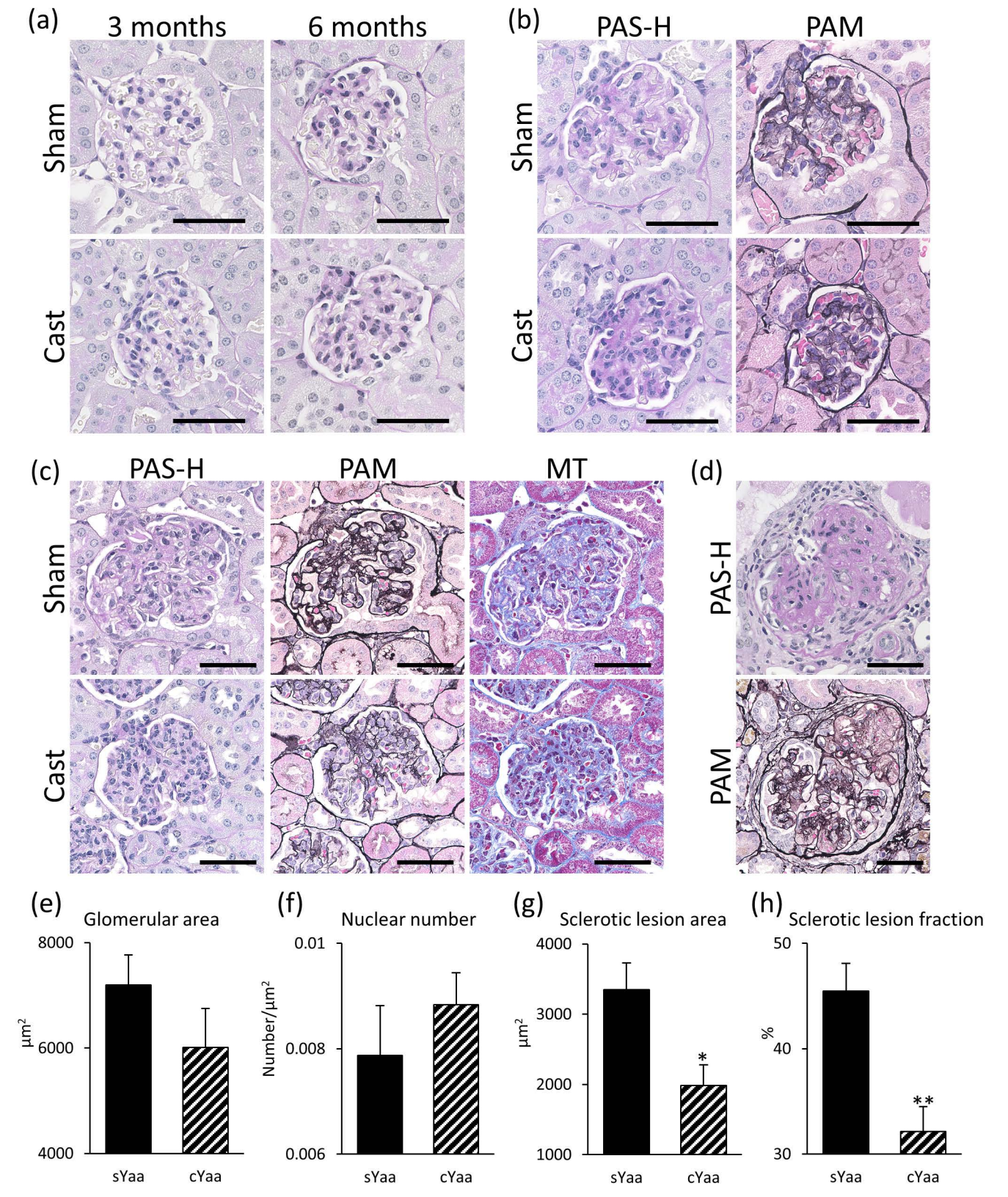
Gene name (accession no.)	Official symbol	Primer sequence (5'-3')	Product size (bp)
Actin beta (NM_007393)	*Actb*	F: TGTTACCAACTGGGACGACA R: GGGGTGTTGAAGGTCTCAAA	165
Nephrosis 1, nephrin (NM_019459)	*Nphs1*	F: ACCTGTATGACGAGGTGGAGAG R: TCGTGAAGAGTCTCACACCAG	218
Nephrosis 2, podocin (NM_130456)	*Nphs2*	F: AAGGTTGATCTCCGTCTCCAG R: TTCCATGCGGTAGTAGCAGAC	105
Synaptopodin (NM_177340.2)	*Synpo*	F: CATCGGACCTTCTTCCTGTG R: TCGGAGTCTGTGGGTGAG	90

