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**Studies on molecular pathogenesis
of murine autoimmune glomerulonephritis
- an early sign to the progression of chronic kidney disease
indicated by injured podocytes -**

自己免疫性糸球体腎炎モデルマウスの分子病態に関する研究

- 慢性腎臓病の早期進行サインとしての足細胞傷害 -

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Abbreviations

Actb: actin beta

Actn4: actinin alpha 4

Aim2: absent in melanoma 2

Aqp1: aquaporin 1

Aqp2: aquaporin 2

B6: C57BL/6

B6.MRL: B6.MRL-(*D1Mit202-D1Mit403*)

BMP: Bone morphogenetic protein

BUB: blood–urine barrier

BUN: blood urea nitrogen

BXSB: BXSB/MpJ-*Yaa*⁺ (male and female)

BXSB-*Yaa*: BXSB/MpJ-*Yaa* (male)

C3: complement component 3

Ccl: chemokine (C-C motif) ligand

CD: collecting duct

Cd3e: cluster of differentiation 3 epsilon

Cd68: cluster of differentiation 68

Chr: chromosome

CKD: chronic kidney disease

Cre: creatinine

Cxcl2: chemokine (C-X-C motif) ligand 2

DAMPs: danger-associated molecular patterns

DIG: digoxigenin

dsDNA: double strand DNA

DT: distal tubule

EDTA: ethylenediaminetetraacetic acid

EMT: epithelial-to-mesenchymal transition

ESRD: end-stage renal disease

Fcεr1g: Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide

Fcγr: Fc gamma receptor

FP: foot process

GBM: glomerular basement membrane

GFR: glomerular filtration rate

GL: glomerular lesion

GN: glomerulonephritis

HE: hematoxylin-eosin

HMGB1: high-mobility group box 1

IC: immune-complex

Ifi: interferon activated gene

Ifnb1: interferon, beta 1

Ifnγ: interferon, gamma

IgG: immunoglobulin G

Il10: interleukin 10

Il1b: interleukin ,1 beta

IL-1F6: interleukin-1 family, member 6

Il1rn: interleukin-1 receptor antagonist

Itln1: Intelectin-1

Lbw1-7: lupus NZB x NZW 1

LED: luminal epithelial decuduation

Lprm1-5: lymphoproliferation modifier 1-5

LPS: lipopolysaccharide

Mag: MRL autoimmune glomerulonephritis

miRNA: micro RNA

MMP: matrix metalloproteinase

Mndal: myeloid nuclear differentiation antigen like

mRNA: messenger ribonucleic acid

Nba1-3: New Zealand Black autoimmunity 1-3

Nhlh1: nescient helix loop helix 1

Nphs1: Nphrosis 1 (known as nephrin)

Nphs2: Nphrosis 2 (known as podocin)

NZB/WF1: (NZB X NZW) F1

PAMPs: pathogen-associated molecular patterns

PAS-H: periodic acid Schiff-hematoxylin

PAM-H: periodic acid methenamine silver-hematoxylin

PB: phosphate buffer

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFA: paraformaldehyde

PT: proximal tubule

Ptprc: Protein tyrosine phosphatase, receptor type, C

Pyhin1: pyrin and HIN domain family, member 1

RT-PCR: reverse transcription polymerase chain reaction

Sbw1,2: splenomegaly-NZB x NZW 1, 2

SD: slit diaphragm

SDS: sodium lauryl sulfate

Serpinb7: serpin peptidase inhibitor, clade B

Slamf9: SLAM family member 9

Slc12a1: solute carrier family 12, member 1

SLE: systemic lupus erythematosus

Sle1-6: systemic lupus erythematosus susceptibility 1-6

SM: Sternheimer-Malbin

Synpo: Synaptopodin

TEM: transmission electron microscope

Tgfb: transforming growth factor beta

TRITC: tetramethylrhodamine

TIL: tubulointerstitial lesion

TLR: Toll-like receptor

Tnfa: tumor necrosis factor

uACR: urinary albumin creatinine ratio

Vwf: von Willebrand factor

Wt1: Wilms tumor 1

Yaa: Y-linked autoimmune acceleration

Indexes

Preface	1
Chapter 1 Renal pathology and urinary cellular patterns in autoimmune glomerulonephritis.....	5
Introduction	6
Materials and Methods	9
Results.....	16
Discussion.....	20
Summary	27
Tables and Figures	29
Chapter 2 Podocyte injuries in autoimmune glomerulonephritis	39
Introduction	40
Materials and Methods	43
Results.....	50
Discussion.....	55
Summary	61
Tables and Figures	62
Chapter 3 Genetic factors contributing to autoimmune glomerulonephritis in BXSB/MpJ-Yaa mice.....	74
Introduction	75
Materials and Methods	78
Results.....	82

Discussion.....	87
Summary	93
Tables and Figures	95
Chapter 4 Toll-like receptor contribution to podocyte injury in autoimmune glomerulonephritis - Can TLR8 be a novel biomarker for podocyte injury? -	108
Introduction	109
Materials and Methods	112
Results.....	117
Discussion.....	122
Summary	127
Tables and Figures	129
Conclusion	140
References.....	144
Acknowledgements	164
Conclusion in Japanese.....	165

Preface

Recently, the rising number of elderly individuals in national populations has caused an increase in age-related diseases such as chronic kidney disease (CKD) (Lysaght, 2002). CKD is defined as a progressive loss of renal function, determined by a decline in glomerular filtration rate (GFR), which is associated with irreversible pathological changes in kidney (Smyth et al., 2014). Patients with progressive CKD have an increased risk of cardiovascular diseases, hospitalization, and death (Sarnak et al., 2003; Nakai et al., 2006; Tonelli et al., 2006). In addition, patients with CKD show a progressive loss of renal function, and eventually develop end-stage renal disease (ESRD) requiring renal replacement therapies including chronic dialysis and renal transplantation (Smyth et al., 2014). The worldwide prevalence of patients with ESRD is steadily increasing, raising one of the most serious public health problems. In veterinary medicine, the companion animals with CKD are also increasing because of their aging, especially approximately 30% of cats over 15 years of age show CKD sign (Bartlett et al., 2010). Therefore, understanding the molecular pathophysiology of CKD is critically important for the development of novel diagnostic and therapeutic methods to improve the morbidity and mortality of companion animals as well as human.

Common etiologies for CKD include diabetes, hypertension, polycystic kidney disease, chronic pyelonephritis, and chronic glomerulonephritis (GN) (Lysaght, 2002; Smyth et al., 2014). Especially, chronic GN is one of the major CKDs that is primarily caused by certain infections, drugs, and systemic disorders such as systemic lupus

erythematosus (SLE) (Kriz and LeHir, 2005; Nakai et al., 2006), and has annually led more than 20% of CKD patients in Japan to dialysis (Uchida, 2011). SLE is an autoimmune disease characterized by autoantibody production and immune-complex (IC) deposition that result in multiorgan tissue inflammation and damage (Ahearn et al., 2012). In kidney, IC accumulation in glomerulus causes SLE-related autoimmune GN, which is also known as lupus nephritis. More than half of patients with SLE develop lupus nephritis, and it is one of the most common and severe complications of SLE because it directly affects the prognosis of SLE patients (Lech and Anders, 2013).

The most suitable strategy for CKD control would be the establishment of a noninvasive diagnostic method as well as novel therapeutic methods because kidney is a nonregenerative organ. Although the levels of proteinuria, blood urea nitrogen (BUN), and serum creatinine (Cre) had been generally used for the diagnostic markers of CKD progression in human and animals, these have limitations as biomarkers for CKD in respect of sensitivity and specificity (Finco and Duncan, 1976; Fassett et al., 2011; Smyth et al., 2014). Thus, recent studies have been focusing on the identification of more promising biomarkers for CKD progression. Briefly, Sato et al. demonstrated that urinary levels of podocyte specific mRNAs such as *Nphs1* and *Nphs2* mark progression of glomerular disease (Sato et al., 2009). Ichimura et al. showed that injured proximal tubules (PTs) drastically increased the expression of Kidney injury molecule 1 (KIM-1), which is a transmembrane protein with uncertain function, and that urinary KIM-1 would be predictor of tubulointerstitial lesions (TILs) (Ichimura et al., 1998; Ichimura et

al., 2004). Furthermore, Yanagita et al. elucidated the upregulation of uterine sensitization-associated gene-1 (USAG1), which is one of the Bone morphogenetic protein (BMP) antagonists abundantly expressed in distal tubules (DTs), as a biomarker for renal fibrosis (Yanagita et al., 2006; Tanaka et al., 2010). Interestingly, the USAG1 antibody was also suggested to be a novel therapeutic drug for renal disease (Yanagita, 2006; Tanaka et al., 2008). These studies strongly suggest that the identification of novel biomarkers which correctly reflect the renal pathological condition can lead the development of effective diagnostic and therapeutic methods for CKD.

In this thesis, to identify novel diagnostic and therapeutic targets, the author investigated the molecular pathogenesis of CKD by using murine models, mainly BXSB/MpJ-*Yaa* (BXSB-*Yaa*) mouse, especially focusing on chronic GN caused by autoimmune disease. In first Chapter, the author investigated the renal histopathology and urinary cellular patterns of BXSB-*Yaa* mice and found the urinary mRNAs indicating the injuries of podocytes and renal tubules. In second Chapter, the author especially focused on the glomerular damage as an early pathological event of CKD and emphasized the clinical importance of podocyte injury in chronic GN by analyzing BXSB-*Yaa* mice as well as another murine model. Third Chapter explored the genomic factors affecting the chronic GN pathogenesis including podocyte injury in BXSB-*Yaa* mice. Based on the data of third Chapter, fourth Chapter focused on Toll-like receptor (TLR) family and identified TLR8 as the aggravating factor of podocyte injury in chronic GN. In conclusion, podocyte injury could be an early sign of CKD progression, and the author found TLR8 as a novel diagnostic and therapeutic target of CKD.

Contents of this research were published in the following articles.

1. Kimura, J., Ichii, O., Otsuka, S., Kanazawa, T., Namiki, Y., Hashimoto, Y., Kon, Y. 2011. Quantitative and qualitative urinary cellular patterns in murine glomerulonephritis. *PLoS One*, **6**: e16472.
2. Kimura, J., Ichii, O., Otsuka, S., Sasaki, H., Hashimoto, Y., Kon, Y. 2013. Close relations between podocyte injuries and membranous proliferative glomerulonephritis in autoimmune murine models. *Am. J. Nephrol.*, **38**: 27-38.
3. Kimura, J., Ichii, O., Nakamura, T., Horino, T., Otsuka, S., Kon, Y. 2014. BXSB-type genome causes murine autoimmune glomerulonephritis; pathological correlation between telomeric region of chromosome 1 and *Yaa*. *Genes Immun.*, **15**: 182-189.
4. Kimura, J., Ichii, O., Miyazono, K., Nakamura, T., Horino, T., Otsuka-Kanazawa, S., Kon, Y. 2014. Overexpression of Toll-like receptor 8 correlates with the progression of podocyte injury in murine autoimmune glomerulonephritis. *Sci. Rep.*, **4**: 7290.

Chapter 1

Renal pathology and urinary cellular patterns

in autoimmune glomerulonephritis

Introduction

Lack of renal disease control is an inevitable problem in clinical medicine because the kidney is a nonregenerative organ. The global population of patients with ESRD has recently been increasing (Lysaght, 2002). Several studies have indicated that CKD is strongly associated with ESRD progression (Sarnak et al., 2003; Nakai et al., 2006; Tonelli et al., 2006), and the rapid increase in the number of patients with CKD has become a worldwide public health problem.

Chronic GN, which begins with glomerular lesions (GLs), is one of the major CKDs that is primarily caused by certain infections, drugs, and systemic disorders such as SLE (Kriz and LeHir, 2005; Nakai et al., 2006). In the early stages of chronic GN, glomerular IC depositions cause GLs, such as blood-urine barrier (BUB) disruption, which lead to ultrafiltration of plasma proteins or protein-associated factors (Kriz and LeHir, 2005). Chronic GLs are thought to be converted into TILs by ultrafiltration of several proteins and inflammatory cytokines or local hypoxia (Kriz and LeHir, 2005). Eventually, chronic GN progresses to ESRD through a final common pathway in which progressive interstitial fibrosis is associated with tubular atrophy and peritubular capillary loss (Kriz and LeHir, 2005).

Recent studies have attempted to discover new biomarkers for the development of a new diagnostic strategy for CKD control, in which tissue injury markers such as inflammatory cytokines, chemokines, or slit diaphragm (SD) molecules are noted (Kovesdy and Kalantar-Zadeh, 2009; Skoberne et al., 2009). The most suitable strategy for CKD control is the establishment of a noninvasive diagnostic method that can detect

pathological conditions at the early stages; however, no protocol currently satisfies this requirement. Further, the pathological features of CKD differ among animals, especially dogs mainly showed GL rather than TIL, and reverse is true in cats (Ichii et al., 2011). These differences also complicate the clinicopathological diagnosis and research of CKD in veterinary medicine.

It has recently been suggested that loss of nephron constituent cells results in deterioration of renal function. The pathological correlations between podocyte loss and GLs are suggested in human and animal models (Kim et al., 2001; White et al., 2002; Dalla Vestra et al., 2003; Matsusaka et al., 2005; Wharram et al., 2005; Wiggins, 2007). Hara et al. detected podocytes and their fragments in the urine of patients with several glomerular diseases (Hara et al., 1998; Nakamura et al., 2000; Hara et al., 2001; Hara et al., 2005; Hara et al., 2007). Moreover, Sato et al. demonstrated that podocyte mRNAs were detected in the urine of rats administered with drugs (Sato et al., 2009). On the other hand, Ichii et al. demonstrated a correlation between distal tubular epithelial damage and TILs in murine chronic GN models, showing luminal epithelial decidualization (LED; the term “decidualization” means the dropping of epithelia into lumen) (Ichii et al., 2010b). These reports suggest that damaged renal parenchymal cells are dropped into the urine as renal disease progresses. However, no study has reported on the quantitative and qualitative details of urinary cells derived from spontaneous animal models.

As the model for SLE-related autoimmune GN, MRL/MpJ-*lpr*, NZB/WF1, and BXSB-*Yaa* mice are widely used and these strains develop systemic autoimmune diseases such as increase of serum autoantibodies and vasculitis as well as chronic GN.

Especially, BXSB-*Yaa* mice carry the mutant gene located on the chromosome Y (Chr.Y), designated as *Yaa* (Y-linked autoimmune acceleration), and male mice show more severe GN than females (Izui et al., 1988). Andrews et al. demonstrated the deposits of ICs such as IgG and C3 in glomeruli from BXSB-*Yaa* mouse kidneys (Andrews et al., 1978), indicating that BXSB-*Yaa* mice can be used as a representative model of human lupus nephritis and animal autoimmune GN; however, little has been known about the detailed molecular pathogenesis in this model mouse strain.

In Chapter 1, the author investigated the chronic GN pathology and urinary cytology in BXSB-*Yaa* mice. The author found BXSB-*Yaa* mice clearly showed membranous proliferative GN. Further, the author's results indicate that injured renal parenchymal cells, including podocyte, DTs, and collecting ducts (CDs), fall into the urine as chronic GN progresses.

Materials and Methods

Ethical statement

This study was carried out as part of a research project entitled “Analysis of the MRL/MpJ mouse phenotypes.” This project includes the analysis of disease models such as autoimmune disease, CKD, and urogenital organ disease to develop new diagnosis methods. This research was approved by the Institutional Animal Care and Use Committee, which is convened at the Graduate School of Veterinary Medicine, Hokkaido University (approval no. 09-0129). The investigators adhered to *The Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine* (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International).

Animals and Sample Preparations

Male BXSB-*Yaa* mice ($n = 12$) and C57BL/6 (B6) mice ($n = 5$) at ages of 3–6 months were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and were maintained under specific pathogen-free conditions. The mice were subjected to deep anesthesia (pentobarbital sodium 60 mg/kg administered intraperitoneally), and urine was collected by bladder puncture to avoid contamination by lower urinary tract cells. Bladder urine was collected, and the animals were euthanized by exsanguination from the carotid arteries; subsequently, humoral and organ samples were collected.

Serological and Urinary Analysis

For renal function evaluation, serum BUN and Cre levels in all animals were determined using BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. Urinary albumin was detected by sodium dodecyl sulphate (SDS) -polyacrylamide gel electrophoresis. Briefly, 3 μ L of urine and 1 μ g of bovine serum albumin were heated at 65°C for 5 min in 2 $\times$ SDS sample buffer [100 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% bromophenol blue, 12% 2-mercaptoethanol] and loaded on 12% polyacrylamide gel (e-PAGEL; ATTO Corporation, Tokyo, Japan). Electrophoresis was performed at 150 V in Tris-glycine buffer [25 mM Tris (pH 8.3), 192 mM glycine] containing 0.1% SDS for 2 h. Gels were stained with Quick CBB PLUS (Wako Pure Chemical Industries).

Cytology of Urinary Cells

Two staining methods were performed to observe and identify urinary cell morphology. First, 100 μ L of urine was immediately centrifuged at 200 $\times$ g for 5 min. Ninety microliters of supernatant urine was then removed, and 200 μ L of 4% paraformaldehyde (PFA) was added. Urinary cells fixed by 4% PFA were centrifuged at 200 $\times$ g for 5 min, and 190 μ L of supernatant was removed. The remaining 20 μ L of urine sediments was placed on a glass slide, dried, and stained with hematoxylin-eosin (HE). Second, 100 μ L of freshly obtained urine was

centrifuged at $200\times g$ for 5 min, and 90 μL of supernatant was removed. The remaining 10 μL of urine sediments was stained with Sternheimer-Malbin (SM) stain. After staining, the urine sediments were placed on a glass slide and a coverslip was gently applied. The number of cells per field was counted and averaged in at least 5 fields of the HE-stained samples, and urinary cells were characterized using the 2 staining techniques mentioned above.

Reverse Transcription and Polymerase Chain Reaction

For mRNA expression examination, total RNA from urine was purified using the SV Total RNA Isolation System (Promega, Madison, WI, USA). DNase-treated total RNAs were synthesized to cDNAs by a reverse transcription (RT) reaction by using the ReverTra Ace reverse transcriptase enzyme (Toyobo, Osaka, Japan) and oligo dT primers (Invitrogen, Carlsbad, CA, USA). Each cDNA, adjusted to 1.0 $\mu\text{g/mL}$, was used for the polymerase chain reaction (PCR) reaction with Ex Taq (Takara Bio, Tokyo, Japan) and the appropriate primer pairs as shown in Table 1-1. Nested PCR reactions were performed using 1/20 volume of the first PCR products with the primer pairs designed at the inside of the sequence between the first primer pairs. The amplified samples were electrophoresed with 1% agarose gel containing ethidium bromide and finally photographed under an ultraviolet lamp.

Histological Analysis

The kidney samples for histology were fixed by 4% PFA in 0.1 M phosphate buffer (PB; pH 7.4) at 4°C overnight. Paraffin sections (2 µm thick) were then prepared and stained with periodic acid Schiff-haematoxylin (PAS-H). To assess the severity of GN, cell number in one glomerulus was counted.

Immunohistochemical and Immunofluorescence Analyses

Immunostaining for Wilms tumor 1 (WT1) and interleukin-1 family, member 6 (IL-1F6) was performed according to the following procedure. The paraffin sections were deparaffinized and incubated in citrate buffer (pH 6.0) for 20 min at 105°C for antigen retrieval. After cooling, slides were soaked in methanol containing 3% H₂O₂ for 15 min at room temperature to remove internal peroxidase. After being washed, sections were blocked by 10% normal goat serum (for WT1) or 10% normal donkey serum (for IL-1F6) for 60 min at room temperature and incubated with rabbit polyclonal IgG antibodies for WT1 (1:1000; Calbiochem/EMD, Darmstadt, Germany) or goat polyclonal antibodies for IL-1F6 (1:400; R&D Systems, Minneapolis, MN, USA) overnight at 4°C. After washing 3 times in phosphate-buffered saline (PBS), sections were incubated with biotin-conjugated goat anti-rabbit IgG antibodies (SABPO® kit, Nichirei, Tokyo, Japan) for WT1 or donkey anti-goat IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for IL-1F6 for 30 min at room temperature, washed, and incubated with streptavidin-biotin complex (SABPO® kit) for 30 min. The sections were then incubated with 3,3'-diaminobenzidine tetrahydrochloride-H₂O₂

solution. Finally, the sections were slightly counterstained with hematoxylin. Immunostaining for C3 was performed according to the following procedure. In brief, deparaffinized 2- μ m-thick paraffin sections were incubated in 0.1% pepsin/0.2 M HCl for 5 min at 37°C for antigen retrieval. After being washed, sections were pretreated with 0.25% casein/0.01 M PBS for 60 min at room temperature and incubated with goat polyclonal IgG antiserum for C3 (1:800; MP Biomedicals, Solon, OH, USA) overnight at 4°C. After washing 3 times with PBS, the sections were incubated with tetramethylrhodamine (TRITC) -labeled rabbit anti-goat IgG antibodies (1:200; Zymed/Invitrogen) for 30 min at room temperature and washed again. For nuclear staining, sections were incubated with Hoechst 33342 (1:200; Wako Pure Chemical Industries) for 30 min. Finally, the sections were examined under a confocal laser scanning microscope (LSM700; Zeiss, Thornwood, NY, USA).

In Situ Hybridization Analysis

cRNA probes for C3 were synthesized in the presence of digoxigenin (DIG) -labeled UTP by using a DIG RNA Labeling Kit in accordance with the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). The primer pairs for making each probe are shown in Table 1-1. Deparaffinized, proteinase K-digested sections were incubated with a prehybridization solution and then with hybridization buffer containing 50% formamide, 10 mM Tris-HCl (pH 7.6), 200 mg/mL RNA, 1 $\times$ Denhardt's solution (0.02% bovine serum albumin, 0.02%

polyvinylpyrrolidone, and 0.02% Ficoll PM400; Amersham Pharmacia, Uppsala, Sweden), 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA (pH 8.0), and sense or antisense RNA probe (final concentration, 0.2 µg/mL) for 24 h at 58°C. The sections were then incubated with 0.2% polyclonal sheep anti-DIG Fab fragments conjugated to alkaline phosphatase (1:400; Nucleic Acid Detection Kit, Roche Diagnostics) for 24 h at room temperature. The signal was detected by incubating the sections with a color substrate solution (Roche Diagnostics) containing nitroblue tetrazolium/X-phosphate in a solution composed of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl₂ in a dark room overnight at room temperature.