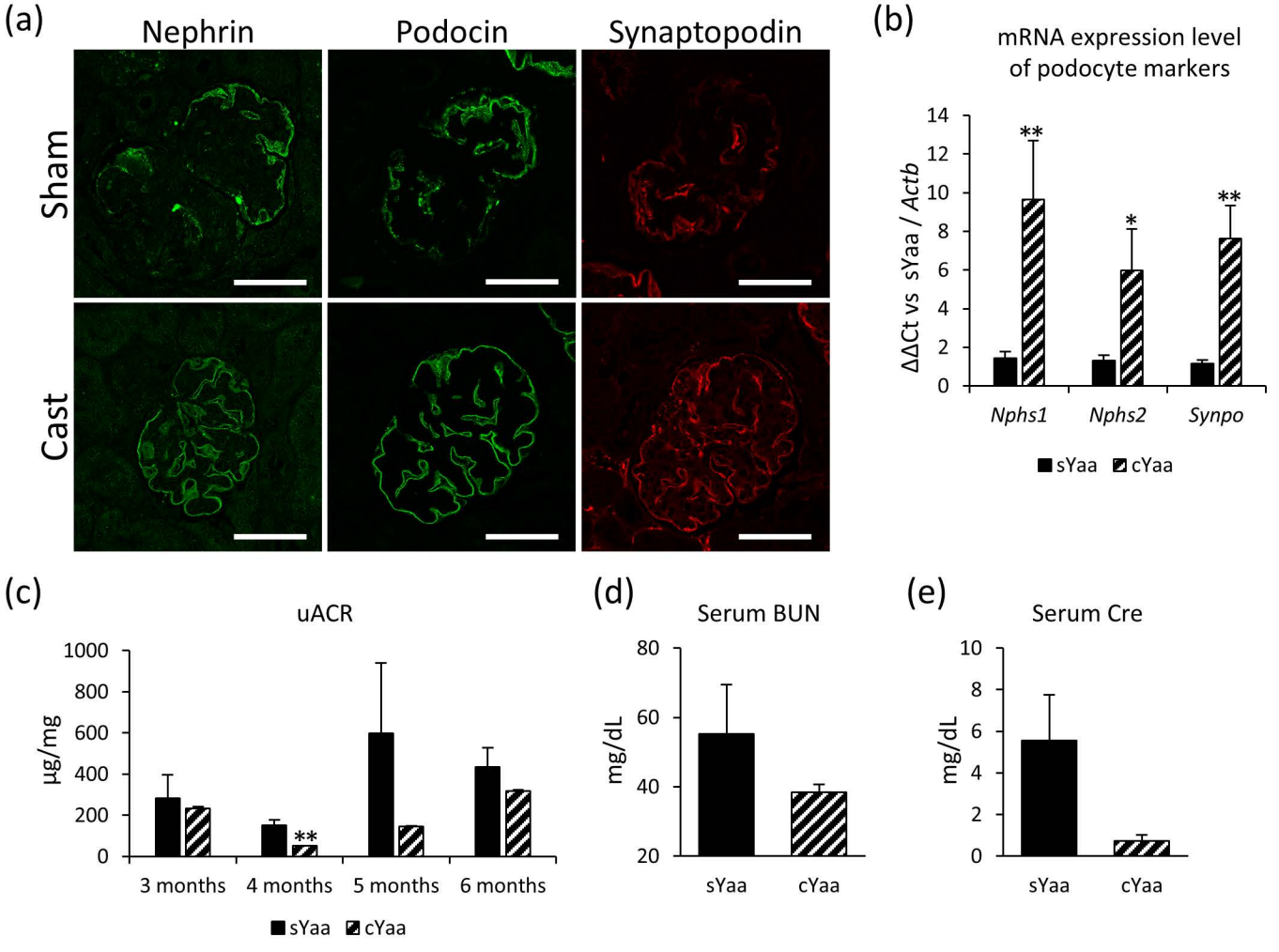
Table 3. Correlation analysis among examined parameters in 6-month-old Yaa.