PCR Array Analysis

To identify the factors that exacerbate the disease, PCR array analysis was performed and the relative expression of 84 inflammatory cytokines, chemokines, and their receptors were examined. Total RNAs were purified from the kidneys of 3-month-old male B6 and BXSB-*Yaa* mice, which were stored in RNeasy lysis solution (Qiagen, Crawley, UK), using TRIzol reagent (Invitrogen). After purification of the total RNAs with an RNeasy Micro Kit (Qiagen, Germantown, USA), the RNAs were treated with Turbo DNase (Ambion) for DNA digestion and then repurified. Five micrograms of total RNA was synthesized to cDNA by using the RT² PCR Array First Strand Kit (SuperArray, Frederick, MD, USA). PCR array analysis was performed using 10

μL of cDNA solution, Mouse Inflammatory Response and Autoimmunity PCR RT² ProfilerTM PCR Array (SuperArray), and a MX 3000 thermal cycler (Stratagene, La Jolla, CA, USA).

Statistical analysis

Results were expressed as the mean $\pm$ standard error and statistically analyzed using a nonparametric Mann–Whitney *U* test ($P < 0.05$). The correlation between 2 parameters was analyzed using Spearman's correlation test ($P < 0.05$).

Results

Cytological Observation of Urinary Cells

To assess the number and morphology of urinary cells, urine sediment smears were examined using HE and SM stains. The urinary cell numbers in BXSB-*Yaa* mice were significantly higher than those in B6 mice (Figs. 1-1a-c). In the urine from BXSB-*Yaa* mice, several kinds of urinary cells were observed: small round cells (Figs. 1-1d and g), homogeneous and amorphous cell components (Figs. 1-1e and h), and columnar cells with basophilic cytoplasm (Figs. 1-1f and i). Among these cell types, small round cells showed an aggregation pattern, and this was also observed in control mice.

Correlations between Urinary Cell Number and Renal Pathology

BXSB-*Yaa* mice showed membranous proliferative GN and TILs, namely, the expansion of mesangial matrix, proliferation of mesangial cells, dilated tubules by urinary casts, and perivascular cell infiltration (Figs. 1-2a and b). Glomerular cell number was used as an index of membranous proliferative GN and was comparable to urinary cell number, suggesting that the number of urinary cells significantly increased with glomerular damage score (Fig. 1-2c). In addition, urinary cell number significantly correlated with urinary albumin; however, no correlation was detected with BUN and Cre (Figs. 1-2d-f).

Identification of Urinary Cell Types

For urinary cell identification, urinary mRNA detection was performed. Markers of renal parenchymal cells, including podocytes (*Wt1*, *Nphs1*, *Nphs2*, *Actn4*); mesangial cells (*Serp1b7*); vascular endothelium (*Vwf*); proximal tubular epithelial cells (*Aqp1*); distal tubular epithelial cells (*Slc12a1*); and CD epithelial cells (*Aqp2*), including T-cells (*CD3e*), B-cells (*Ptprc*), and macrophages (*CD68*), were used. Table 1-2 shows that the expression of *Wt1*, *Nphs1*, *Actn4*, *Slc12a1*, and *Aqp2* was detected in the urine from BXSB-*Yaa* mice at a high rate. In addition to markers of renal epithelium, *Vwf* was detected in a few urine samples from BXSB-*Yaa* mice. Although perivascular infiltration of inflammatory cells was observed in BXSB-*Yaa* mouse kidneys (Fig. 1-2a), inflammatory cell markers were not detected in BXSB-*Yaa* mouse urine.

Histological Evidence of Renal Parenchymal Cell Loss

To confirm the urinary decidualization of podocytes, DT epithelium, and CD epithelium, immunohistochemical analysis of WT1 (podocyte marker) and IL-1F6 (marker of damaged DT and CD) was performed. WT1 was localized in podocyte nuclei in the glomerulus, and the number of glomerular WT1-positive cells in BXSB-*Yaa* mouse kidneys was significantly lower than that in B6 mouse kidneys (Figs. 1-3a and b). Additionally, there was a significant inverse correlation between urinary cell number and the number of glomerular WT1-positive cells in BXSB-*Yaa* mouse kidneys (Fig. 1-3c). IL-1F6 was localized in epithelial cells from DTs and CDs showing tubular dilations or epithelial decidualization (Fig. 1-3d

and e). The number of urinary cells was significantly correlated with the number of IL-1F6–positive tubules (Fig. 1-3f).

Selection of Inflammatory Markers for Detection of Urinary Cells

For the development of inflammatory urine cell markers derived from the kidney, a PCR array targeting 84 inflammatory cytokines and chemokines and their receptors was analyzed using kidneys from male B6 and BXSB-*Yaa* mice. Genes of the chemokine (C-X-C motif) ligand (*Cxcl*) and chemokine (C-C motif) ligand (*Ccl*) and their receptors (*Ccr* and *Cxcr*) were upregulated in BXSB-*Yaa* mice. The author found that interleukin-10 (*Il10*), chemokine (C-X-C motif) ligand 2 (*Cxcl2*), complement component 3 (*C3*), and interleukin-1 receptor antagonist (*Il1rn*) showed particularly high expression levels in BXSB-*Yaa* mice (Table 1-3). Among 4 highly upregulated mRNAs (*Il10*, *Cxcl2*, *C3*, and *Il1rn*), the expression of *C3* was detected in the urine from BXSB-*Yaa* mice at a high rate (Table 1-4). All results detecting pathological parameters and urinary cell patterns in the urine from BXSB-*Yaa* and B6 mice were summarized in Table 1-5.

Localization of C3-Producing Cells in the Kidney

Immunofluorescence analysis of the kidney showed that C3 protein was localized in the glomerulus, tubular epithelial cells, and vascular endothelium (Figs. 1-4a and b). To examine whether C3 protein was synthesized or deposited, *in situ* hybridization of *C3* mRNA was also performed. Figs. 1-4c and d shows that

positive reactions were detected in the epithelia of cortical renal tubules. Several positive tubules tended to localize in the same cortical regions. Furthermore, the colocalization of *C3* mRNA and its protein was confirmed by the serial sections (Figs. 1-4e and f).

Discussion

It has been clinically recognized that cells derived from the kidney, such as cellular casts, appear in the urine of patients with renal disease, and these cell components indicate the pathological conditions of the kidney. In the present study, the author elucidated a significant positive correlation between urinary cell number and indices of renal pathology, such as glomerular damage score and urinary albumin levels, by using spontaneous animal models.

In humans, a prominent histological feature of chronic GN is cellular hyperplasia in the glomerulus as well as glomerular inflammatory diseases in experimental conditions caused by both proliferation of mesangial cells and infiltration of leukocytes (Schocklmann et al., 1999). However, the present study suggests that urinary decidual cells were derived from podocytes, but not mesangial cells or inflammatory cells. Recent studies have indicated that infiltrated inflammatory cells produce various reactive oxygen species, pro-inflammatory cytokines, matrix metalloproteinases (MMPs), and transforming growth factors (TGFs), which modulate local response and increase inflammation (Galkina and Ley, 2006). In particular, factors such as TGF- β and MMPs were reported to play an important role in the loss of cell adhesion molecules through the E-cadherin and integrin families (Catania et al., 2007; Peinado et al., 2007). These findings suggest that cellular hyperplasia in the glomerulus and subsequent inflammatory reactions might contribute to the detachment of podocytes but not of other cells.

Common pathological characteristics between human and animal models in

the case of glomerular diseases, such as chronic GN and diabetic nephropathy, include urinary leakage of proteins, such as albumin (albuminuria), caused by disruption of the BUB (Ryan and Karnovsky, 1975; Reiser et al., 2002). In the present study, urinary cell number correlated with urinary albumin but not with BUN and Cre levels, indicating that urinary albumin correlated with urine cell number rather than serological values showing renal function. Although measurement of BUN and Cre levels is the most widely used method for renal diagnosis in the clinical field, neither BUN nor Cre can be used as a precise indicator of renal function because of a lack of sensitivity and specificity (Finco and Duncan, 1976). The present and previous studies suggest that evaluation of urinary cell number is a sensitive and specific method for diagnosing renal pathology, especially BUB disruption. Furthermore, cell number in the urine also seemed to be a more specific marker than urinary albumin because the latter could be elevated by contamination of the urine with secretions from various glands in the lower urinary tract, such as sex accessory glands.

Identification of urinary cells could lead to a detailed understanding of renal pathological conditions. From microscopic observation, the urinary cell numbers in BXSB-*Yaa* mice were significantly higher than those in B6 mice. However, excessive decidualization of bladder epithelial cells increases the number of urinary cells. Bladder epithelial cells are characterized as transitional epithelium. Therefore, these cells dropping into the urine might show an aggregation pattern. The author considers the small round cells to be bladder epithelial cells because

this cell type exhibited an aggregation pattern and was also observed in control mice. In addition, these cells were observed less frequently than the other cell types. From these findings, the author also inferred that the homogeneous and amorphous cell components or the columnar cells with basophilic cytoplasm might be derived from the kidney. To identify the details of these cell types, the author performed urine PCR analysis for cell-specific genes.

In urine PCR analysis, *Wt1*, *Nphs1*, *Actn4*, *Slc12a1*, and *Aqp2* mRNA were detected in BXS^B-Yaa mouse urine at a high rate. Furthermore, these mRNAs tended to be detected in the urine from mice with more severe renal conditions. On the other hand, the expression of *Vwf* was also detected in a few BXS^B-Yaa mouse urine samples. Although endothelial cells from glomerular capillaries might drop into the urine because of glomerular epithelium (podocyte) damage, the expression of podocyte marker was not detected in *Vwf*-positive urine. In addition, the indices of renal damage from their kidney showed low levels. Therefore, the author considered the bladder urine to be contaminated by the endothelial cells as a result of vessel damage by bladder puncture. From these findings, it was strongly suggested that podocytes and DT and CD epithelia fall into urine as the renal pathological condition of GN progresses.

Podocytes, highly differentiated cells lining the outside of the glomerular capillaries, are composed of a body with extending primary processes that further branch into foot processes (FPs) separated by a SD. Recently, it has been suggested that the effacement of podocytes started from disruption of FPs and/or

the SD is associated with the development of proteinuric renal diseases, and their mRNAs are detected in the urine of renal disease patients (Szeto et al., 2005; Wang et al., 2007; Wang et al., 2008). In the present study, molecular and morphological analyses showed that the loss of podocytes (podocytopenia) (Lemley et al., 2002) or urinary excretion of podocytes (Hara et al., 1998; Nakamura et al., 2000; Hara et al., 2001; Hara et al., 2005; Hara et al., 2007) was associated with progression of renal pathology in GN. Interestingly, among various markers for podocytes, *Nphs1* was detected at a high rate. According to Nakatsue et al., urinary *Nphs1* protein, but not *Nphs2* protein, was detected in the urine at the early stages of rabbit Heyman nephritis (Nakatsue et al., 2005), indicating that urinary *Nphs1* protein is a useful tool for early diagnosis in the case of Heyman nephritis, although other podocyte markers such as *Wt1*, *Nphs2*, and *Actn4* are suggested to be helpful for the diagnosis of various kidney diseases (Szeto et al., 2005; Wang et al., 2007; Wang et al., 2008). These data therefore suggest that injured podocytes have different patterns of protein expression in each kidney disease, indicating that evaluation of podocyte decidualization using appropriate podocyte markers in each renal disease is essential for accurate and early diagnosis.

Ichii et al. demonstrated the correlation between TILs and LED in the distal segment (Ichii et al., 2010b), indicating that epithelia from damaged DT and CD expressing IL-1F6 fall into the urine. In the present study, DT and CD markers, but not PT markers, were detected in the urine from BXS^B-*Yaa* mice. IL-1F6 is known

as a member of the IL-1 gene family, and its product has been identified as a member of the IL-1 cytokine family, which regulates inflammation by mediating the expression of various cytokines, chemokines, nitric oxide synthases, and MMPs (Barksby et al., 2007). In the kidney, IL-1F6 is associated not only with cellular infiltrations but also with changes in epithelial morphology (Ichii et al., 2010b). In epithelial cells, the downregulation of epithelial markers and upregulation of mesenchymal markers are known as epithelial-to-mesenchymal transitions (EMTs), and the transitions of renal tubular epithelial cells have been shown to cause the progression of interstitial fibrosis (Barksby et al., 2007). Furthermore, it is suggested that PT epithelia that undergo EMT migrate to the tubulointerstitial space as transformed matrix-producing cells (Iwano et al., 2002). On the other hand, injured DT and CD epithelia are reported to fall into the tubular lumen but not into the tubulointerstitial space. From these findings, the author inferred that the EMT mechanism might differ between the proximal and distal segments; namely, injured PT cells undergoing EMT eventually migrate to the tubulointerstitial space, whereas injured DT and CD cells move to LED. These findings suggest that evaluation of urinary DT and CD epithelia leads to the prognosis of TILs.

For PCR array analysis, the upregulations of chemokines and their receptors were elucidated in BXSB-*Yaa* mouse kidneys. *Il10*, *Cxcl2*, *C3*, and *Il1rn* were highly upregulated in the BXSB-*Yaa* mouse kidney; interestingly, *C3* in particular was detected in BXSB-*Yaa* mouse urine. The complement system is the major

effector of the humoral arm of the immune system. C3, which plays a pivotal role in the complement cascade, is the most abundant complement protein in the circulation. The majority of C3 is synthesized in the liver, but numerous other tissue sources of complement have been discussed. In the kidney, various cell types are capable of producing C3 *in vitro* and *in vivo* (Ueki et al., 1987; Brooimans et al., 1991; Sacks et al., 1996). Furthermore, recent reports have indicated that C3 synthesized without liver may be a more important mediator of inflammation and immunological injury in the kidney than plasma C3 derived from the liver (Sheerin et al., 2008). The author's immunofluorescence and *in situ* hybridization analyses showed that damaged nephrons synthesized C3 mRNA and protein. Furthermore, C3 mRNA was detected in the urine from BXSB-*Yaa* mice at a high rate. These results suggest that damaged cortical tubular epithelia synthesizing C3 fall into the urine.

The present study showed that some BXSB-*Yaa* mouse nephrons showing local C3 synthesis tended to become TILs. Once the complement cascade is activated by the presence of C3, generation of a sublytic concentration of C5b-9 alters renal epithelial cell function, inducing morphological changes, upregulation of collagen gene expression, and production of inflammatory cytokines (David et al., 1997; Abe et al., 2004). The data further suggest that C3a decreases the expression of E-cadherin protein and increases the expression of both α -smooth muscle actin protein and collagen type I mRNA in tubular epithelial cells (Tang et al., 2009). These findings suggest that C3 plays an important role in the EMT. The

EMT of tubular epithelial cells may lead to subsequent TILs, including LEDs and interstitial fibrosis.

In conclusion, the author demonstrated that urinary cells reflect renal disease progression, such as podocyte effacement and DT/CD tubule damage, suggesting that a system for detecting urinary cells is a useful tool for the early, noninvasive diagnosis of several renal diseases. The evaluation of urinary cellular patterns might have some advantages to diagnose CKD in each animal species because their pathological features such as GL and TIL differed among species. Additional studies for the development of a detection system for urinary cells are necessary to further animal and human health.

Summary

The kidney is a nonregenerative organ composed of numerous functional nephrons and CDs. Glomerular and tubulointerstitial damages decrease the number of functional nephrons and cause anatomical and physiological alterations resulting in renal dysfunction. It has recently been reported that nephron constituent cells are dropped into the urine in several pathological conditions associated with renal functional deterioration. The author investigated the quantitative and qualitative urinary cellular patterns in a murine GN model and elucidated the correlation between cellular patterns and renal pathology.

Urinary cytology and renal histopathology were analyzed in BXSB-*Yaa* (chronic GN model) and B6 (control) mice. Urinary cytology revealed that the number of urinary cells in BXSB-*Yaa* mice changed according to the histometric score of GN and urinary albumin; however, no correlation was detected for the levels of BUN and Cre. The expression of specific markers for podocytes, DTs, and CDs was detected in BXSB-*Yaa* mouse urine. Cells immunopositive for WT1 (podocyte marker) and IL-1F6 (damaged DT and CD marker) in the kidney significantly decreased and increased in BXSB-*Yaa* versus B6 mice, respectively. In the PCR array analysis of inflammatory cytokines and chemokines, *Il10*, *Cxcl2*, *C3*, and *Il1rn* showed relatively higher expression in BXSB-*Yaa* mouse kidneys than in B6 mouse kidneys. In particular, the highest expression of *C3* mRNA was detected in the urine from BXSB-*Yaa* mice. Furthermore, *C3* protein and mRNA were localized in the epithelia of damaged nephrons.

These findings suggest that injured epithelial cells of the glomerulus, DT, and CD are dropped into the urine, and that these patterns are associated with renal pathology progression.

Tables and Figures

Table. 1-1. Summary of specific gene primers

Genes (accession)	Primer sequence (5'-3') F: forward, R: reverse	Product size (bp)	Primer sequence (5'-3') F: forward, R: reverse	Product size (bp)	Application	Specific expression cell
<i>Wt1</i> (NM_144783)	F: GCATGACCTGGAATCAGATG R: TCTCTCGCAGTCCTTGAAGTC	383	F: GGTATGAGAGTGAGAACCACACG R: AGATGCTGACCGGACAAGAG	137	Urine RT-PCR	Podocyte
<i>Nphs1</i> (NM_019459)	F: GATCCAGGTCTCCATCACTACC R: AAGGCCATGTCCTCATCTTC	432	F: AGGAGGATCGAATCAGGAATG R: GCGATATGACACCTCTTCCAG	161	Urine RT-PCR	Podocyte
<i>Nphs2</i> (NM_130456)	F: GACCAGAGGAAGGCATCAAG R: GTCATGTCATCTAAGGCAACC	496	F: AAGGTTGATCTCCGTCTCCAG R: TTCCATGCGGTAGTAGCAGAC	105	Urine RT-PCR	Podocyte
<i>Actn4</i> (NM_021895)	F: TCCAGGACATCTCTGTGGAAG R: AAGGCATGGTAGAAGCTGGAC	340	F: CCTTCAATGCACTCATCCAC R: TGTCCTCAGCATCCAACATC	147	Urine RT-PCR	Podocyte
<i>Vwf</i> (NM_011708)	F: ACAAGTGTCTGGCTGAAGGAG R: CACTGCATGGCGTTGATG	316	F: TGCTGTGACACATGTGAGGAG R: GCACATCCTCGATGTCAATG	160	Urine RT-PCR	Endothelium
<i>Serpinb7</i> (NM_027548)	F: GGCCTTCACCAAGACTGATAC R: CCAGAGGCAATTCCAGAGAG	390	F: ACCAATGCAGGTTCTTGAGC R: CTCCTATTGGTCCAGTCCATC	129	Urine RT-PCR	Mesangial cell
<i>Aqp1</i> (NM_007472)	F: GCATTGAGATCATTGGCACTC R: CATCCAGGTCATACTCCTCCAC	351	F: GCTGGCGATTGACTACACTG R: ACTGGTCCACACCTTCATGC	199	Urine RT-PCR	PT epithelium
<i>Slc12a1</i> (NM_183354)	F: CCACAAAGATTTGACCACTGC R: CACCAAGGCACAACATTTCTC	325	F: CAGAAGTGAAGCAGTCAAGG R: AGGAGGAAGGTTCTTGCTCAG	179	Urine RT-PCR	DT epithelium
<i>Aqp2</i> (NM_009699)	F: CCATGTCTCCTTCCTTCGAG R: GGAGCAGCCGGTGAAATAG	310	F: CGCCATCCTCCATGAGATTAC R: TCAGGAAGAGCTCCACAGTC	110	Urine RT-PCR	CD epithelium
<i>Cd3e</i> (NM_007648)	F: CCATCTCAGGAACCAAGTGTAGAG R: CATAGTCTGGGTTGGGAACAG	417	F: TGCCTCAGAAGCATGATAAGC R: TTGGCCTTCCTATTCTTGCTC	244	Urine RT-PCR	T-cell
<i>Ptpcr</i> (NM_011210)	F: GAGGTGTCTGATGGTGCAAG R: TCATCTGATTCAAGGCTCACTCTC	336	F: TGGAGGCTGAATACCAGAGAC R: TGCTCATCTCCAGTTCATGC	153	Urine RT-PCR	B-cell
<i>Cd68</i> (NM_009853)	F: TGGATTCAAACAGGACCTACATC R: CTGGTAGGTTGATTGTCGTCTG	388	F: CTACATGGCGGTGGAATACA R: CAATGATGAGAGGCAGCAAG	263	Urine RT-PCR	Macrophage
<i>IL10</i> (NM_010548)	F: TGCTATGCTGCCTGCTCTTAC R: CGGTTAGCAGTATGTTGTCCAG	186	-	-	Urine RT-PCR	-
<i>Cxcl2</i> (NM_009140)	F: TCAAGAACATCCAGAGCTTGAG R: TCCAGGTCAGTTAGCCTTGC	170	-	-	Urine RT-PCR	-
<i>C3</i> (NM_009778)	F: TGCAGACTGAACAGAGAGCAG R: CTCACAACACTTCCGAAGACC	134	-	-	Urine RT-PCR	-
<i>C3</i> (NM_009778)	F: CACTGGACCCAGAGAAGCTC R: GGATGTGGCCTCTACGTTGT	866	-	-	<i>In situ</i> hybridization	-
<i>Il1rn</i> (NM_031176)	F: TTGTGCCAAGTCTGGAGATG R: TCTAGTGTGTGCAGAGGAACC	174	-	-	Urine RT-PCR	-

Primer sequences given on the left column are for the first PCR, and those on the right column are for the second PCR. PT: proximal tubule, DT: distal tubule, CD: collecting duct)

Table 1-2. Expression of various nephron constituent cell markers in the urine from 12 BXSB-*Yaa* mice

Marker/Case		1	2	3	4	5	6	7	8	9	10	11	12	Ratio (%)
Podocyte	<i>Wt1</i>	+	-	-	-	-	-	-	-	-	-	+	+	25.0
	<i>Nphs1</i>	+	-	-	+	-	-	+	-	++	-	+	-	41.7
	<i>Nphs2</i>	-	-	-	-	-	-	-	-	-	-	-	-	0
	<i>Actn4</i>	+	-	-	-	-	-	-	-	+	-	-	+	25.0
Endothelium	<i>Vwf</i>	-	-	-	-	-	+	-	+	-	+	-	-	25.0
Mesangial cells	<i>Serpina1</i>	-	-	-	-	-	-	-	-	-	-	-	-	0
PT epithelium	<i>Aqp1</i>	-	-	-	-	-	-	-	-	-	-	-	-	0
DT epithelium	<i>Slc12a1</i>	+	+	+	+	+	+	+	+	+	+	+	+	100
CD epithelium	<i>Aqp2</i>	+	+	+	+	+	+	+	+	+	+	+	++	100
T-cell	<i>Cd3e</i>	-	-	-	-	-	-	-	-	-	-	-	-	0
B-cell	<i>Ptprc</i>	-	-	-	-	-	-	-	-	-	-	-	-	0
Macrophage	<i>Cd68</i>	-	-	-	-	-	-	-	-	-	-	-	-	0

++, detected at first PCR; +, detected at second PCR; -, not detected.

Table 1-3. Summary of the results of PCR array analysis targeting aggravating factors of chronic GN

Ranking expressing higher level	Symbol	Accession no.	BXSB-Yaa / B6
1	<i>Il10</i>	NM_010548	8.75
2	<i>Cxcl2</i>	NM_009140	8.46
3	<i>C3</i>	NM_009778	5.58
4	<i>Il1rn</i>	NM_031176	4.53
5	<i>Cxcl1</i>	NM_008176	4.03
6	<i>C3ar1</i>	NM_009779	3.41
7	<i>Il8rb</i>	NM_009909	3.36
8	<i>Ccl2</i>	NM_011333	3.36
9	<i>Ccl17</i>	NM_011332	3.20
10	<i>Ccl7</i>	NM_013654	3.14
11	<i>Ccr3</i>	NM_009914	3.10
12	<i>Il23r</i>	NM_144548	3.07
13	<i>Tlr2</i>	NM_011905	2.87
14	<i>C4b</i>	NM_009780	2.83
15	<i>Ccl3</i>	NM_011337	2.83
16	<i>Tnf</i>	NM_013693	2.81
17	<i>Il1b</i>	NM_008361	2.77
18	<i>Itgb2</i>	NM_008404	2.58
19	<i>Ccl11</i>	NM_011330	2.51
20	<i>Ccl8</i>	NM_021443	2.51
21	<i>Il23a</i>	NM_031252	2.51
22	<i>Ccl4</i>	NM_013652	2.41
23	<i>Ccr2</i>	NM_009915	2.33
24	<i>Ccr1</i>	NM_009912	2.33
25	<i>Fos</i>	NM_010234	2.23
26	<i>Il6</i>	NM_031168	2.11
27	<i>Ltb</i>	NM_008518	1.99
28	<i>Tlr7</i>	NM_133211	1.79
29	<i>Cxcl5</i>	NM_009141	1.78
30	<i>Ccl20</i>	NM_016960	1.77

Values are fold increase compared to B6 mice.

Table 1-4. Summary of results showing the expression of *Il10*, *Il1rn*, *C3*, and *Cxcl2* mRNAs in the urine from BXS^B-*Yaa* mice

Marker/Case	1	2	3	4	5	6	7	8	9	10	11	12	Ratio (%)
<i>Il10</i>	-	-	-	-	-	-	+	-	-	-	-	-	8.3
<i>Il1rn</i>	-	-	-	-	-	-	-	-	+	-	-	-	8.3
<i>C3</i>	+	-	-	+	-	+	+	-	+	-	-	-	41.7
<i>Cxcl2</i>	-	-	-	-	-	-	-	-	-	-	-	-	0

+, positive; -, negative.

Table 1-5. Summary of results detecting pathological parameters and urinary cell patterns in the urine from 12 BXSB-*Yaa* mice and 5 B6 mice.

	Urinary cell number	Glomerular damage score	Urinary albumin (mg/ml)	BUN (mg/dl)	Cre (mg/dl)	WT1 positive cell number	IL-1F6 positive tubule number	Podocyte marker	mRNA expression		
									DT/CD marker	Other marker	Inflammatory cytokine
BXSB- <i>Yaa</i> case 1	34.3	261	5.72	51.1	0.05	7.0	61.0	<i>Nphs1, Wt1, Actn4</i>	<i>Aqp2, Slc12a1</i>	-	<i>C3</i>
BXSB- <i>Yaa</i> case 2	8.0	70	0.74	28.6	0.40	9.2	0.0	-	<i>Aqp2, Slc12a1</i>	-	-
BXSB- <i>Yaa</i> case 3	11.2	85	0.50	84.4	1.17	8.0	32.0	-	<i>Aqp2, Slc12a1</i>	-	-
BXSB- <i>Yaa</i> case 4	16.5	198	2.19	97.8	1.37	5.0	8.0	<i>Nphs1</i>	<i>Aqp2, Slc12a1</i>	-	<i>C3</i>
BXSB- <i>Yaa</i> case 5	4.5	84	0.00	21.1	0.35	9.6	1.0	-	<i>Aqp2, Slc12a1</i>	-	-
BXSB- <i>Yaa</i> case 6	8.0	115	0.00	24.4	0.32	10.0	10.0	-	<i>Aqp2, Slc12a1</i>	<i>Vwf</i>	<i>C3</i>
BXSB- <i>Yaa</i> case 7	27.1	289	1.83	40.8	0.43	3.8	57.0	<i>Nphs1</i>	<i>Aqp2, Slc12a1</i>	<i>Serpina7</i>	<i>C3, Il10</i>
BXSB- <i>Yaa</i> case 8	12.0	113	0.00	18.8	0.14	10.6	1.0	-	<i>Aqp2, Slc12a1</i>	<i>Vwf</i>	-
BXSB- <i>Yaa</i> case 9	15.0	192	1.75	44.7	1.52	6.2	36.0	<i>Nphs1, Actn4</i>	<i>Aqp2, Slc12a1</i>	-	<i>C3, Il1rn</i>
BXSB- <i>Yaa</i> case 10	3.6	121	0.00	37.9	1.10	9.8	2.5	-	<i>Aqp2, Slc12a1</i>	<i>Vwf</i>	-
BXSB- <i>Yaa</i> case 11	12.4	191	0.14	49.5	1.34	5.7	4.5	<i>Nphs1, Wt1</i>	<i>Aqp2</i>	-	-
BXSB- <i>Yaa</i> case 12	no data	261	5.71	129.8	2.31	3.8	22.0	<i>Actn4, Wt1</i>	<i>Aqp2, Slc12a1</i>	-	-
Average	13.9	156	1.55	52.4	0.86	7.4	19.6	/	/	/	/
B6 case1	1.3	3.0	0.12	44.1	0.37	19.0	0.0	<i>Wt1</i>	<i>Aqp2</i>	-	-
B6 case2	2.5	0.0	0.00	30.7	0.09	15.2	0.0	-	-	-	-
B6 case3	2.5	5.0	0.00	39.6	0.58	14.8	0.0	-	-	-	-
B6 case4	2.5	6.0	0.00	52.1	0.75	12.6	0.0	-	<i>Aqp2, Slc12a1</i>	-	-
B6 case5	1.6	3.0	0.00	32.8	0.55	13.0	0.0	-	-	-	-
Average	2.1	3.4	0.024	39.9	0.47	14.9	0.0	/	/	/	/

DT, distal tubule; CD, collecting duct; -, negative; /, not applicable.

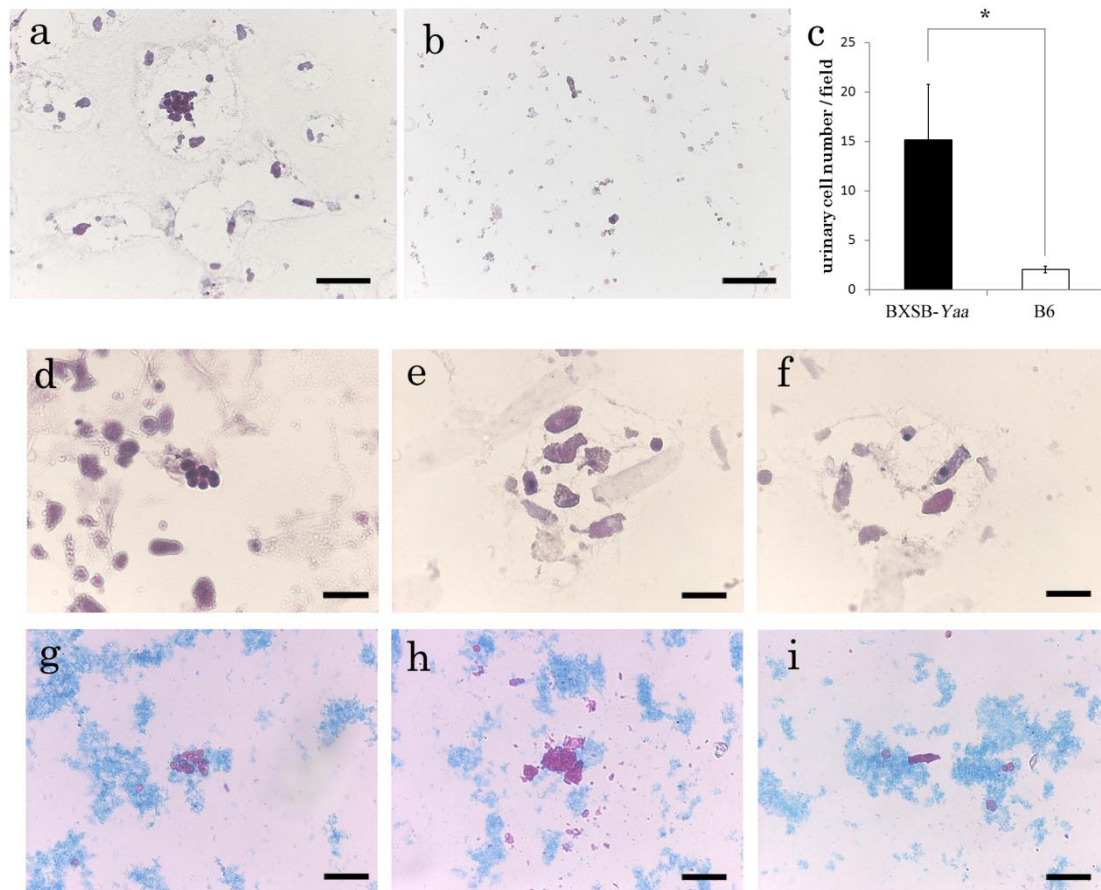


Figure 1-1. Cytology and the number of urinary cells in BXSB-Yaa and B6 mice.

(a and b) Comparison of urinary smears from BXSB -Yaa (a) and B6 (b) mice. Bar = 50 μ m. Urinary cell numbers in BXSB-Yaa mice are higher than those in B6 mice.

(c) Numbers of urinary cells in BXSB-Yaa and B6 mice. *, significantly different from B6 mice (Mann-Whitney *U* test, $P < 0.05$); $n = 11$.

(d-i) Morphology of urinary cells in BXSB-Yaa mice stained with HE (d-f) and SM (g-i). Small round cells (d and g), homogeneous and amorphous cell components (e and h), and columnar cells (f and i) are observed. Bars = 50 μ m.

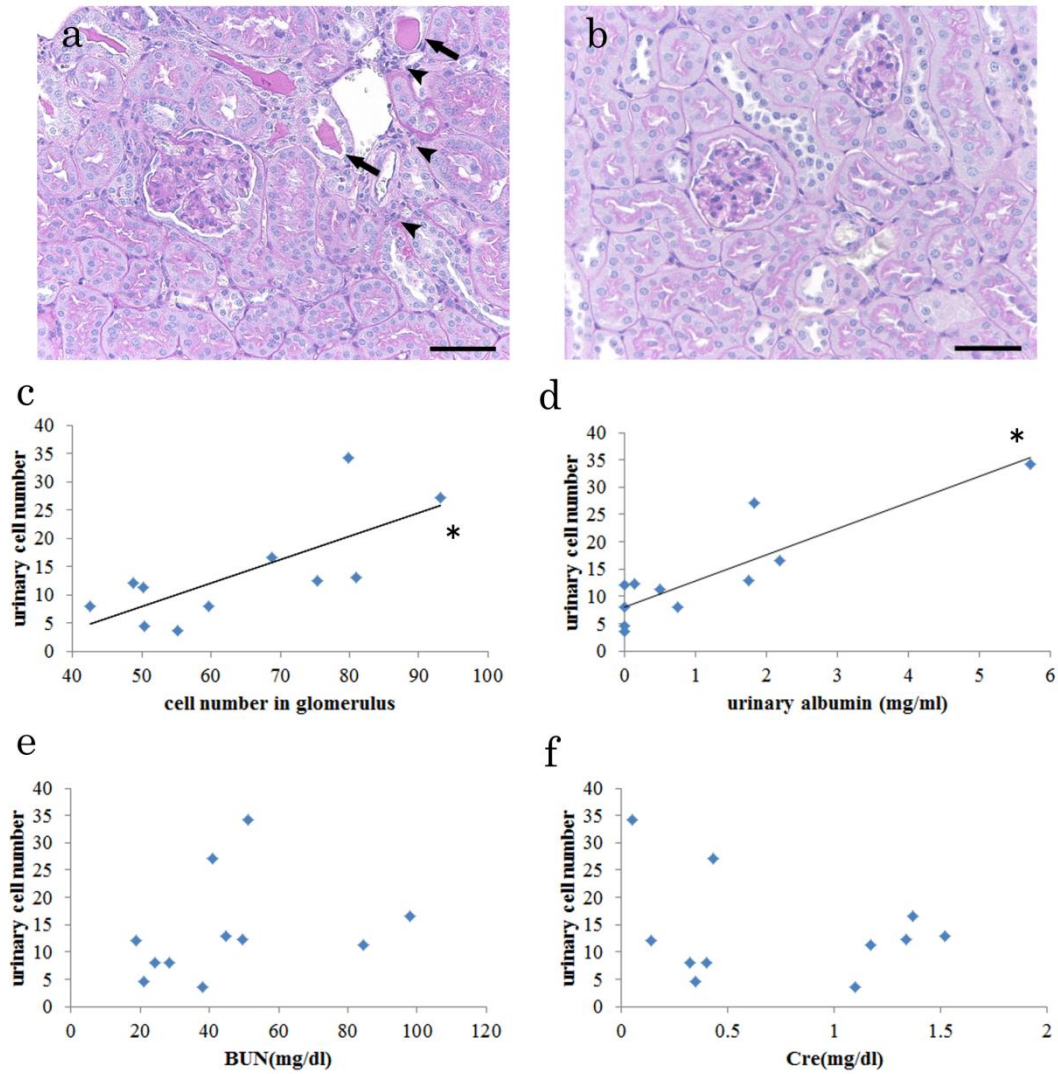


Figure 1-2. Comparison between renal condition and urinary cell number.

(a and b) Representative PAS-H-stained kidney sections from BXSb-Yaa (a) and B6 (b) mice. Expansion of mesangial matrix, proliferation of mesangial cells, dilation of tubules by urinary cast (arrow), and perivascular cell infiltration (arrowhead) are observed in the BXSb-Yaa kidney. Bars = 50 μ m.

(c) Relationship between glomerular damage score and urinary cell number. $P < 0.05$, $r = 0.802$ (Spearman's correlation test); $n = 11$.

(d) Relationship between urinary albumin and urinary cell number. $P < 0.05$, $r = 0.837$ (Spearman's correlation test); $n = 11$.

(e) Relationship between BUN level and urinary cell number; $n = 11$.

(f) Relationship between Cre level and urinary cell number; $n = 11$. BUN and serum Cre levels do not correlate with urinary cell number.

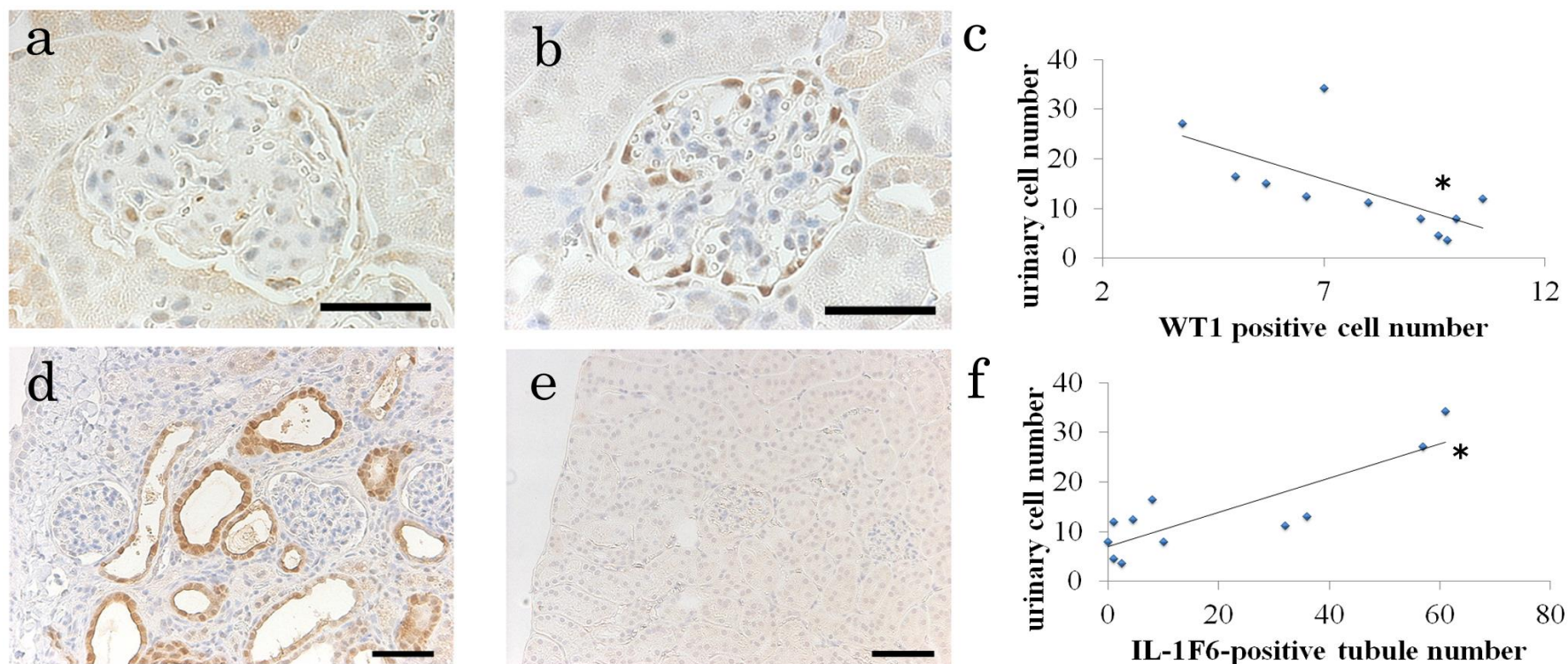


Figure 1-3. Localization of WT1 and IL-1F6 proteins and urinary cell number.

(a and b) Immunohistochemistry of BXSB-Yaa (a) and B6 (b) mice kidneys. WT1-positive reactions are observed in podocyte nuclei. The number of WT1-positive cells in the BXSB-Yaa kidney is higher than that in the B6 mice kidney. Bars = 50 μ m.

(c) Relationship between the number of WT1-positive cells and urinary cell number. $P < 0.05$, $r = -0.662$ (Spearman's correlation test); $n = 11$.

(d and e) Immunohistochemistry for IL-1F6 in BXSB-Yaa (d) and B6 (e) mice kidneys. IL-1F6-positive reactions are observed in damaged tubules. Bars = 50 μ m.

(f) Relationship between the number of IL-1F6-positive tubules and urinary cell number. $P < 0.05$, $r = 0.712$ (Spearman's correlation test); $n = 11$.

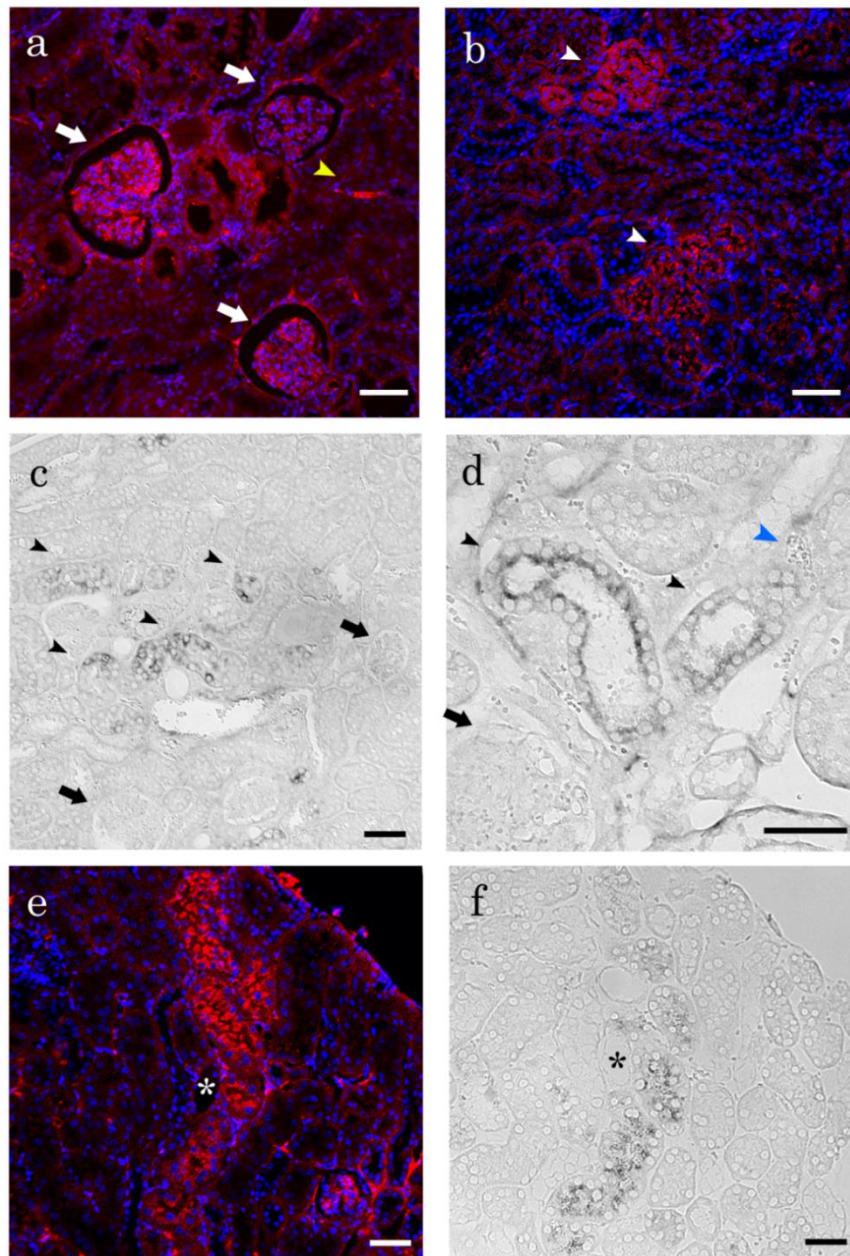


Figure 1-4. C3 protein and C3 mRNA expression in the urine and kidneys from BXSb-Yaa mice.