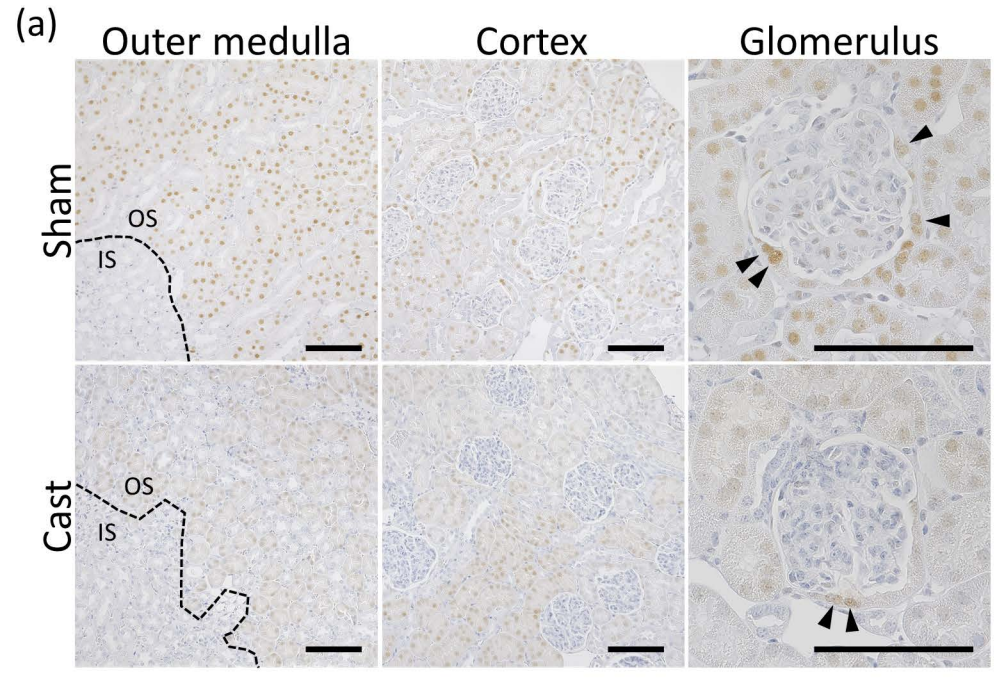
			Autoimmune disease		Activation of PECs		
	Parameters		Spleen weight	Serum dsDNA level	CD44-positive PEC number	CD44-positive renal corpuscle ratio	Total PEC number
Sclerotic lesion	Sclerotic lesion area	ρ	0.515*	0.003	0.863**	0.851**	0.649**
		P	0.029	0.991	<0.001	<0.001	0.004
	Sclerotic lesion fraction	ρ	0.302	-0.124	0.655**	0.638**	0.645**
		P	0.223	0.649	0.003	0.004	0.004
Glomerular function	uACR	ρ	0.612	0.297	0.794**	0.818**	0.527
		P	0.060	0.405	0.006	0.004	0.117
Activation of PECs	CD44-positive PEC number	ρ	0.417	0.196	-	0.994**	0.723**
		P	0.085	0.468	-	<0.001	0.001
	CD44-positive renal corpuscle ratio	ρ	0.475*	0.210	0.994**	-	0.719**
		P	0.047	0.434	<0.001	-	0.001

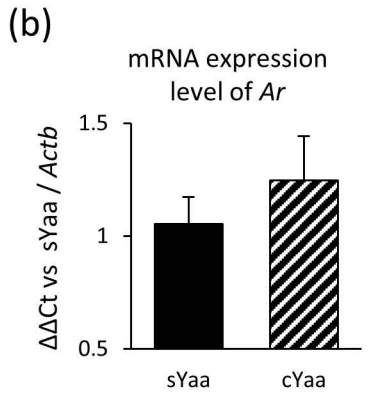
*$P < 0.05$, ** $P < 0.01$. ρ: Spearman's rank correlation coefficient, n = 18. uACR: urinary albumin creatinine ratio, PEC: parietal epithelial cell

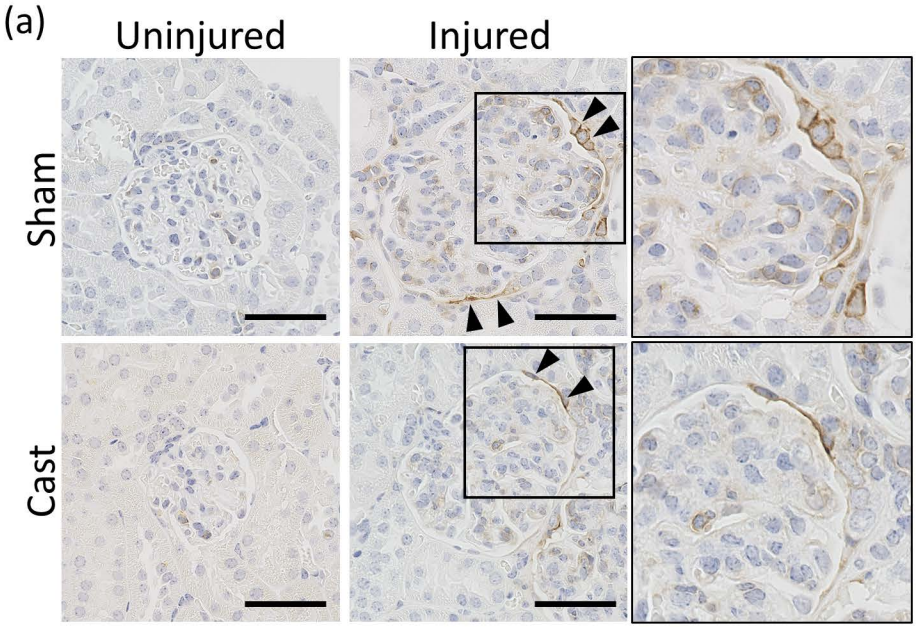

(a)
Testosterone
ng/mL
5
4
3
2
1
0
3 months
6months
sBXSB cBXSB sYaa cYaa
(b)
Body weight
g
25
20
15
3 months
6 months
sBXSB cBXSB sYaa cYaa
(c)
Spleen weight
g
0.5
0.25
0
3 months
6 months
sBXSB cBXSB sYaa cYaa
(d)
The ratio of spleen weight to body weight
%
3.0
1.5
0.0
3 months
6 months
sBXSB cBXSB sYaa cYaa
(e)
Serum anti-dsDNA antibody level
mU/mL
10^8
10^4
1
3 months
6 months
sBXSB cBXSB sYaa cYaa

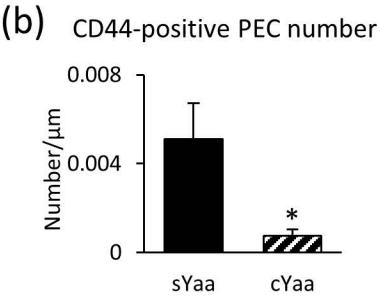

(a)
3 months
6 months
Sham
Cast
(b)
PAS-H
PAM
Sham
Cast
(c)
PAS-H
PAM
MT
Sham
Cast
(d)
PAS-H
PAM
(e)
Glomerular area
8000
6000
4000
μm²
sYaa
cYaa
(f)
Nuclear number
0.01
0.008
0.006
Number/μm²
sYaa
cYaa
(g)
Sclerotic lesion area
4000
3000
2000
1000
μm²
*
sYaa
cYaa
(h)
Sclerotic lesion fraction
50
40
30
%
**
sYaa
cYaa