(a and b) Immunofluorescence for C3. Positive C3 reactions are observed in the glomerular capillary rete (a, white arrow), tubular epithelial cells (b, white arrowhead), and vascular endothelia (a, yellow arrowhead). Additionally, several C3-positive tubules tend to be localized in the same cortical regions (b). Bars = 50 μ m.

(c and d) *In situ* hybridization for C3 mRNA. Positive reactions are observed in the cytoplasm of tubular epithelial cells (c, black arrowhead). Similar to C3 protein staining, several C3 mRNA-positive tubules are localized in the same cortical regions. On the other hand, the glomerulus (black arrow) and vascular endothelium (blue arrowhead) are not stained (c and d). Bars = 50 μ m.

(e and f) Immunohistochemistry and *in situ* hybridization for C3 protein and C3 mRNA in serial sections. C3 protein (e) and C3 mRNA (f) show colocalization in the same tubules. *, the same vessel. Bars = 50 μ m.

Chapter 2

Podocyte injuries

in autoimmune glomerulonephritis

Introduction

CKD is one of the most serious public health problems because it is strongly associated with not only ESRD but also cardiovascular diseases (Tonelli et al., 2006). Thus, understanding the pathophysiology of CKD for the identification of novel therapeutic and diagnostic targets is important to improve the morbidity and mortality of patients.

In Chapter 1, the author clarified that BXS^B-*Yaa* mice show the damage of epithelium composing glomerulus (podocytes) and tubulointerstitium (DTs and CDs) with proteinuria in GN progression. Recent studies have indicated that CKD often begins with GLs leading to subsequent TILs (Kriz and LeHir, 2005; Nakai et al., 2006). Briefly, from early stage chronic GN, increased glomerular cells and IC depositions were observed in GLs with proteinuria caused by the ultrafiltration of plasma proteins (Kriz and LeHir, 2005). Chronic GLs with increased urinary protein (proteinuria) could trigger the formation of TILs and eventually progress to interstitial fibrosis leading to ESRD (Kriz and LeHir, 2005). Especially, progressive podocyte injury, also called podocytopathy, has been suggested to be one of early and crucial events in the pathogenesis of GLs in major CKD primary diseases such as diabetic nephropathy and renal sclerosis (Kretzler, 2005; Diez-Sampedro et al., 2011). Therefore, in second Chapter, the author focused on podocyte injury rather than TIL as an earlier sign of CKD progression.

Podocytes are highly differentiated epithelial cells lining the outside of

glomerular capillaries, and their FPs regulate BUB by the formation of a SD. Yi et al. indicated that a decrease in the expression of SD molecules and effacement of FPs were associated with the development of renal sclerosis (Yi et al., 2007). According to Pagtalunan et al., the number of podocytes in the glomerulus could be used as indices of podocyte injury in patients with diabetic nephropathy (Pagtalunan et al., 1997). In a CKD animal study targeting dogs, Ichii et al. have also clarified that the immune-positive levels of glomerular SD molecules, especially nephrin and actinin alpha 4 (ACTN4), negatively correlated with serum Cre levels, and nephrin mRNA expression in the kidneys of CKD dogs was significantly lower than that in normal animals and negatively correlated with serum Cre (Ichii et al., 2011). Several studies have indicated that the signaling pathway through Notch, TGF- β , and angiotensin II play crucial roles in podocyte injuries (Schiffer et al., 2001; Niranjana et al., 2008; Zhang et al., 2010; Lavoie et al., 2012).

Spontaneous chronic GN models, lupus-prone mice such as NZB, (NZB $\times$ NZW) F1 hybrid, BXSB-*Yaa*, and MRL/MpJ-*lpr* are commonly used (Li et al., 2012; Moser et al., 2012; Okamoto et al., 2012). BXSB-*Yaa* mice carry a mutant gene locus *Yaa*, and males show severe chronic GN similar to human and animal (Subramanian et al., 2006). Furthermore, Ichii et al. developed a spontaneous CKD model B6.MRL-(*D1Mit202-D1Mit403*) (B6.MRL) mice, carrying the B6 mouse background and the telomeric regions of Chr. 1 (*D1Mit202-D1Mit403* (68–81 cM)) derived from lupus-prone MRL/MpJ mice (Ichii et al., 2008b). This

congenic region contains the Fas ligand, interferon activated gene 200 (Ifi200) family, and Fc gamma receptor (Fcgr) family, which were strongly associated with the developments of autoimmunity and chronic GN (Ichii et al., 2008b). With age, female B6.MRL mice also develop chronic GN similar to human CKD (Ichii et al., 2008a, b; Ichii et al., 2010b). Therefore, the author considered that BXSB-*Yaa* mice and B6.MRL mice as appropriate models for the investigation of podocyte injury in chronic GN.

In Chapter 2, the author investigated the pathological features of chronic GN using 2 murine models, BXSB-*Yaa* mice and B6.MRL mice, with focus on podocyte injuries. The results showed that these chronic GN models clearly developed podocyte injuries, characterized by the decreased mRNA and protein levels of its functional markers with morphological changes and exacerbation of clinical parameters. The author's data emphasized the clinicopathological importance of podocyte injuries in the progression of chronic GN.

Materials and Methods

Ethics statement

All animal experimentations in Chapter 2 were performed as described in Chapter 1.

Animals and Sample Preparations

Experimental animals were handled as shown in Chapter 1. Male BXSB-*Yaa* and female B6.MRL mice were used as chronic GN model mice at age 4 months and 9–15 months, respectively. As healthy controls, male BXSB and female B6 mice were used at age 4 and 9 months, respectively. B6.MRL mice were created by Ichii et al. (Ichii et al., 2008b), whereas B6, BXSB, and BXSB-*Yaa* mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The urine, serum, and kidneys were collected and processed as shown in Chapter 1. The kidneys were fixed by 4% PFA in 0.1 M PB (pH 7.4) or 2.5% glutaraldehyde in 0.1 M PB at 4°C for histopathological analysis.

Histological Analysis

To assess the severity of GN, semiquantitative glomerular damage scoring was performed as previous study (Ichii et al., 2008b). Briefly, 100 glomeruli per kidney were examined by using PAS-H-stained sections and scored from 0 to +4 according to the following criteria: 0, no recognizable lesion in glomeruli; +1, a little PAS-positive deposition, mild cell proliferation, mild membranous

hypertrophy, and/or partial podocyte adhesion to the parietal layer of the renal corpuscle; +2, segmental or global PAS-positive deposition, cell proliferation, membranous hypertrophy, and/or glomerular hypertrophy; +3, the same as grade 2 with PAS-positive deposition in 50% of regions of glomeruli and/or severe podocyte adhesion to the parietal layer of the renal corpuscle; +4, disappearance of capillary and capsular lumina, global deposition of PAS-positive material, and/or periglomerular infiltration of inflammatory cells and fibrosis, based on the degrees of PAS-positive deposition, cell proliferation, membranous hypertrophy, podocyte adhesion to the parietal layer, disappearance of capillary and capsular lumina, and periglomerular infiltration of inflammatory cells and fibrosis.

Glomerular Isolation

Glomeruli of mice were isolated by a bead perfusion method (Takemoto et al., 2002). Briefly, 40 mL of Hanks' balanced salt solution (HBSS) containing 8×10^7 Dynabeads (Life Technologies, Palo Alto, CA) was perfused from the left ventricle. The kidneys were removed and digested in collagenase (1 mg/mL collagenase A [Roche, Basel, Switzerland] and 100 U/mL deoxyribonuclease I [Life Technologies] in HBSS) at 37°C for 30 min. The digested tissue was gently pressed through a 100- μ m cell strainer (BD Falcon, Franklin Lakes, NJ, USA) using a flattened pestle, and the cell suspension was centrifuged at $200 \times g$ for 5 min. The cell pellet was resuspended in 2 mL HBSS. Finally, glomeruli containing

Dynabeads were gathered by a magnetic particle concentrator (Life Technologies).

Serological and Urinary Analysis

For the evaluation of the systemic autoimmune condition, serum levels of anti-double strand DNA (dsDNA) antibody were measured using Mouse Anti-dsDNA Ig's (Total A+G+M) ELISA kit (Alpha Diagnostic International, San Antonio, TX, USA). For the evaluation of renal function, serum BUN and Cre levels in all animals were measured using Fuji DriChem 7000v (Fujifilm, Tokyo, Japan). Urinary albumin creatinine ratio (uACR) was determined using Albuwell M and The Creatinine Comparison (Exocell, Philadelphia, PA, USA).

Immunohistochemistry and Histoplanimetry

Immunostaining for nephrin, podocin, synaptopodin, CD3, and B220 was performed according to the procedure shown in Table 2-1. Details of the procedures are described in the following. For antigen retrieval, deparaffinized sections were incubated in citrate buffer (pH 6.0) for 20 min at 105°C for nephrin and podocin (Dako Target Retrieval Solution, pH 9; DAKO, Glostrup, Denmark), for 20 min at 105°C for synaptopodin and CD3, or in 0.1% pepsin/0.2 M HCl for 5 min at 37°C for B220. After cooling, slides were soaked in methanol containing 3% H₂O₂ for 15 min at room temperature. After washing, sections were blocked by 10% normal goat serum for nephrin and podocin, blocking solution A (mouse stain kit; Nichirei, Tokyo, Japan) for synaptopodin,

or 0.25% casein/0.01 M PBS for CD3 and B220 for 60 min at room temperature. Then, sections were incubated with rabbit polyclonal antibodies for nephrin (1:500; Immuno-Biological Laboratories, Gunma, Japan), rabbit polyclonal antibodies for podocin (1:800; Immuno-Biological Laboratories), mouse monoclonal antibodies for synaptopodin (1:50; Fitzgerald, MA, USA), rabbit polyclonal antibodies for CD3 (1:150; Nichirei), or rat monoclonal antibodies for B220 (1:1000; Cedarlane, Ontario, Canada) overnight at 4°C. After washing 3 times in phosphate-buffered saline (PBS), sections were incubated with biotin-conjugated goat anti-rabbit IgG antibodies for nephrin, podocin, and CD3 (SABPO kit, Nichirei) or goat anti-rat IgG antibodies for B220 (1:200; Caltag Medsystems, Buckingham, UK) for 30 min at room temperature, washed, and incubated with streptavidin-biotin complex (SABPO kit, Nichirei) for 30 min. For synaptopodin, sections were incubated with blocking solution B (mouse stain kit, Nichirei) for 10 min at room temperature, washed, and incubated with Simple Stain Mouse MAX-PO(M) (mouse stain kit, Nichirei) for 10 min. All sections were then incubated with 3,3'-diaminobenzidine tetrahydrochloride–H₂O₂ solution. Finally, the sections were counterstained with hematoxylin.

Quantifications of positive immunohistochemical reactions of SD molecules were performed as described previously (Ichii et al., 2011). Briefly, the glomerulus area and black pixels of positive reaction were measured, and the number of pixels per area was calculated for each SD molecule by means of BZII-Analyzer (Keyence, Osaka, Japan). To evaluate T-cell and B-cell

infiltration into the glomeruli, the number of CD3- and B220-positive cells was counted. In these measurements, 20 glomeruli were counted in 1 kidney section in each group (n = 5), and the values were expressed as means.

Immunofluorescence

Immunofluorescence for nephrin, podocin, and synaptopodin was performed according to the procedure shown in Table 2-1. Details of the procedures are described in the following. After the deparaffinization, antigen retrieval was performed by same method as in immunohistochemistry. After being washed, sections were blocked by 5% normal donkey serum for 60 min at room temperature. Incubation with primary antibodies was performed by same method as in immunohistochemistry. After washing with PBS, the sections were incubated with Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500, Life Technologies) for nephrin and podocin, or Alexa Fluor 488-labeled donkey anti-mouse IgG antibodies (1:500, Life Technologies) for synaptopodin for 30 min at room temperature and washed again. For nuclear staining, sections were incubated with Hoechst 33342 (1:2000; Dojindo, Kumamoto, Japan) for 5 min. Finally, the sections were examined under a fluorescence microscope (BZ-9000, Keyence).

Electron Microscopy

Ultrastructural analysis with a transmission electron microscope (TEM) was

performed according to the following procedure. After fixation with 2.5% glutaraldehyde in 0.1 M PB for 4 h, small pieces of kidney tissue were fixed with 1% osmium tetroxide in 0.1 M PB for 2 h, dehydrated by graded alcohol, and embedded in epoxy resin (Quetol 812 Mixture; Nisshin EM, Tokyo, Japan). Ultrathin sections (70 nm) were double stained with uranyl acetate and lead citrate. All samples were observed under a JEOL transmission electron microscope (JEM-1210; JEOL, Tokyo, Japan). Ultrastructural analysis with a scanning electron microscope (SEM) was performed according to the following procedure. Quarter sizes of glutaraldehyde-fixed kidneys were kept in 2% tannic acid for 1 h at 4°C and postfixed with 1% osmium tetroxide in 0.1 M PB for 1 h. The specimens were dehydrated through graded alcohol, transferred into 3-methylbutyl acetate, and dried using an HCP-2 critical point dryer (Hitachi, Tokyo, Japan). The dried specimens were sputter-coated with Hitachi E-1030 ion sputter coater (Hitachi), and then examined on an S-4100 SEM (Hitachi) with an accelerating voltage of 20 kV.

Reverse Transcription and Real-time PCR

For the examination of mRNA expression, total RNA from isolated glomeruli was purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNAs were synthesized to cDNAs by a reverse transcription (RT) reaction by using ReverTra Ace reverse transcriptase enzyme (Toyobo, Osaka, Japan) and random dT primers (Promega, Madison, WI). Each cDNA was used for

real-time PCR with Brilliant III SYBR Green QPCR master mix on Mx3000P (Agilent Technologies, La Jolla, CA, USA). The expression levels of genes were normalized to actin beta (*Actb*) as a housekeeping gene. The appropriate primer pairs are shown in Table 2-2.

Statistical Analysis

Results were statistically analyzed as described in Chapter 1.

Results

Clinical Parameters of Chronic GN Models

As the index of the systemic autoimmune condition, the serum anti-dsDNA antibody level of BXSB-*Yaa* and B6.MRL mice was significantly higher than that of BXSB and B6 mice, respectively (Table 2-3). BXSB-*Yaa* mice showed significantly higher anti-dsDNA antibody levels than B6.MRL mice. In the indices of renal functions, including BUN, Cre, and uACR, every parameter of BXSB-*Yaa* and B6.MRL mice was higher than that of BXSB and B6 mice, respectively. Significant differences between controls and chronic GN models were observed in uACR. The uACR in BXSB-*Yaa* mice was significantly higher than that in B6.MRL mice.

Glomerular Histopathology in Chronic GN Models

Both BXSB-*Yaa* and B6.MRL mice developed membranous proliferative GN characterized by glomerular hypertrophy, increased mesangial cells and their matrix, and thickening of the glomerular basement membrane (GBM) (Figs. 2-1b and d, Table 2-4). Proliferative and membranous lesions, in particular, were more severe in BXSB-*Yaa* and B6.MRL mice, respectively. No GLs were observed in the controls (Figs. 2-1a and b, Table 2-4). To assess the glomerular infiltration of immune cells, immunohistochemistry for CD3 (T-cell marker) and B220 (B-cell marker) was performed. CD3-positive cells were observed in the glomerulus of BXSB-*Yaa* and B6.MRL mice as well as in the renal interstitium (Figs. 2-1f and h),

but not in controls (Figs. 2-1e and g). Although few B220-positive cells were observed in the glomerulus of BXS^B and BXS^B-*Yaa* mice (Figs. 2-1i and j), they were scarcely observed in B6 and B6.MRL mice (Figs. 2-1k and l). In histoplanimetry, the number of CD3-positive cells in the glomerulus was significantly higher in both chronic GN models than in each control (Fig. 2-1m). In relation to the histological observation, the number of B220-positive cells in the glomerulus was significantly higher in BXS^B and BXS^B-*Yaa* mice than in B6 and B6.MRL mice, respectively. No significant difference was observed between the controls and the chronic GN models (Fig. 2-1m).

Glomerular Ultrastructure in Chronic GN Models

To analyze the morphological changes of podocytes in chronic GN, the glomerular ultrastructure of chronic GN models was compared with that of the controls by TEM and SEM. Under TEM observation, the controls showed clear cytotrabecula (Cyto, which also known as primary process) and cytopodium (FP, which is also known as secondary process) (Figs. 2-2a, c, f, and h). In both chronic GN models, FPs showed irregular arrangements with hypertrophy and partial fusions (Figs. 2-2b, d, e, g, i, and j). Furthermore, in chronic GN models, the GBM was thickened and wrinkled, and high electron-dense deposits resembling ICs were observed in the double-counteracted GBM of the subendothelial regions (Figs. 2-2b, d, e, g, i, and j). At higher magnifications, the liner SD was clearly observed between FPs of control mice (Figs. 2-2f and h). In both chronic GN models, FP

effacement was clearly observed, but the SD and the 3-layer structure of the GBM were unclear (Figs. 2-2g, i, and j).

Under SEM observation, the width of the cytotrabecula was increased in chronic GN models compared with the controls and the FPs were unclear in the former podocytes (Figs. 2-2k–t). At higher magnifications, the engagements of each FP were irregular, and a microvillus-like process was observed in the surface of podocytes in both chronic GN models (Figs. 2-2q, s, and t). In B6.MRL mouse podocytes, these morphological changes were observed in TEM and SEM and severer at 15 months than at 9 months.

Localization and Expression of SD Molecules in Chronic GN Models

For the evaluation of podocyte injury, immunohistochemistry and immunofluorescence for SD molecules (nephrin, podocin, and synaptopodin) were performed (Figs. 2-3). Linear-positive reactions for nephrin, podocin, and synaptopodin were observed along the glomerular capillary rete in controls (Figs. 2-3a, c, e, g, i, and k). In contrast, those in the chronic GN models tended to be faint, partially showed granular patterns, and localized to the glomerular edge rather than the center (Figs. 2-3b, d, f, h, j, and l). In immunohistoplanimetry, the relative positive areas of all SD molecules in the glomerulus were significantly smaller in both chronic GN models than in controls (Fig. 2-3m).

Furthermore, to evaluate the relation between immune cell infiltration and podocyte injuries, immune cell number (B220- and CD3-positive cells) and the

relative SD molecule–positive area (nephrin, podocin, synaptopodin) in the glomerulus were analyzed (Table 2-5). No significant correlation was detected between both parameters.

mRNA Expression of Podocyte Functional Markers in Chronic GN Models

For further investigation of podocyte injury, the mRNA expression of the functional markers was evaluated by quantitative PCR by using glomeruli isolated by bead perfusion methods (Fig. 2-4). As markers of functional proteins, *Nphs1*, *Nphs2*, and *Synpo* (SD molecules); *Actn4*, *Cd2ap*, and *Myh9* (cytoskeletal proteins); and *Podxl* (the major constituent of podocyte glycocalyx) were examined. The mRNA expression levels of all podocyte functional markers except *Myh9* were significantly decreased in BXSB-*Yaa* mice compared with BXSB mice. In the early chronic GN stage of B6.MRL mice (9 months), the expression levels of *Nphs2*, *Actn4*, and *Myh9* tended to be higher than those of B6 mice; the reverse was true for *Nphs1*, *Synpo*, and *Cd2ap*. In the late chronic GN stage of B6.MRL mice (12 months), the expression levels of all functional marker mRNAs were significantly lower compared with B6 mice.

Furthermore, the relations between clinical parameters and the mRNA expression levels of podocyte functional markers were evaluated (Table 2-6). Serum anti-dsDNA levels were negatively correlated with the values of *Nphs1* and *Actn4* in BXSB-*Yaa* mice, and with *Nphs1* and *Podxl* in B6.MRL mice. Furthermore, uACR levels were negatively correlated with the mRNA expression

levels of all functional markers in both BXSB-*Yaa* and B6.MRL mice.

Discussion

SLE is a representative autoimmune disease showing the deposition of ICs or direct autoantibody deposition to systemic organs, including the kidney, leading to complement activation, Fc receptor ligation, and subsequent inflammation (Tsokos, 2011). In particular, SLE-related chronic GN (lupus nephritis) is one of the most serious SLE complications since it is a major predictor of poor prognosis (Waldman and Appel, 2006; Tsokos, 2011). In lupus nephritis, it has been suggested that glomerular IC depositions cause GLs such as mesangial cell proliferation and GBM thickening, which lead to BUB disruption, as in other chronic GNs such as IgA nephropathy and drug-induced GN (Ichii et al., 2008b; Ichii et al., 2010b; Nowling and Gilkeson, 2011). BXS^B-Yaa and B6.MRL mice are spontaneous murine models of autoimmune-mediated chronic GN (Ichii et al., 2008b; Ichii et al., 2010b). The present study clarified that both chronic GN models had clearly elevated serum anti-dsDNA antibody levels and developed membranous proliferative GN with IC depositions and altered podocyte morphology, such as FP effacement and the appearance of microvillus-like processes. Furthermore, the glomerular mRNA levels of podocyte functional markers were significantly decreased and negatively correlated with uACR in the chronic GN models. Little has been known about podocyte injury in chronic GN, which is one of the major primary diseases leading to CKD and subsequent ESRD. The present study clarified that the decrease of podocyte functional mRNA expression levels in chronic GN closely correlated with the morphological changes of podocytes as

well as glomerular dysfunction indicated by increased uACR. Importantly, the pathological mechanism of podocyte injuries was mainly investigated by using drug-induced glomerular disease models but not spontaneous models such as BXSB-*Yaa* or B6.MRL mice. The podocyte injuries in spontaneous models would more clearly reflect those in human clinical cases than the drug induced glomerular disease models. Recent experimental and clinical studies have revealed that podocytes exhibit various structural changes, including FP effacement, cell body attenuation, pseudocyst formation, hypertrophy, cytoplasmic accumulation of lysosomal elements, and detachment from the GBM, under several pathological situations (Kriz et al., 1998). Among these changes, FP effacement is the most representative change in podocyte shape, which is closely correlated with progressive proteinuria (Inokuchi et al., 1996; Shirato, 2002). Furthermore, an increase in podocyte microvilli represents another morphologic alteration during both experimental and human nephrotic syndrome, with unknown mechanisms and significance (Hara et al., 2010). These findings suggested that immune-mediated chronic GN involves podocyte injury with morphological change leading to BUB disruption, similar to other glomerular diseases.

Interestingly, there was a difference in glomerular pathological features between BXSB-*Yaa* and B6.MRL mice; proliferative and membranous lesions were more severe in BXSB-*Yaa* and B6.MRL mice, respectively. Furthermore, the populations of glomerulus-infiltrating immune cells were different between these models; B-cells were localized in the glomerulus of BXSB-*Yaa* mice, but not

B6.MRL mice. Because BXSB mice also had B-cells in the glomerulus, the BXSB mouse genomic background rather than the *Yaa* mutation might have a role in the infiltration of B-cells in the glomerulus. Studies on lupus models have demonstrated that infiltrating B-cells in the kidney secrete antibodies with various antigen specificities, contributing increased *in situ* ICs (Espeli et al., 2011). Recent reports have suggested that depleting B-cells either before or after disease onset prevented and/or delayed the onset of nephritis in several different lupus model mice (Bekar et al., 2010; Ramanujam et al., 2010). Furthermore, lupus-prone MRL/MpJ-*lpr* mice, which express a mutant transgene encoding surface immunoglobulin (meaning that their B-cells are unable to secrete antibodies), still develop nephritis (Chan et al., 1999). These reports indicated that B-cells can play some roles not only in antibody productions and the activation of pathogenic T-cells but also in secreting pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6), contributing to the development of GLs. However, the author's study demonstrated that no significant correlation was observed between T-cells or B-cells and SD protein expression levels in the glomerulus of chronic GN models. From these findings, the presence and functional activation of infiltrating B-cells in the glomerulus might exacerbate the glomerular proliferative lesions in BXSB-*Yaa* mice, not primarily contributing to podocyte injury.

Recently, it has been suggested that FP effacement, the response of the podocyte to injury, is dependent on the disruption of the actin cytoskeletal network

as an initial event (Shirato et al., 1996; Asanuma et al., 2005). In several glomerular diseases of human and mice, it has been implicated that the molecular framework of process consists of actin filaments and cytoskeleton proteins such as ACTN4, CD2-associated protein (CD2AP), and myosin, heavy chain 9, non-muscle (MYH9), and FP effacement is caused by rearrangements of this molecular framework (Shirato et al., 1996; Asanuma et al., 2005; Durvasula and Shankland, 2006; Asanuma et al., 2007). Indeed, the author's data indicated that the glomerular mRNA expression levels of actin-associated cytoskeletal proteins, including *Actn4* and *Cd2ap*, decreased in chronic GN models compared with controls. Interestingly, the glomerular *Actn4* mRNA expression in both chronic GN models strongly and negatively correlated with uACR. In contrast, the glomerular *Myh9* expression level of B6.MRL mice in the early stage (age 9 months) was significantly higher than that of controls. From these findings, altered glomerular mRNA expression levels of cytoskeletal proteins might indicate cytoskeletal rearrangements associated with chronic GN progression and may be associated with the effacement of FPs and formation of microvillus-like processes in podocytes.

Several factors, including genetic, mechanical, and immunological stresses, and toxins, were suggested to be the causes of FP effacement associated with cytoskeletal rearrangement and redistribution of SD proteins (Asanuma and Mundel, 2003). In the present study, chronic GN podocytes showed not only morphological changes but also abnormalities in SD protein localization; nephrin,

podocin, and synaptopodin were localized to the cell body as well as FP, showing a granular pattern. More recent studies indicated that Rho-kinases play a pivotal role in the organization of the actin cytoskeleton of podocytes (Asanuma et al., 2006; Shibata et al., 2006; Meyer-Schwesinger et al., 2012). Asanuma *et al* suggested that Rho-kinase regulates actin–myosin-containing stress fibers in the podocyte cell body (Asanuma et al., 2006). Meyer-Schwesinger et al. demonstrated that increased activation of Rho-kinases leads to cytoskeletal rearrangement in the course of antibody-mediated podocyte injury, culminating in FP effacement, proteinuria, and detachment into the urine, and that this could be prevented by Rho-kinase inhibition (Meyer-Schwesinger et al., 2012). Furthermore, *in vitro* and *in vivo* studies have revealed that proinflammatory cytokines such as IL-1 β and TNF- α are involved in Rho-kinase pathway activation (Hiroki et al., 2004; Kanda et al., 2006). From these findings, Rho-kinase abnormality, which is caused by the glomerular exposure of internal or blood proinflammatory cytokines, including IL-1 β and TNF- α , might be a trigger for podocyte injury in chronic GN. The determination of the immune-mediated pathological pathway of podocyte injuries would lead the regulation of animal and human chronic GN.

In conclusion, although there was a small difference in glomerular pathological features between BXSB-*Yaa* and B6.MRL mice, both chronic GN models clearly developed podocyte injuries characterized by decreased expression of functional markers, with morphological changes and aggravation of clinical parameters. The author considered that injured podocyte can be an early diagnostic

and therapeutic target of chronic GN which begins with GLs.

Summary

Chronic GN is a major primary cause of CKD. In this Chapter, the author focused on podocyte injury as crucial and early pathological event of chronic GN, leading to CKD.

The autoimmune-prone mouse strains, BXSB-*Yaa* and B6.MRL, were used as the chronic GN models. Both models developed membranous proliferative GN with albuminuria and elevated serum anti-dsDNA antibody levels. BXSB-*Yaa* and B6.MRL mice showed severe proliferative lesions with T- and B-cell infiltrations and membranous lesions with T-cell infiltrations, respectively. FP effacement and microvillus-like structure formation were observed ultrastructurally in the podocytes of both chronic GN models. Furthermore, both chronic GN models showed a decrease in immune-positive areas of nephrin, podocin, and synaptopodin in the glomerulus, and in the mRNA expression of *Nphs1*, *Nphs2*, *Synpo*, *Actn4*, *Cd2ap*, and *Podxl* in the isolated glomerulus. Significant negative correlations were detected between serum anti-dsDNA antibody levels and glomerular *Nphs1* expression, and between uACR and glomerular expression of *Nphs1*, *Synpo*, *Actn4*, *Cd2ap*, or *Podxl*.

Taken collectively, chronic GN models clearly developed podocyte injuries characterized by the decreased expression of podocyte functional markers with altered morphology. These data emphasized the clinicopathological importance of podocyte injuries in the progression of chronic GN.

Tables and Figures

Table 2-1. Summary of immunostaining conditions

Immunohistochemistry		Nephrin	Podocin	Synaptopodin	CD3	B220
First antibody		Rabbit polyclonal antibodies (1:500; IBL, Gunma, Japan)	Rabbit polyclonal antibodies (1:800; IBL)	Mouse monoclonal antibodies (1:50; Fitzgerald, MA, USA)	Rabbit polyclonal antibodies (1:150; Nichirei, Tokyo, Japan)	Rat monoclonal antibodies (1:1000; Cedarlane, Ontario, Canada)
Second antibody		Biotinylated goat anti-rabbit IgG antibodies (SABPO kit, Nichirei)	Biotinylated goat anti-rabbit IgG antibodies (SABPO kit, Nichirei)	Simple Stain Mouse MAX-PO(M) (mouse stain kit, Nichirei)	Biotinylated goat anti-rabbit IgG antibodies (SABPO kit, Nichirei)	Biotinylated goat anti-rat IgG antibodies (Caltag Medsystems, Buckingham, UK)
Immunofluorescence		Nephrin	Podocin	Synaptopodin	CD3	B220
First antibody		Same as in immunohistochemistry	Same as in immunohistochemistry	Same as in immunohistochemistry	/	/
Second antibody		Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500, Life Technologies)	Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500, Life Technologies)	Alexa Fluor 488-labeled donkey anti-mouse IgG antibodies (1:500, Life Technologies)	/	/

Table 2-2. Summary of specific gene primers for real-time PCR

Genes (accession no.)	Primer sequence (5'-3') F: forward, R: reverse	Product size (bp)	Specific function in podocyte
<i>Nphs1</i> (NM_019459)	F: ACCTGTATGACGAGGTGGAGAG R: TCGTGAAGAGTCTCACACCAG	218	SD protein
<i>Nphs2</i> (NM_130456)	F: AAGGTTGATCTCCGTCTCCAG R: TTCCATGCGGTAGTAGCAGAC	105	SD protein
<i>Synpo</i> (NM_177340.2)	F: CATCGGACCTTCTTCCTGTG R: TCGGAGTCTGTGGGTGAG	90	SD protein
<i>Actn4</i> (NM_021895)	F: TCCAGGACATCTCTGTGGAAG R: CATTGTTTAGGTTGGTGACTGG	216	Cytoskeletal protein
<i>Cd2ap</i> (NM_009847)	F: CAAGATGCCTGGAAGACGA R: GCACTTGAAGGTGTTGAAAGAG	177	Cytoskeletal protein
<i>Myh9</i> (NM_022410)	F: AAGGACCAGGCTGACAAGG R: GTCACGACAAATGGCAGGTC	209	Cytoskeletal protein
<i>Podxl</i> (NM_013723)	F: TCCTAAGGCCGTGTATGAGC R: GATGCCATGCAGACGATG	153	Podocyte glycocalyx

SD; slit diaphragm

Table 2-3. Clinical parameters of chronic GN model and control mice

		dsDNA ($\mu\text{g/mL}$)	BUN (mg/dL)	Cre (mg/dL)	uACR ($\mu\text{g/mg}$)
BXSB	4 months	72.35 ± 4.45	29.80 ± 3.50	0.56 ± 0.17	96.02 ± 54.43
BXSB- <i>Yaa</i>	4 months	883.07 $\pm 85.11^{*\dagger}$	38.93 ± 9.92	1.30 ± 1.03	835.20 $\pm 592.33^{*\dagger}$
B6	9 months	61.57 ± 19.08	22.88 ± 1.42	0.12 ± 0.01	2.92 ± 0.40
	9 months	85.01 $\pm 35.86^*$	24.92 ± 5.42	0.21 ± 0.05	29.02 $\pm 21.57^*$
B6.MRL	15 months	407.55 $\pm 166.38^*$	30.24 ± 4.63	0.68 ± 0.56	378.61 $\pm 278.87^*$

Values are mean $\pm$ S.E. dsDNA, double-strand DNA; BUN, serum blood urea nitrogen; Cre, serum creatinine; uACR, urinary albumin creatinine ratio. *Significantly different from each control, $\dagger$ Significantly different from B6.MRL at 15 months (Mann–Whitney U test, $P < 0.05$); $n \geq 5$.

Table 2-4. Quantitative evaluations of glomerular damage

	Glomerulus damage score	Cell number in 1 glomerulus	Thickness of GBM (μm)
BXSB 4 month	34.01 ± 10.45	35.12 ± 2.33	1.98 ± 0.11
BXSB-Yaa 4 month	180.68 $\pm 60.77^*$	67.09 $\pm 9.96^*$	4.01 $\pm 1.69^*$
B6 9month	78.11 ± 21.26	41.12 ± 4.27	2.31 ± 0.08
B6.MRL 15 month	195.96 $\pm 42.53^*$	55.96 $\pm 3.55^*$	5.96 $\pm 0.98^*$

GBM; glomerular basement membrane. Values are mean $\pm$ S.E. *Significantly different from each control (Mann-Whitney U test, $P < 0.05$); $n \geq 5$.

Table 2-5. Relation between histological parameters and expression of podocyte functional proteins

Parameter/protein	BXSB- <i>Yaa</i>			B6.MRL		
	Nephrin	Podocin	Synaptopodin	Nephrin	Podocin	Synaptopodin
CD3-positive cell number	-0.700	-0.600	-0.700	-0.493	0.309	-0.319
B220-positive cell number	-0.308	-0.564	-0.564	-0.030	0.277	-0.577

Values are the Spearman's rank correlation coefficients. $n \geq 5$.

Table 2-6. Relation between clinical parameters and podocyte functional mRNA expression

BXSB- <i>Yaa</i>								B6.MRL						
Parameter /mRNA	SD protein			Cytoskeletal protein			Podocyte glycocalyx	SD protein			Cytoskeletal protein			Podocyte glycocalyx
	<i>Nphs1</i>	<i>Nphs2</i>	<i>Synpo</i>	<i>Actn4</i>	<i>Cd2ap</i>	<i>Myh9</i>	<i>Podxl</i>	<i>Nphs1</i>	<i>Nphs2</i>	<i>Synpo</i>	<i>Actn4</i>	<i>Cd2ap</i>	<i>Myh9</i>	<i>Podxl</i>
sADA	-0.569*	-0.464	-0.420	-0.662**	-0.310	-0.279	-0.437	-0.557*	-0.121	-0.439	-0.421	-0.275	-0.379	-0.578*
BUN	-0.245	-0.159	-0.203	-0.286	-0.144	-0.256	-0.167	-0.066	-0.170	-0.379	-0.159	-0.352	-0.385	-0.324
Cre	-0.295	-0.110	-0.128	-0.195	-0.121	-0.136	-0.191	0.278	0.109	-0.020	0.154	-0.022	-0.199	-0.003
uACR	-0.624**	-0.512*	-0.747**	-0.865**	-0.624*	-0.574*	-0.621**	-0.505*	-0.245	-0.577**	-0.469*	-0.552**	-0.466*	-0.49*

Values are the Spearman's rank correlation coefficients. SD, slit diaphragm; BUN, serum blood urea nitrogen; Cre, serum creatinine; uACR, urinary albumin creatinine ratio. * and **, significantly correlated (Spearman's rank-correlation test, * $P < 0.05$. ** $P < 0.01$); $n \geq 5$.

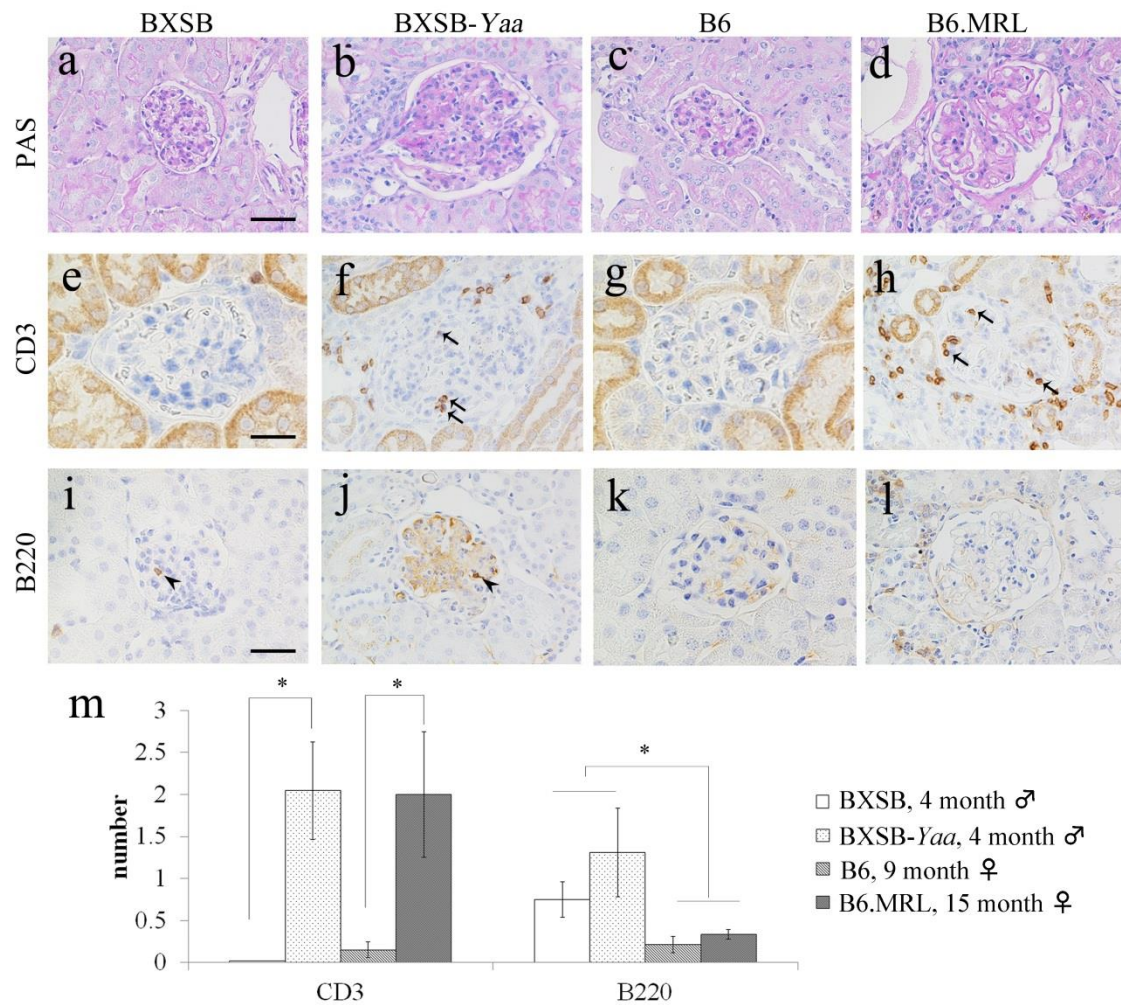


Figure 2-1. Histopathology and immune cell infiltration in chronic GN model mice.

(a–d) Histopathology of renal cortices. BXSB (4 months; a), BXSB-Yaa (4 months; b), B6 (9 months; c), and B6.MRL mice (15 months; d). In the glomerulus, mesangial matrix expansion, mesangial cell proliferation, and periglomerular cell infiltration are observed in the BXSB-Yaa and B6.MRL mice models. PAS-H staining. Bars = 50 μ m.

(e–l) Immunohistochemistry of CD3 (e–h) and B220 (i–l). BXSB (e and i), BXSB-Yaa (f and j), B6 (g and k), and B6.MRL mice (h and k). Bars = 50 μ m. CD3-positive cells are observed in the glomerulus and renal interstitium of BXSB-Yaa and B6.MRL mice (arrows). B220-positive cells are observed in the glomerulus of BXSB and BXSB-Yaa mice (arrowheads), but not in B6 and B6.MRL mice.

(m) Numbers of CD3-positive and B220-positive cells in the glomerulus. BXSB and BXSB-Yaa mice: 4 months. B6 mice: 9 months. B6.MRL mice: 15 months. Values are mean $\pm$ S.E. *, significantly different (Mann–Whitney U test, $P < 0.05$); $n = 5$.

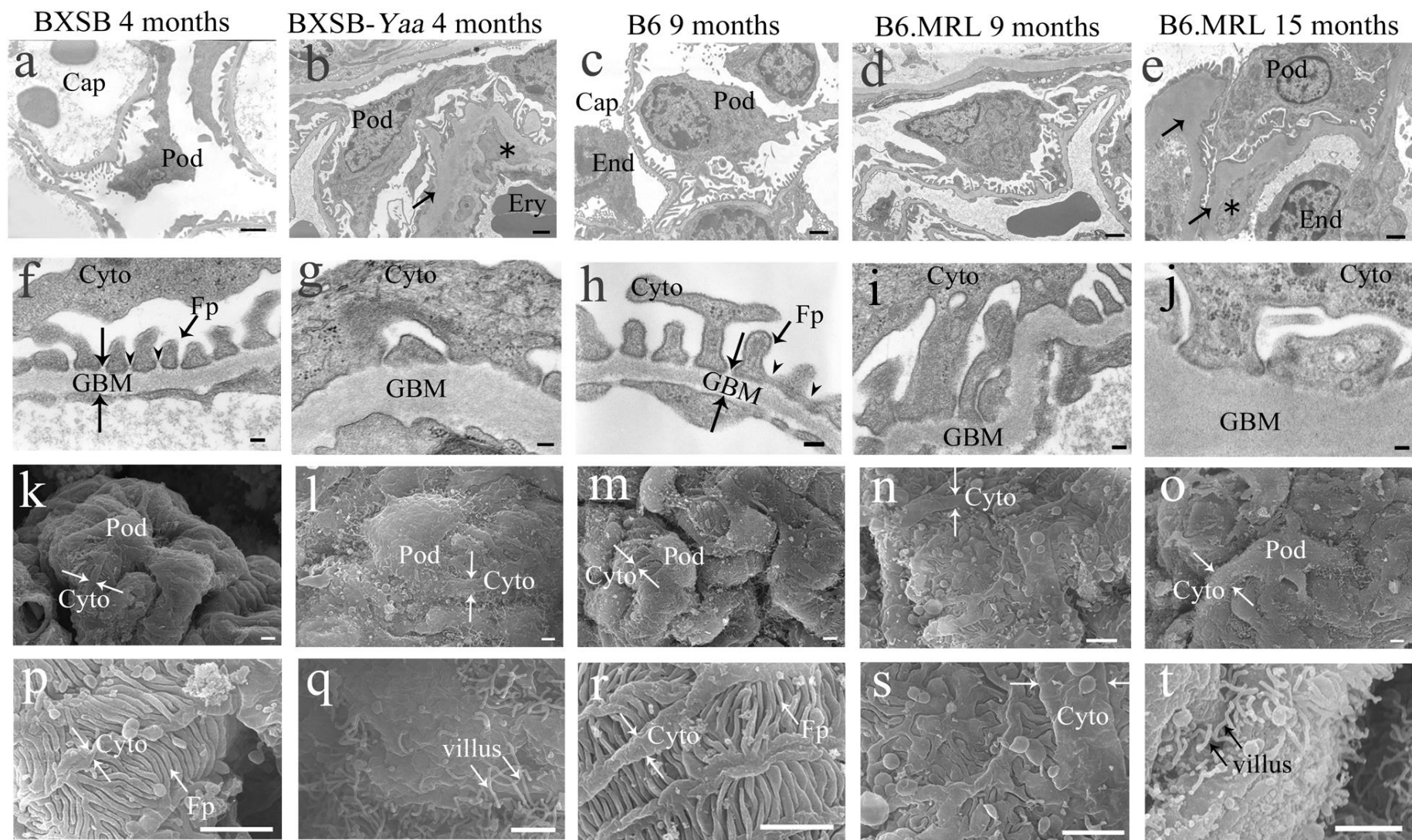


Figure 2-2. Ultrastructural changes of the glomerulus in chronic GN model mice.