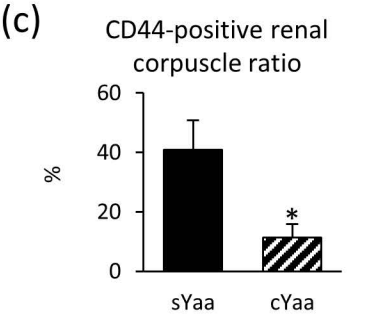

(a)
Nephrin
Podocin
Synaptopodin
Sham
Cast
(b)
mRNA expression level
of podocyte markers
ΔΔCt vs sYaa / Actb
14
12
10
8
6
4
2
0
**
*
**
Nphs1
Nphs2
Synpo
sYaa
cYaa
(c)
uACR
µg/mg
1000
800
600
400
200
0
**
3 months
4 months
5 months
6 months
sYaa
cYaa
(d)
Serum BUN
mg/dL
80
60
40
20
sYaa
cYaa
(e)
Serum Cre
mg/dL
10
8
6
4
2
0
sYaa
cYaa

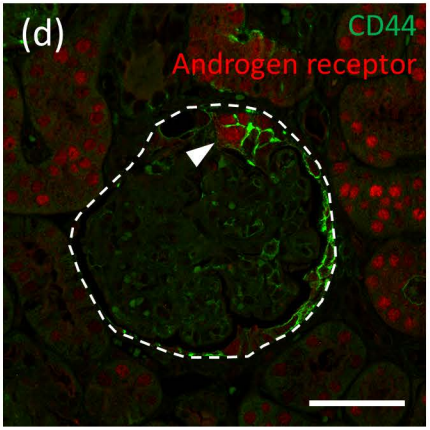

(a)
Outer medulla
Cortex
Glomerulus
Sham
Cast
OS
IS
OS
IS

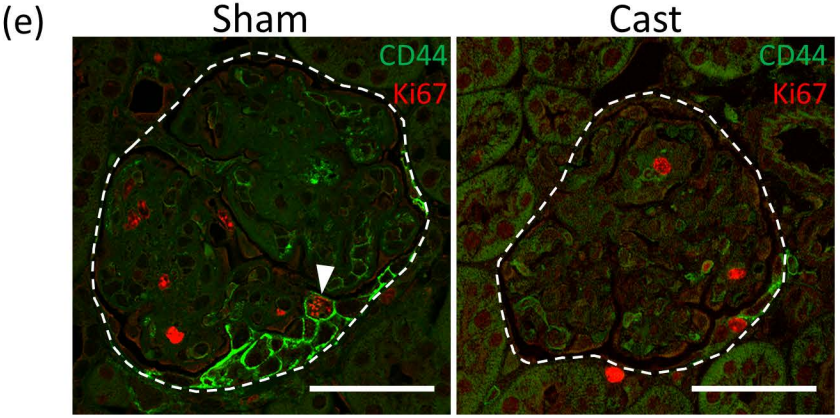

(b)
mRNA expression level of Ar
ΔΔCt vs sYaa / Actb
1.5
1
0.5
sYaa
cYaa

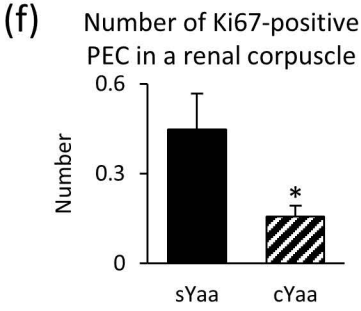

(a)
Uninjured
Injured
Sham
Cast

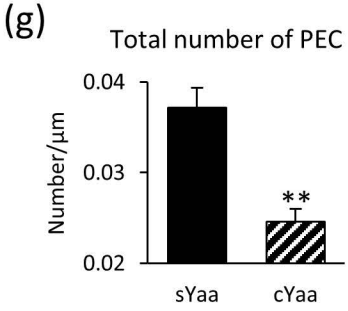

(b)
CD44-positive PEC number
Number/µm
0.008
0.004
0
*
sYaa
cYaa


(c)
CD44-positive renal corpuscle ratio
%
60
40
20
0
*
sYaa
cYaa


(d)
CD44
Androgen receptor


(e)
Sham
Cast
CD44
Ki67
CD44
Ki67


(f)
Number of Ki67-positive PEC in a renal corpuscle
Number
0.6
0.3
0
*
sYaa
cYaa


(g)
Total number of PEC
Number/µm
0.04
0.03
0.02
**
sYaa
cYaa