(a–j) Ultrastructure of the glomerulus under a TEM. BXSB (4 months; a and f), BXSB-*Yaa* (4 months; b and g), B6 (9 months; c and h), B6.MRL (9 months; d and i), and B6.MRL mice (15 months; e and j). Compared with the podocytes (Pod) of BXSB and B6 mice, showing clear cytotrabecula (Cyto, which is also known as primary process) and cytopodium (foot process, Fp, which is also known as secondary process) (a, c, f, and h), the Fps of BXSB-*Yaa* and B6.MRL mice show irregular arrangements with hypertrophy and partial fusions (b, d, e, g, i, and j). The GBM is thickened and wrinkled in BXSB-*Yaa* and B6.MRL mice (b and e, arrows), and high electron-dense deposits are observed in the double-counteracted GBM of the subendothelial regions (b and e, asterisks). The SD is a clear linear pattern between the Fps of BXSB and B6 mice (f and h, arrowheads). In both BXSB-*Yaa* and B6.MRL mice, the Fp is effaced, and the SD as well as the 3-layer structure of the GBM are unclear (g, i, and j). Ery, erythrocyte. Bars = 1 μ m (a–e) and 100 nm (f–j).

(k–t) Ultrastructure of the glomerulus under a scanning electron microscope. BXSB (4 months; k and p), BXSB-*Yaa* (4 months; l and q), B6 (9 months; m and r), B6.MRL (9 months; n and s) and B6.MRL mice (15 months; o and t). The width of Cyto increase in BXSB-*Yaa* and B6.MRL mice (l, n, and o) compared with the controls (k and m) and the Fps are unclear in the former Pod. The engagements of each Fp are irregular, and microvillus-like processes (villi) are observed on the surface of Pod in BXSB-*Yaa* and B6.MRL mice. Bars = 2 μ m.

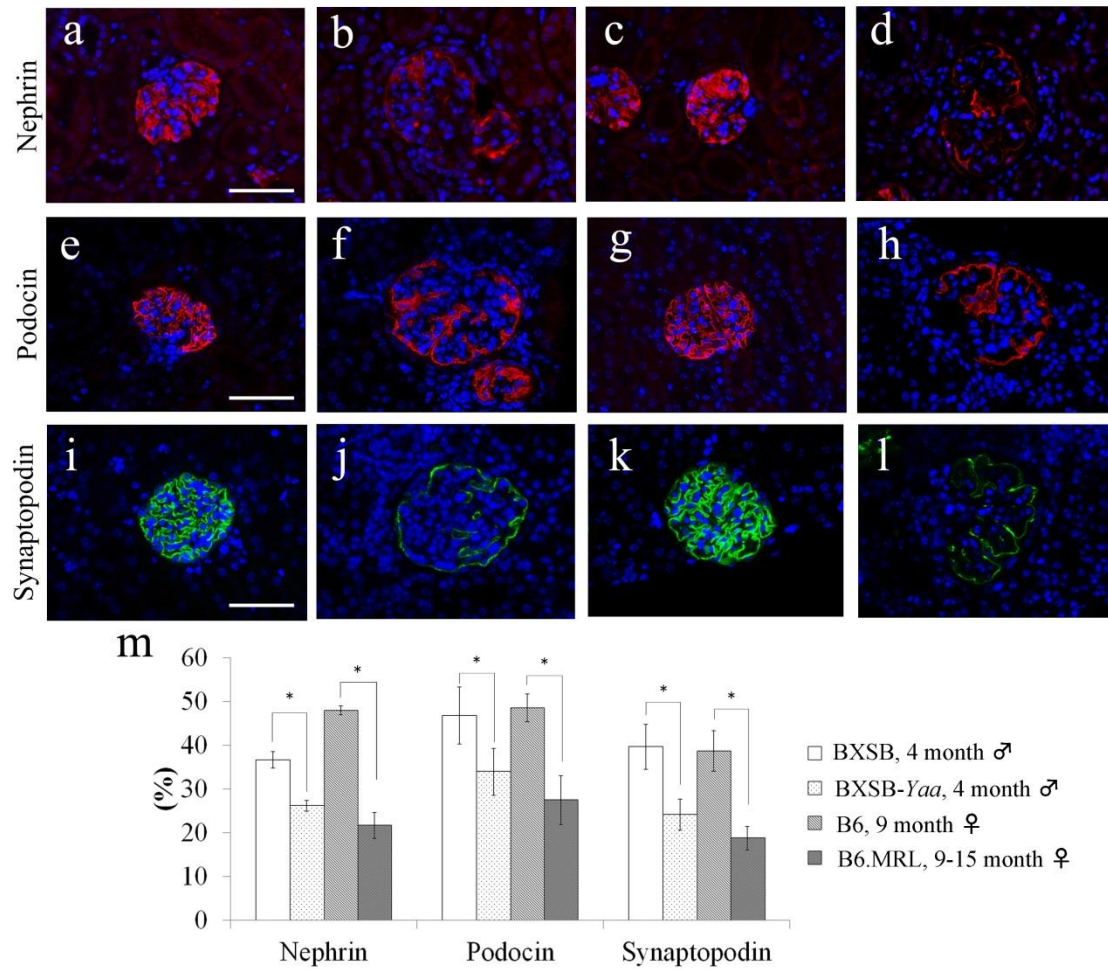


Figure 2-3. Localizations of podocyte functional markers in chronic GN model mice.

(a–l) Immunofluorescence of nephrin (a–d), podocin (e–h), and synaptopodin (i–l). BXSB (4 months; a, e, and i), BXSB-Yaa (4 months; b, f, and j), B6 (9 months; c, g, and k), and B6.MRL mice (15 months; d, h, and l). The positive reactions for nephrin, podocin, and synaptopodin tend to be faint, partially show granular patterns, and localize to the glomerular edge rather than the center in BXSB-Yaa and B6.MRL mice glomeruli. Bars = 50 μ m.

(m) Comparison of SD protein-positive area ratio. The relative immune-positive areas of nephrin, podocin, and synaptopodin in the glomerulus. BXSB and BXSB-Yaa mice: 4 months. B6 mice: 9 months. B6.MRL mice: 15 months. Values are mean $\pm$ S.E. *, significantly different from each control mice (Mann-Whitney *U* test, $P < 0.05$); $n = 5$.

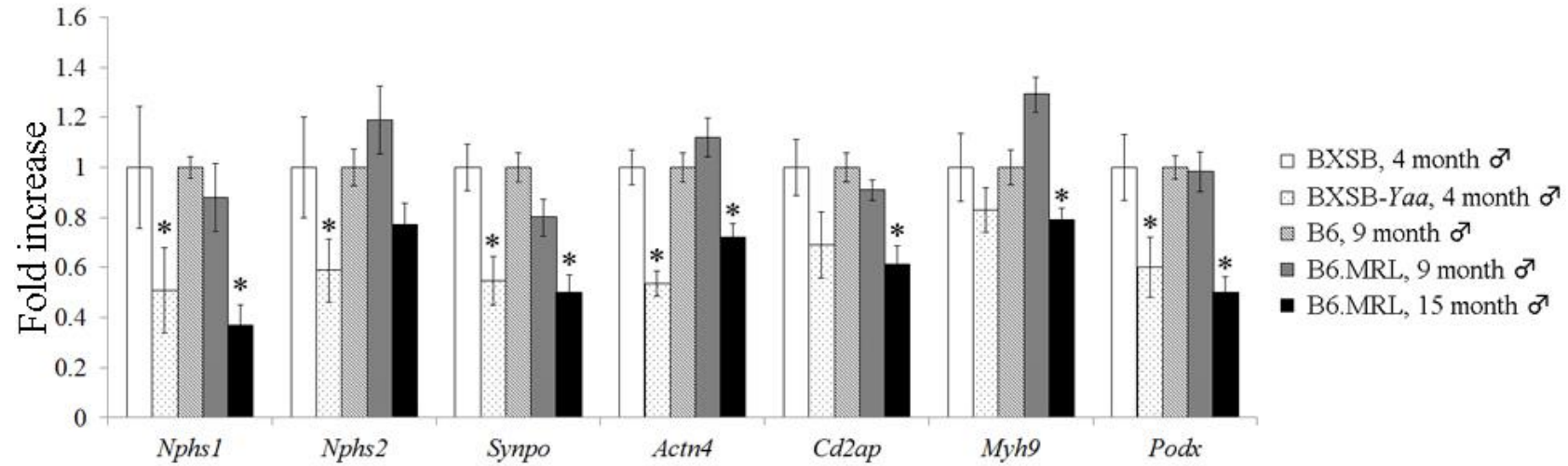


Figure 2-4. mRNA expression levels of podocyte functional markers in chronic GN model mice.

The relative mRNA expression of podocyte functional markers was analyzed by quantitative real-time PCR, using isolated glomerulus samples. BXSB (4 months), BXSB-Yaa mice (4 months), B6 mice (9 months), and B6.MRL mice (9 and 15 months). Each podocyte functional marker was normalized to *Actb*. Values are mean $\pm$ S.E. *, significantly different from each control mice (Mann-Whitney *U* test, $P < 0.05$); $n \geq 4$.

Chapter 3

Genetic factors contributing

to autoimmune glomerulonephritis in BXSB/MpJ-*Yaa* mice

Introduction

Chronic GN is one of the major CKDs that is primarily caused by certain infections, drugs, and SLE (Kriz and LeHir, 2005; Nakai et al., 2006), and its progression was strongly affected by the abnormality of genomic background in patients (Rao et al., 2007). In Chapters 1 and 2, the author investigated the pathology of BXSB-*Yaa* mice as a model of chronic GN and clarified that they develop GLs with decreased functional podocyte proteins, thereby leading to proteinuria subsequent to the formation of TILs. BXSB-*Yaa* mice carry a mutant gene locus *Yaa*, and it was thought that only males show severe chronic GN (Merino et al., 1992). Interestingly, the B6-type background congenic strain carrying *Yaa* does not develop an autoimmune phenotype (Izui et al., 1988). Furthermore, Haywood et al. used male backcrosses between BXSB-*Yaa* and C57BL/10 mice to identify 6 autosomal loci (*Bxs1–6*) that play important roles in susceptibilities of mortality, autoantibody production, splenomegaly, and chronic GN (Haywood et al., 2006). These findings indicate that *Yaa* as well as BXSB mouse genomic background is associated with the disruption of autoimmunity leading GLs.

Recent studies have revealed susceptibility loci linked with the development of SLE in mouse models, such as *Sle1-6*, *Lbw1-7*, *Sbw1-2*, *Nba1-3*, *Lprm1-5*, and *Mag* (Nguyen et al., 2002; Ichii et al., 2008b). *Sle1*, *Nba2*, and *Mag* are linked to the development of membranous proliferative GN progression, and *Lbw7* is linked to autoantibody production and splenomegaly. Interestingly, these loci were mapped to the same telomeric region of Chr.1 in different inbred mouse SLE

models (Nguyen et al., 2002)..

The telomeric region of Chr.1 includes major immune-related genes such as those of the Fc gamma receptor (Fc γ R) family and Ifi200 family (Ichii et al., 2008b). Fc γ Rs recognize the Fc portion of IgG and play crucial roles in determining the response to deposited ICs (Takai, 2005). Immune response activation through Fc γ R is caused by binding of ICs containing IgG to active Fc γ Rs such as Fc γ RIII and Fc γ RIV in mouse (Reth, 1989; Takai, 2005). Fc γ Rs also negatively regulate immune responses; mouse Fc γ RIIB, which is the only inhibitory Fc γ R, takes balance of immune responses to deposited ICs (Reth, 1989; Reiser et al., 2002; Takai, 2005). Ichii et al. previously demonstrated that altered ratios of mouse Fc γ RIIB and Fc γ RIII in the kidney are strongly related to the development of autoimmune-mediated chronic GN in congenic mouse strain B6.MRL (Ichii et al., 2008a). The Ifi200 family, including *Ifi202b*, *Ifi203*, *Ifi204*, and *Ifi205*, consists of genes induced by IFNs (Choubey, 2012). These genes encode highly homologous proteins and are suggested to play important roles in the regulation of cellular growth, differentiation, survival, and death (Ludlow et al., 2008; Zimmerman et al., 2010; Choubey, 2012).

Recent studies using B6.MRL and BXSB-*Yaa* mice clarified that renal *Ifi202b* expression increased with the development of autoimmune-mediated chronic GN (Haywood et al., 2006; Ichii et al., 2010a). In particular, similar to *Sle1*, *Lbw7*, *Nba2*, and *Bxs3* localizes to the telomeric region of BXSB-type Chr.1, which includes the coding regions of *Fcgr2b*, *Fcgr3*, *Fcgr4*, and *Ifi202b* and closely links

to the development of autoantibody production, splenomegaly, and chronic GN (Haywood et al., 2006)

In Chapter 3, to clarify the genomic factors affecting the progression of CKD, the author genetically and pathologically investigated the GLs of BXSB/MpJ (BXSB) mice, not carrying *Yaa* mutation, in addition to those of BXSB-*Yaa* mice. The author's data indicated that the BXSB-type genome causes membranous proliferative GN with significant contribution of the telomeric region of Chr.1, and *Yaa* enhances the expression of chronic GN candidate genes localizing to this locus, thereby leading to severe membranous proliferative GN in males.

Materials and Methods

Ethics statement

All animal experimentations in Chapter 3 were performed as described in Chapter 1.

Animals and sample preparations

Experimental animals were handled as shown in Chapter 1. Female BXSB (2–13 months), female B6 (4–12 months), male BXSB (4 months), and male BXSB-Yaa mice (4 months) were purchased from Japan SLC Inc. (Shizuoka, Japan). The urine, serum, and kidneys were collected and processed as shown in Chapter 1. In addition to PAS-H staining, PFA-fixed paraffin sections were used for periodic acid methenamine silver-hematoxylin (PAM-H) staining. Remaining renal tissue was stored in RNAlater solution (Life Technologies, Carlsbad, CA, USA)

Histological analysis

To assess the severity of chronic GN, semi-quantitative glomerular damage scoring was performed as described in Chapter 2.

Glomerular isolation

Mouse glomeruli were isolated by a bead perfusion method as described in Chapter 2 (Takemoto et al., 2002). The collected glomeruli were used for total

RNA isolation.

Serological and urinary analysis

To evaluate the systemic autoimmune condition and renal function, serum levels of anti-dsDNA antibody, BUN, and Cre and the uACR levels were measured as described in Chapter 2. The BXS^B-Yaa mice were divided into 3 groups depending on their uACR levels: no clinical signs (<100 µg/mg), mild stage (101–250 µg/mg), and severe stage (>250 µg/mg).

Immunohistochemistry and histoplanimetry

Immunostaining for nephrin, podocin, synaptopodin, CD3, and B220 was performed according to the procedure shown in Table 3-1. Details of the procedures are described in Chapter 2. To evaluate T-cell and B-cell infiltration into the glomeruli, the number of CD3-positive and B220-positive cells was counted. In these measurements, 20 glomeruli were counted in one kidney section in each group ($n \geq 3$), and the values were expressed as means.

Quantification of positive immunohistochemical reactions of podocyte functional molecules were performed as described in the following. The glomerulus area and black pixels of a positive reaction were measured, and the number of pixels per area was calculated for each protein using the BZII-Analyzer (Keyence, Osaka, Japan).

Immunofluorescence

Immunofluorescence analysis of nephrin, podocin, and synaptopodin was performed according to the procedure shown in Table 3-1 and Chapter 2. The sections were examined using a fluorescence microscope (BZ-9000; Keyence).

Electron microscopy

Ultrastructural analysis with a TEM was performed as described in Chapter 2.

Reverse transcription and Real-time PCR

To examine mRNA expression, total RNA isolation, cDNA synthesis, real-time PCR analysis was performed as described in Chapter 2. The primer pairs are shown in Table 3-2.

Microarray analysis

Total RNA from isolated glomeruli of mild stage male BXSB and BXSB-*Yaa* mice was purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The total RNA was used to profile the expression of 8×60 k mRNA using Agilent's microarray profiling service (DNA Chip Research Inc., Yokohama, Japan). Briefly, RNA samples were checked for RNA integrity using a Bioanalyzer2100 (Agilent Technologies), amplified by the Genome® Complete Whole Genome Kit (Sigma, St Louis, MO, USA), labeled with Cy3,

and hybridized to SurePrint G3 Gene Expression Microarrays (Agilent Technologies). Slides were scanned by a DNA microarray scanner (Agilent Technologies), and the images were digitized using Feature Extraction Ver.9.5.3 (Agilent Technologies). Finally, data were normalized and expressed as fold increase vs. those from BXSb mice with GeneSpringGX11 (Agilent Technologies). The data discussed in this publication have been deposited into NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through the GEO Series accession number GSE 51230.

Statistical analysis

Results were statistically analyzed as described in Chapter 1.

Results

Clinical Parameters of Female BXSB Mice

The author analyzed the spleen/body weight ratio, serum levels of anti-dsDNA antibody, BUN levels, serum Cre levels, and uACR in female BXSB mice at 2, 7, 10, and 13 months of age to assess the systemic autoimmune conditions and renal functions (Table 3-3). The spleen/body weight was significantly higher at 13 months than that at 2 months. Although no significant increase was detected in the BUN and Cre levels between 2 months of age and other ages, the uACR and anti-dsDNA antibody levels significantly increased from 7 months onward. All examined parameters were the highest at 13 months of ages.

Glomerular Histopathology in Female BXSB Mice

The glomerular histopathology in female BXSB mice was examined in the kidney sections stained by PAS-H or PAM-H. Female BXSB mice developed membranous proliferative GN from 7 months of age, which was characterized by glomerular hypertrophy, increased mesangial cells and their matrix (Figs. 3-1b-d), and thickening of the GBM (Figs. 3-1f-h). Furthermore, the GBM of female BXSB mice formed spike-like structures, and mesangial interposition was observed between the double contouring GBM at 10 and 13 months of age (Figs. 3-1g and h). No GLs were observed at 2 months of age (Figs. 3-1a and e).

The glomerulus damage score (Fig. 3-1i) and cell number in one

glomerulus (Fig. 3-1j) significantly increased from 7 months, and the thickness of the GBM (Fig. 3-1k) increased from 10 months of age.

Unlike female BXSB mice, male BXSB mice develop neither autoimmune symptoms nor renal disease at 12 months of age (Fig. 3-2).

Immune Cell Infiltration of the Glomerulus of Female BXSB Mice

The glomerular infiltration of immune cells in female BXSB mice was assessed by immunohistochemistry for CD3 (T-cell marker) and B220 (B-cell marker). CD3-positive cells were observed in the glomerulus as well as the tubulointerstitium at 13 months (Fig. 3-3b) but not at 2 months of age (Fig. 3-3a). Comparatively, B220-positive cells were scarcely observed in female BXSB mice at 2 and 13 months of age (Figs. 3-3c and d). In histoplanimetry, the number of CD3-positive cells in the glomerulus was significantly higher at 13 months than at 2 months of age (Fig. 3-3e). Consistent with the histological observation, the number of B220-positive cells in the glomerulus was quite low at both 2 and 13 months of age.

Glomerular Ultrastructure in Female BXSB Mice

The glomerular ultrastructure of female BXSB mice was analyzed at 2 and 10 months by using TEM. Female BXSB mice at 2 months of age showed clear cytopodium, which is also known as FP or secondary process (Figs. 3-4a and c). At 10 months of age, FPs showed irregular arrangements with hypertrophy and

partial fusions (Figs. 3-4b and d). Furthermore, at 10 months of age, the GBM was thickened and wrinkled (Figs. 3-4b and d), and high electron-dense deposits resembling ICs were observed at the subendothelial regions (Fig. 3-4b, asterisk) and in the double-counterstained GBM (Fig. 3-4d).

Podocyte Injuries in Female BXSB Mice

The localization of podocyte functional molecules (nephrin, podocin, and synaptopodin) was examined by immunostaining (Fig. 3-5). Linear-positive immunofluorescence reactions for nephrin, podocin, and synaptopodin were observed along the glomerular capillary rete in female BXSB mice at 2 months (Figs. 3-5a, c, and e). In contrast, those at 13 months of age tended to be faint, showed partial granular patterns, and localized to the glomerular edge rather than the center (Figs. 3-5b, d, and f). In histoplanimetry, the relative positive areas of these molecules in the glomerulus were significantly smaller at 13 months than at 2 months (Fig. 3-5g). On the other hand, no apoptotic podocyte was detected in the glomerulus at any age by immunohistochemistry for single strand DNA (data not shown).

Chronic GN Candidate Gene Expression in the Kidney of Female BXSB Mice

Fig. 3-6a is a schematic of murine Chr.1. The common region of autoimmune GN-associated loci (indicated by black bars) such as *Sle1*, *Lbw7*, *Nba2*, and *Mag* include the autoimmune-mediated chronic GN candidate genes

such as the FcγR family including *Fcgr2b* and *Fcgr3*, and the Ifi200 family including *Ifi202b* (Haywood et al., 2006; Ichii et al., 2008a; Ichii et al., 2010a). The expression levels of *Fcgr2b* and *Fcgr4* were significantly lower, and that of *Fcgr3* was significantly higher in the kidney of female BXSB than of female B6 mice (Figs. 3-6b–d). Furthermore, the renal *Fcgr3/Fcgr2b* ratio of female BXSB mice was significantly higher than that of female B6 mice (Figs. 3-6e). The renal *Ifi202b* expression level in female BXSB mice was significantly higher than that of female B6 mice (Figs. 3-6f).

Contribution of Yaa to the Expression of Genes Coded by Telomeric Region of Chr.1 in Male BXSB Mice

Table 3-4 shows the clinical parameters of male BXSB and male BXSB-*Yaa* mice at 4 months of age. Because male BXSB-*Yaa* mice developed membranous proliferative GN with individual differences, they were divided into three groups (no clinical sign, mild stage, and severe stage) according to the uACR levels (Table 3-4). Male BXSB-*Yaa* mice in the mild group showed significantly increased uACR levels, but not BUN and Cre compared to male BXSB mice (Table 3-4). uACR levels of male BXSB-*Yaa* mice were drastically elevated in the severe stage group compared to the other groups as well as female BXSB mice (Table 3-3).

To analyze the contributions of *Yaa* to the expression of genes localizing to the telomeric region of Chr.1, the author comprehensively compared the

glomerular mRNA expression between male BXSB and male BXSB-*Yaa* mice in the mild group by microarray analysis (Table 3-5). Table 3-5 lists only the genes showing significantly changed expression in male BXSB-*Yaa* mice compared to male BXSB mice. In particular, genes belonging to the Fc γ R family (*Fcgr2b*, *Fcgr3*, and *Fcgr4*) and Ifi200 family (*Ifi202b*, *Ifi203*, and *Ifi204*) were significantly upregulated in male BXSB-*Yaa* compared to male BXSB mice.

Discussion

SLE is an autoimmune disorder involving the deposition of IC or direct autoantibody deposition onto multiple organ systems, including the kidney, skin, lung, heart, hematopoietic system, and brain (Oke and Wahren-Herlenius, 2013). SLE-related membranous proliferative GN, known as lupus nephritis, which leads to severe persistent proteinuria, chronic renal failure, and ESRD, remains one of the most severe complications of SLE and is associated with significant morbidity and mortality (Oke and Wahren-Herlenius, 2013). It has been suggested that the glomerular IC depositions in lupus nephritis cause GLs such as mesangial cell proliferation and GBM thickening, which lead to proteinuria and subsequent TILs (Kriz and LeHir, 2005; Oke and Wahren-Herlenius, 2013). BXSB-*Yaa* mouse strain is one of the common spontaneous murine models of autoimmune-mediated membranous proliferative GN, and it has been considered that only the males show severe membranous proliferative GN because of the *Yaa* gene locus on the Chr. Y (Kawano et al., 2013). In Chapters 1 and 2, the author previously demonstrated that male BXSB-*Yaa* mice at 3-4 months of age clearly show membranous proliferative GN associated with glomerular IC depositions; no lesions were observed in females at the same age. Therefore, the pathophysiology of the BXSB-*Yaa* strain has been reported in males but not females.

The present study clarified that female BXSB mice clearly develop autoantibody production and membranous proliferative GN with mild albuminuria characterized by glomerular hypertrophy, increased mesangial cells and their

matrix, and thickening of GBM at 7 months of age. At 10–13 months, these GLs become severe with glomerular IC depositions and podocyte injuries. These findings demonstrated for the first time that aged female BXSB mice clearly develop membranous proliferative GN similar to human lupus nephritis and indicated the BXSB-type genome could cause autoimmune-mediated membranous proliferative GN without the contribution of *Yaa*.

In relation to several clinical and experimental autoimmune diseases, sex hormones or chromosomes are considered to be directly responsible for sex-related differences of SLE-related symptoms such as typical skin lesions, serositis, and renal disease; more serious deterioration is observed in females than in males (Bouman et al., 2005). Ichii et al. also reported the sex-related differences of autoimmune mediated membranous proliferative GN in B6.MRL mice as a result of sex hormones (Ichii et al., 2009). Indeed, unlike female BXSB mice, male BXSB mice that do not carry *Yaa* develop neither autoimmune symptoms nor renal disease at 12 months of age (Fig. 3-2). Without *Yaa* contribution, the BXSB-type genome might cause more severe autoimmune-mediated membranous proliferative GN in females than in males because of sex-related differences in immunity.

Bxs3, a chronic GN susceptibility locus on Chr.1 of BXSB-*Yaa* mice, contains the coding genes of FcγRs, which are key molecules in the pathogenesis of chronic GN in both mouse and human (Fujii et al., 2003; Ichii et al., 2008a; Zhou et al., 2010). Briefly, the author have demonstrated that the congenic mice containing the MRL/MpJ-type *Fcgr* locus develop imbalanced expression of inhibitory *Fcgr2b*

and active *Fcgr3* in the kidney and immune organs, which influences the pathogenesis of autoimmune-mediated membranous proliferative GN (Ichii et al., 2008a). In the present study, *Fcgr2b* downregulation and *Fcgr3* upregulation were demonstrated in female BXSB mouse kidneys. In the mouse kidney, Fc γ R-positive reactions were detected in the glomerular mesangial regions (Ichii et al., 2008a). Glomerular mesangial cells participate in the regulation of the GFR and in glomerular pathogenesis (Floege et al., 1994). Furthermore, Radeke et al. demonstrated that normal murine mesangial cells mainly expressed Fc γ RII rather than Fc γ RIII, and that stimulation by proteins such as IFN γ caused downregulation of Fc γ RII, activation of Fc γ RIII, and induction of chemokine productions (Radeke et al., 2002). Interestingly, previous sequence analysis identified a haplotype defined by deletions in the *Fcgr2b* promoter region that is present in major lupus-prone mouse strains including NZB, BXSB-*Yaa*, and MRL/MpJ (Jiang et al., 1999; Xiu et al., 2002). According to Pritchard et al., the polymorphism is associated with reduced expression of Fc γ RIIB and subsequent failure of Fc γ RIIB-mediated negative immune regulation of activated lymphocytes, thereby leading to immune hyperactivity in these strains (Pritchard et al., 2000). From these findings, the altered balance of inhibitory and active Fc γ Rs in mesangial cells might also contribute to the pathogenesis of membranous proliferative GN in female BXSB mice through altered local production of chemokines or cytokines.

The *Bxs3* locus also contains *Ifi200* family genes (Haywood et al., 2006), and elevated *Ifi202* expression was reported in the kidneys of several GN models such

as NZB/WF1, MRL/MpJ-*lpr*, and B6.MRL mice (Ichii et al., 2010a). In the present study, *Ifi202* was also overexpressed in the kidneys of female BXSB mice. Furthermore, previous microarray analysis revealed that *Ifi202* is overexpressed in male B10.*Yaa.Bxs2/3* mice, which possess the telomeric region of BXSB-type Chr.1 (Haywood et al., 2006). Therefore, *Ifi202* might also be a candidate gene for autoimmune-mediated GN susceptibility in female BXSB mice.

The *Ifi202* protein is generally induced by IFNs in cells such as bone marrow, fibroblasts, lymphocytes, and myoblasts, and has been suggested to contribute to the regulation of cellular differentiation, proliferation, and survival by controlling the activities of several transcription factors (Xin et al., 2003; Ludlow et al., 2008; Zimmerman et al., 2010; Choubey, 2012). In the present study, the number of T-cells increased in female BXSB mouse glomeruli. Chen et al. demonstrated that the stimulation of both a mouse T-cell hybridoma cell line (2B4.11) and T-cells derived from lupus-prone NZB upregulate the *Ifi202* expression in part through the JNK/c-Jun pathway (Chen et al., 2008). On the basis of these findings, the author proposed that the close correlation between renal overexpression of *Ifi202* and infiltrating inflammatory cells exacerbates the pathological condition of membranous proliferative GN in female BXSB mice similar to other lupus-prone mice.

In Chapter 2, the author observed infiltrating B-cells in the glomerulus of male BXSB-*Yaa* mice, although they were scarce in the lupus-prone B6.MRL (Chapter 2) and female BXSB mice. Therefore, the author considered that the *Yaa*

locus contributes to glomerular B-cell infiltration leading to the severe membranous proliferative GN in male BXSB-*Yaa* mice. Recently, the *Yaa* mutation was identified to be a translocation from the telomeric end of the Chr. X onto the Chr. Y (Subramanian et al., 2006). The duplicated segment contains at least 19 genes, including the TLR family and phosphoribosyl pyrophosphate synthetase; *Tlr7* gene duplication plays a crucial role in the activation of auto-reactive B-cell, thereby contributing to the *Yaa*-mediated enhancement of the autoimmune phenotype (Subramanian et al., 2006). Studies of lupus models have demonstrated that infiltrating B-cells in the kidney secrete antibodies with various antigen specificities, which contribute to increase *in situ* ICs (Espeli et al., 2011). Interestingly, it has also been suggested that infiltrating B-cells play a role in secreting pro-inflammatory cytokines such as TNF- α , IL-6, and IFNs, thereby contributing to the local development of autoimmune diseases, including inflammatory bowel disease, experimental autoimmune encephalomyelitis, and collagen-induced arthritis (Chan et al., 1999; Harris et al., 2005; Bekar et al., 2010; Fujimoto, 2010; Ramanujam et al., 2010; Fillatreau, 2013).

In addition, the chronic GN candidate genes coded by the telomeric regions of Chr.1, including *Fcgr3* and *Ifi202b*, were upregulated in the kidney of male BXSB-*Yaa* mice in the mild stage. Interestingly, the overexpression of *Fcgr4* was also noted in male BXSB-*Yaa* mice, but not male and female BXSB mice. In mice, Fc γ RIV has been more recently identified as an active Fc γ R, which is homologous with human Fc γ RIIIA, and suggested to have similar functions as mouse Fc γ RIII

(Seeling et al., 2013). Taken collectively, these findings suggest that *Yaa* contributes to the hyper-activation of auto-reactive B-cells infiltrating the glomerulus and the further overexpression of the telomeric regions of Chr.1 including *Fcgr3*, *Fcgr4*, and *Ifi202b* in mesangial cells and infiltrating T-cells, thereby contributing to the exacerbation of the autoimmune status and subsequent severe membranous proliferative GN in male BXS*B-Yaa* mice.

In conclusion, the BXS*B*-type genome causes membranous proliferative GN with strong contribution from the telomeric region of Chr.1, and *Yaa* enhances the expression of genes localizing to this locus. These data would contribute to the clarification of the genetic mechanism in the progression of CKD, and the provision of crucial genetic loci carrying candidate molecules which should be clarified in next Chapter.

Summary

The pathogenesis of chronic GN is strongly affected by the abnormality of genomic background in patients. The author clarified the pathological feature of GLs of BXSB-*Yaa* mice, a model of chronic GN, in Chapter 1 and 2. However, the pathological contribution of BXSB-type genome in GN progression remains unclear.

In this Chapter, the author showed that female BXSB mice develop age-related membranous proliferative GN without *Yaa*, a mutated locus on Chr. Y of BXSB-*Yaa* mice contributing the development of autoimmunity. Female BXSB mice clearly developed membranous proliferative GN characterized by increased mesangial cells, thickening of the GBM, double contouring and spike formation of GBM with T-cell infiltrations, and podocyte injuries corresponding with increased autoantibody production and albuminuria. On the other hand, male BXSB mice developed neither autoimmune symptom nor GN. Analysis of the renal levels of the *Fcgrs* and *Ifi200* family genes, which are membranous proliferative GN candidate genes localized to the telomeric region of Chr.1, showed that *Fcgr2b* levels decreased, whereas *Fcgr3* and *Ifi202b* levels increased in female BXSB mice compared to healthy B6 mice. Furthermore, in isolated glomeruli, microarray analysis revealed that *Fcgr3*, *Fcgr4*, and *Ifi202b* expression was higher in male BXSB-*Yaa* than in male BXSB mice.

These findings indicate that the BXSB-type genome causes membranous proliferative GN with strong contribution from the telomeric region of Chr.1, and

Yaa enhances the expression of genes localizing to this locus. These data would contribute to clarify the genetic mechanism in the progression of CKD and provide the crucial genetic loci carrying candidate molecules which should be clarified in next Chapter.

Tables and Figures

Table 3-1. Summary of immunostaining conditions

Immunohistochemistry		Nephrin	Podocin	Synaptopodin	CD3	B220
First antibody		Rabbit polyclonal antibodies (1:500; IBL, Gunma, Japan)	Rabbit polyclonal antibodies (1:800; IBL)	Mouse monoclonal antibodies (1:50; Fitzgerald, MA, USA)	Rabbit polyclonal antibodies (1:150; Nichirei, Tokyo, Japan)	Rat monoclonal antibodies (1:1000; Cedarlane, Ontario, Canada)
Second antibody		Biotinylated goat anti-rabbit IgG antibodies (SABPO kit, Nichirei)	Biotinylated goat anti-rabbit IgG antibodies (SABPO kit, Nichirei)	Simple Stain Mouse MAX-PO(M) (mouse stain kit, Nichirei)	Biotinylated goat anti-rabbit IgG antibodies (SABPO kit, Nichirei)	Biotinylated goat anti-rat IgG antibodies (Caltag Medsystems, Buckingham, UK)
Immunofluorescence		Nephrin	Podocin	Synaptopodin	CD3	B220
First antibody		Same as immunohistochemistry	Same as immunohistochemistry	Same as immunohistochemistry	/	/
Second antibody		Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500, Life Technologies)	Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500, Life Technologies)	Alexa Fluor 488-labeled donkey anti-mouse IgG antibodies (1:500, Life Technologies)	/	/

Table 3-2. Summary of specific gene primers for real-time PCR

Genes (accession no.)	Primer sequence (5'-3') F, forward; R, reverse	Product size (bp)
<i>Fcgr2b</i> (TV1: NM001077189.1 TV2: NM_010187.2)	F: CTGAGGCTGAGAATACGATC R: GTGGATCGATAGCAGAAGAG	307
<i>Fcgr3</i> (NM010188.4)	F: ATCACTGTCCAAGATCCAGC R: GCCTTGAAGCTGGTGATCCTA	346
<i>Fcgr4</i> (NM_144559.2)	F: GGCATTCAAGCTGGTCTCCA R: GCAATAGCCAGCCCATATGG	280
<i>Ifi202b</i> (TV1: NM_008327 TV2: NM_011940)	F: GGCAATGTCCAACCGTAACT R: TAGGTCCAGGAGAGGCTTGA	125
<i>β-Actin</i> (NM007393)	F: TGTTACCAACTGGGACGACA R: GGGGTGTTGAAGGTCTCAAA	165

TV, Transcript Variant.

Table 3-3. Clinical parameters of female BXSB mice

	Spleen/ body weight	dsDNA ($\mu\text{g/mL}$)	BUN (mg/dL)	Cre (mg/dL)	uACR ($\mu\text{g/mg}$)
2 month	0.50 ± 0.062	164.13 ± 36.82	25.70 ± 4.20	0.10 ± 0.00	12.61 ± 5.20
7 month	0.54 ± 0.038	377.07 $\pm 62.42^*$	23.20 ± 4.56	0.15 ± 0.029	17.89 $\pm 2.60^*$
10 month	0.56 ± 0.037	373.23 $\pm 29.29^*$	25.40 ± 2.69	0.18 ± 0.025	27.36 $\pm 5.45^*$
13 month	0.80 $\pm 0.38^*$	585.28 $\pm 35.02^*$	22.80 ± 1.53	0.10 ± 0.00	80.04 $\pm 14.91^*$

Values are mean $\pm$ S.E. dsDNA, double-strand DNA antibody level; BUN, serum blood urea nitrogen level; Cre, serum creatinine level; uACR, urinary albumin creatinine ratio. * Significantly different from 2 month BXSB mice (Mann-Whitney U -test, $P < 0.05$); $n \geq 3$.

Table 3-4. Clinical parameters of renal function in male BXSB-*Yaa* and BXSB mice at 4 months of age

	BUN (mg/dL)	Cre (mg/dL)	uACR (μ g/mg)
BXSB	29.80 $\pm$ 3.50	0.56 $\pm$ 0.17	43.94 $\pm$ 6.93
BXSB- <i>Yaa</i> (no clinical signs)	31.93 $\pm$ 5.92	0.40 $\pm$ 0.10	74.64 $\pm$ 8.01
BXSB- <i>Yaa</i> (mild stage)	24.10 $\pm$ 1.52	0.25 $\pm$ 0.05	187.84 $\pm$ 38.23*
BXSBJ- <i>Yaa</i> (severe stage)	56.43 $\pm$ 24.61*	3.10 $\pm$ 2.70*	1953.14 $\pm$ 1506.69*

Values are mean $\pm$ S.E. dsDNA, double-strand DNA antibody level; BUN, serum blood urea nitrogen level; Cre, serum creatinine level; uACR, urinary albumin creatinine ratio. * Significantly different from control BXSB mice (Mann–Whitney *U*-test, $P < 0.05$); $n \geq 3$.

Table 3-5. Microarray analysis targeting the genes localized to the telomeric region of Chr. 1.

Regulation	Symbol	Accession no.	P-value	BXSB-Yaa /BXSB
Up	<i>Fcgr4</i>	NM_144559	0.00021	45.70
Up	<i>Fcer1g</i>	NM_010185	0.00040	13.70
Up	<i>Itln1</i>	NM_010584	0.00073	10.35
Up	<i>Al607873</i>	NM_001204910	0.00308	9.50
Up	<i>Arhgap30</i>	NM_001005508	0.00033	7.92
Up	<i>Cd48</i>	NM_007649	0.00176	7.75
Up	<i>Fcgr3</i>	NM_010188	0.01988	7.34
Up	<i>Ifi202b</i> (TV1)	NM_008327	0.01687	5.16
Up	<i>Ifi202b</i> (TV2)	NM_011940	0.01828	4.93
Up	<i>Slamf9</i>	NM_029612	0.00167	4.77
Up	<i>Pyhin1</i>	NM_175026	0.00693	4.71
Up	<i>Fcgr2b</i>	NM_001077189	0.01155	4.42
Up	<i>Nhlh1</i>	NM_010916	0.03302	2.89
Up	<i>Aim2</i>	NM_001013779	0.02655	2.54
Up	<i>Ifi204</i>	NM_008329	0.04011	2.36
Up	<i>Mndal</i>	NM_001170853	0.01352	1.73
Up	<i>Ifi203</i>	NM_001045481	0.04582	1.52

Values are fold increase compared to BXSB mice. Only significantly up-regulated genes coded by the telomeric region of murine Chr. 1 are listed in the table. TV, transcript variant. Student-*t* test, $P < 0.05$. n = 3.

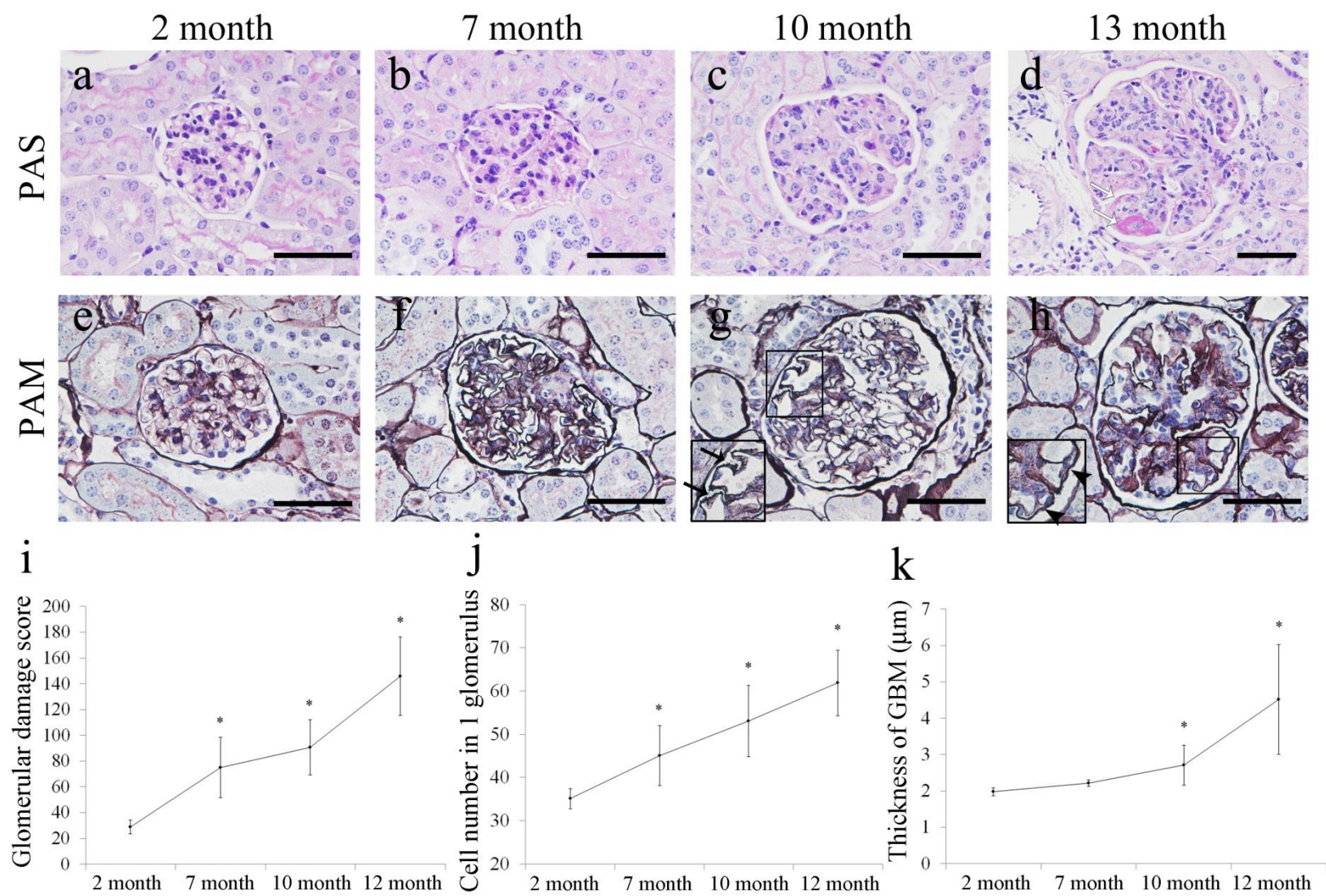


Figure 3-1. Histopathology of female BXSB mice

(a–d) Histopathology of glomeruli in PAS-H staining sections of female BXSB mice. Compared to 2 months (a), mild mesangial matrix expansion and mesangial cell proliferation are observed at 7 months (b). In addition to these lesions, glomerular hypertrophy and cell infiltration are prominent at 10 months (c). At 13 months (d), glomerular proliferative lesions as well as membranous lesions are severe, and PAS-positive materials (white arrows) are observed near the GBM. Bars = 50 μ m.

(e–h) Histopathology of glomeruli in the PAM-H staining sections of female BXSB mice. Compared to 2 months (e), hypertrophy and wrinkling of GBM are observed at 7 months (f). Spike-like structure on GBM (inset, black arrows) is observed at 10 months (g). At 13 months (h), double-contoured GBM and mesangial interposition (inset, black arrowheads) are clearly observed. Bars = 50 μ m.

(i–k) Histological parameters of glomerular damage. Semi-quantification of the glomerulus damage score (i). Cell number per glomerulus (j). Thickness of the GBM (k). Values are mean $\pm$ S.E. *, significantly different from 2 months (Mann-Whitney *U*-test, $P < 0.05$); $n \geq 3$.

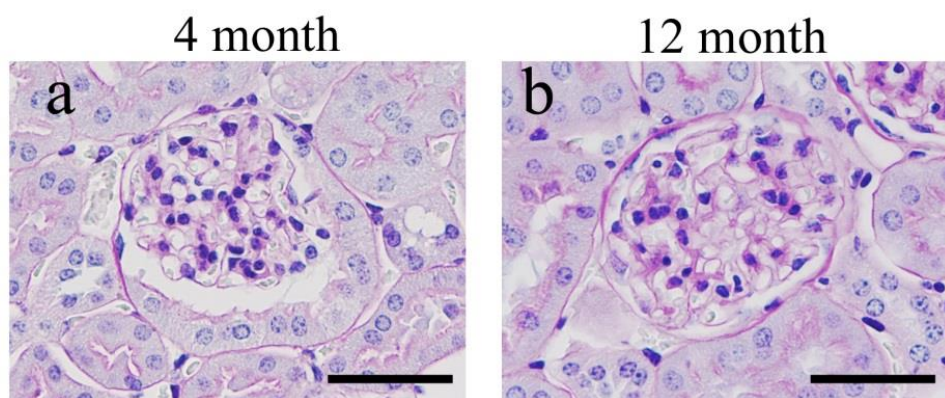


Figure 3-2. Histopathology of male BXSB mice

(a and b) Histopathology of glomeruli in PAS-H staining sections from male BXSB-*Yaa* mice. No GLs were observed at 4 (a) and 12 (b) months. Bars = 50 μm.

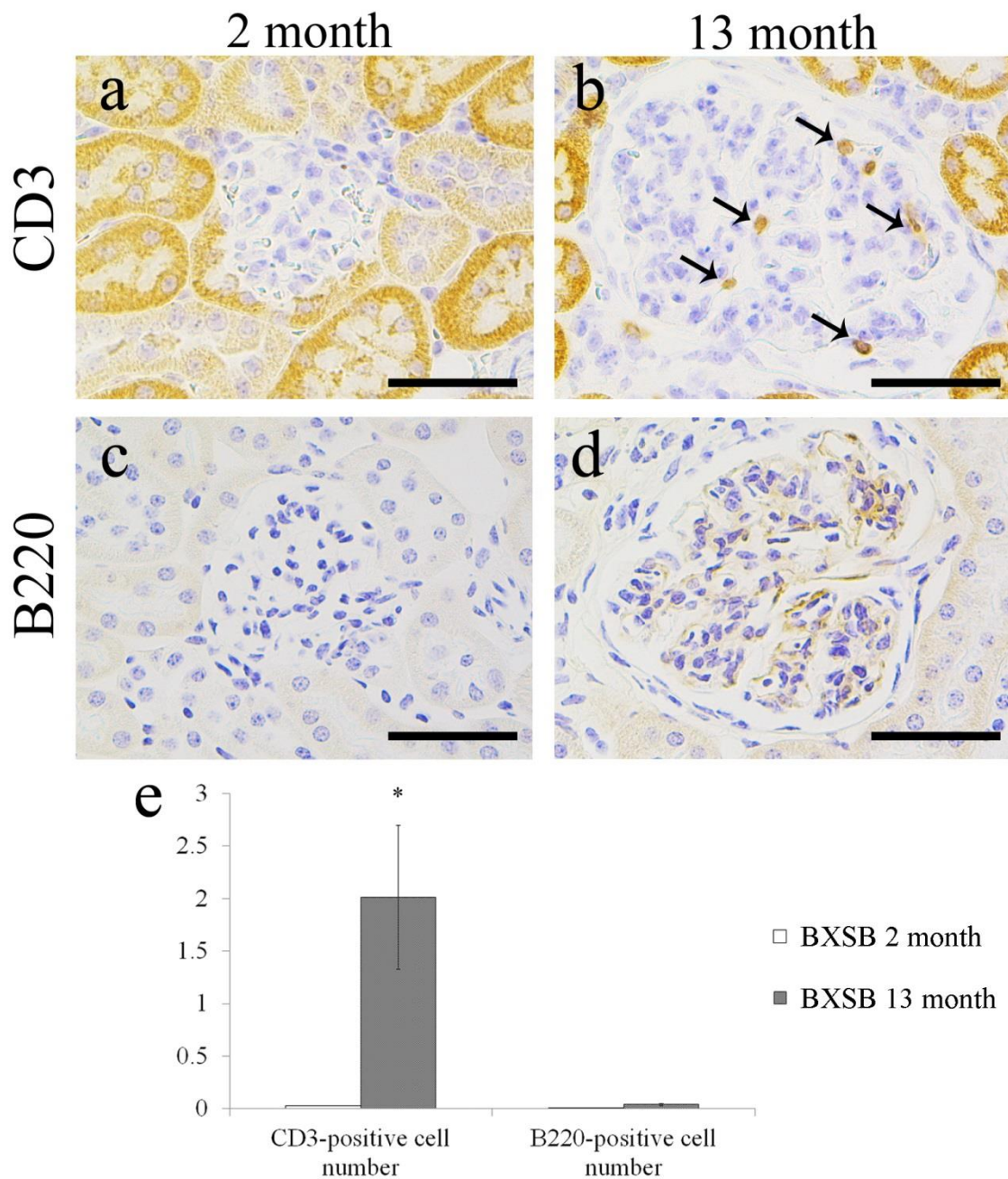


Figure 3-3. Immune cell infiltration in female BXSB mice glomerulus

(a–d) Immunohistochemistry of CD3 and B220 in the kidneys of female BXSB mice. CD3-positive cells are observed in the glomerulus at 13 months (b, arrows) but not 2 months (a). No B220-positive cells are observed at either 2 or 13 months (c and d). Bars = 50 μ m.

(e) The numbers of CD3-positive and B220-positive cells in the female BXSB mouse glomerulus. Values are mean $\pm$ S.E. *, significantly different from 2 months (Mann-Whitney *U*-test, $P < 0.05$); $n \geq 4$.

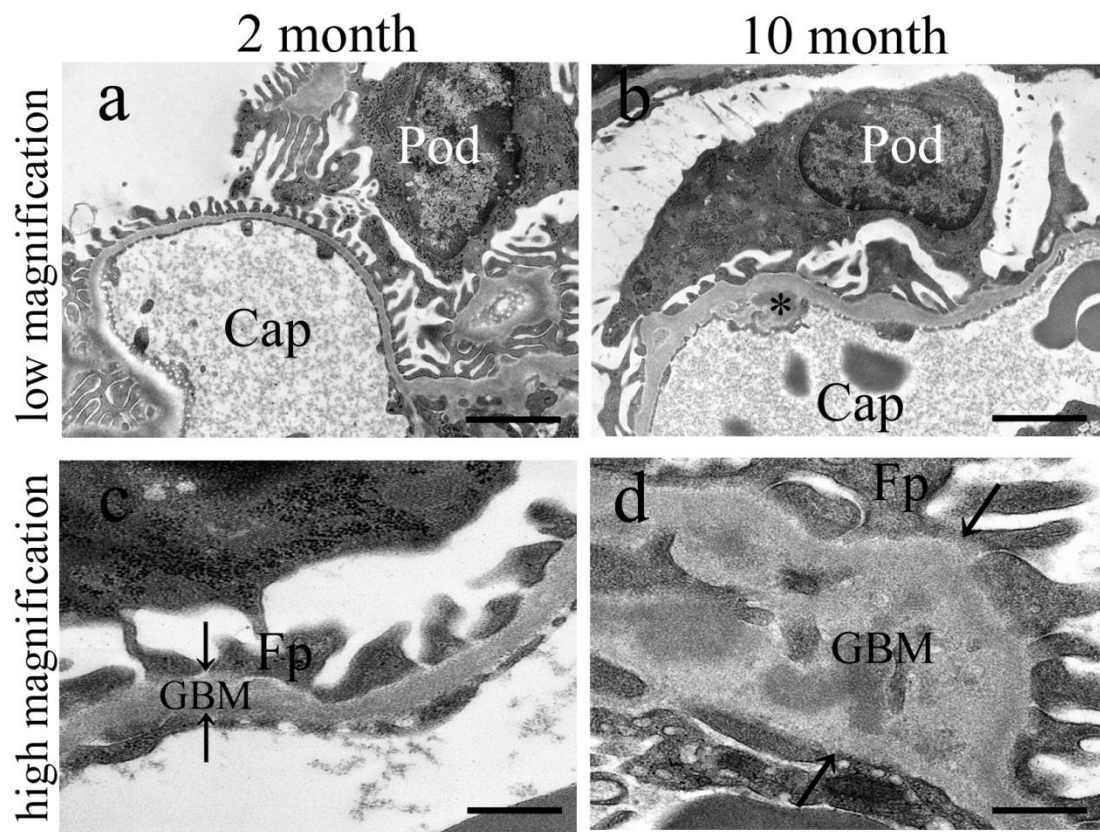


Figure 3-4. Ultrastructural changes of the female BXSB glomerulus

(a–d) Ultrastructure of female BXSB mouse glomeruli using a TEM. The podocytes (Pod) at 2 months show clear cytopodium (foot process, Fp) (a). At 10 months (b and d), the Fps show irregular arrangements with hypertrophy and partial fusions. Compared to the GBM at 2 months (c), the GBM at 10 months is thickened and wrinkled (b and d, arrows), and high electron-dense deposits are observed in the subendothelial regions (b, asterisks) and double-contoured GBM (d). Cap, capillary. Bars = 2 μ m (a, b) and 500 nm (c, d).

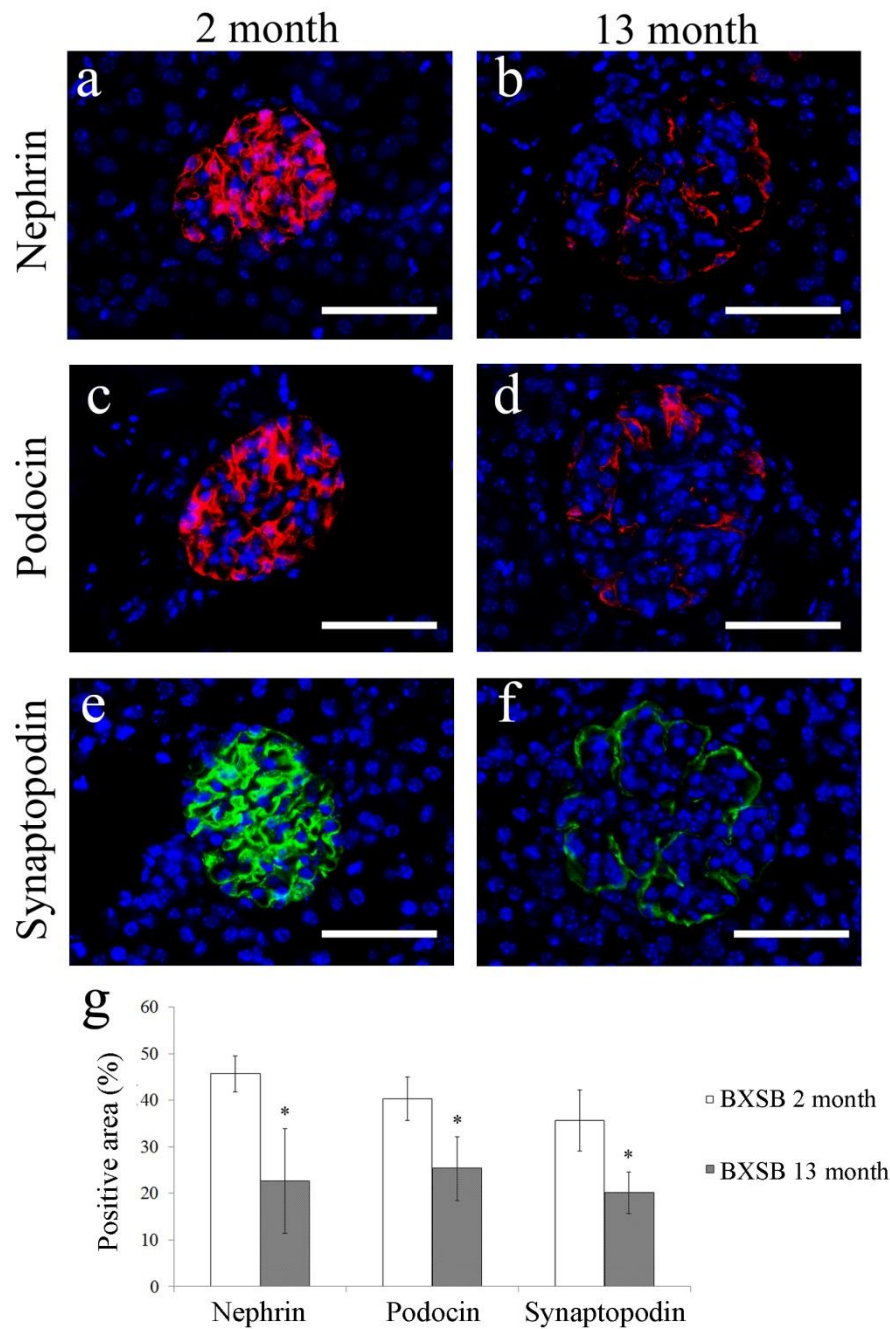


Figure 3-5. Localizations of podocyte functional markers in the female BXSB glomerulus

(a–f) Immunofluorescence of nephrin, podocin, and synaptopodin in female BXSB mouse kidneys. At 2 months, the linear positive reactions of nephrin (a), podocin (b), and synaptopodin (e) are observed along the glomerular capillary rete. At 13 months, the positive reactions for nephrin (b), podocin (d), and synaptopodin (f) are faint, partially show granular patterns, and localize to the glomerular edge rather than the center. Bars = 50 μm.

(g) Comparison of podocyte functional protein-positive area ratio. The relative immune-positive areas of nephrin, podocin, and synaptopodin in the glomerulus. Values are mean ± S.E. *, significantly different from 2 months (Mann-Whitney *U*-test, $P < 0.05$); $n \geq 3$.

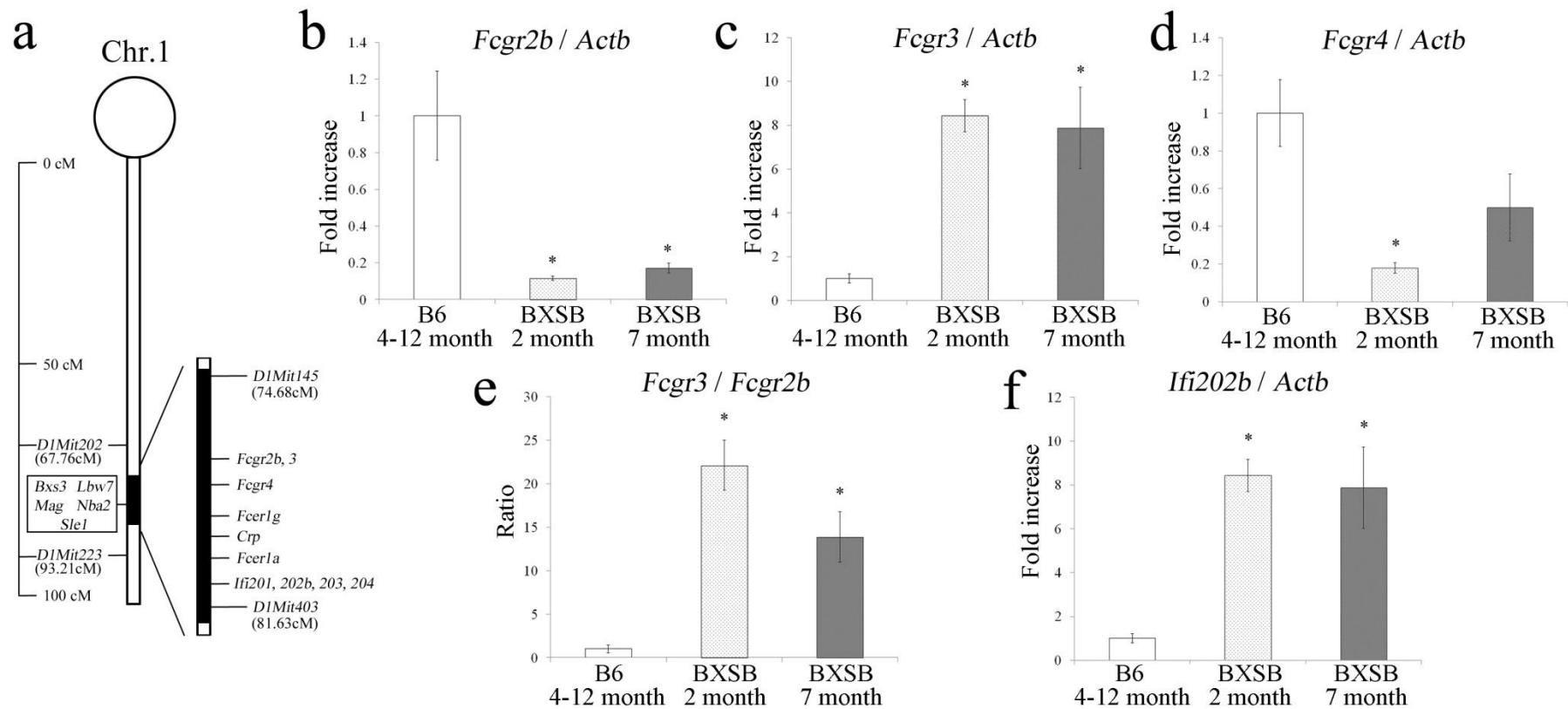


Figure 3-6. Candidate gene expression coded by the telomeric region of Chr. 1 in the female BXSB kidney

(a) Schematic representing the telomeric region of mouse Chr.1. Black area indicates the common region of the autoimmune and GN susceptibility locus in several lupus-prone models such as *Bxs3*, *Lbw7*, *Mag*, *Nba2*, and *Sle1*. The telomeric region of Chr.1 includes the major immune-related genes of the Fc gamma receptor (*Fcgr*) family and *Ifi200* family.

(b–f) The relative mRNA expression of the *Fcgr* family and *Ifi202b* genes in the female BXSB kidney. The expression levels are normalized using *Actb*. Values are mean $\pm$ S.E. *, significantly different from the control B6 mice (Mann-Whitney *U*-test, $P < 0.05$); $n \geq 4$.

Chapter 4

Toll-like receptor contribution

to podocyte injury in autoimmune glomerulonephritis

- Can TLR8 be a novel biomarker for podocyte injury? -

Introduction

In Chapter 3, the author has elucidated the pathological interactions between the immune-associated genes on Chr. 1 and the genetic locus on Chr. Y in the glomerular pathogenesis of BXSB-*Yaa* mice. Although the GN candidate genes on Chr.1 were reported in several studies (Nguyen et al., 2002; Henry and Mohan, 2005; Ichii et al., 2009), those on *Yaa* locus were still unknown. The *Yaa* mutation is a translocation from the telomeric end of the Chr. X onto the Chr. Y, and this duplicated segment plays a crucial role in the activation of auto-reactive B-cells, thereby contributing to the *Yaa*-mediated enhancement of the autoimmune phenotype in male BXSB-*Yaa* mice (Subramanian et al., 2006). In fact, the *Yaa* locus contained several immune-associated genes including TLR family members (Subramanian et al., 2006).

TLRs are expressed on the plasma membrane or intracellular vesicular membrane of not only hematopoietic but also non-hematopoietic cells (Kawai and Akira, 2010). Their function has been characterized as an innate immune sensor to recognize danger signals to the host arising from pathogen-associated molecular patterns (PAMPs) including flagellin, lipopolysaccharide (LPS), and nucleic acids derived from bacteria, mycobacteria, mycoplasma, fungi, and viruses (Kawai and Akira, 2010). Previous studies identified 12 members of the TLR family (TLR1-9 and TLR11-13) in mice and 10 (TLR1-10) in humans (Kawai and Akira, 2010). TLRs activated by their own pathogenic ligands

enhance inflammatory cytokine expression mainly through the NF- κ B pathway to provide the host defense (Kawai and Akira, 2010).

In addition, the TLR-mediated immune system has been shown to play important roles in the pathogenesis of non-infectious injury through the interaction between TLRs and their endogenous ligands (Baccala et al., 2009; Garraud and Cognasse, 2010; Shichita et al., 2012; Mersmann et al., 2013). Mersmann et al. suggested that endogenous high-mobility group box 1 (HMGB1) contributes to myocardial injury through activation of TLR2 signaling (Mersmann et al., 2013). Recently, Shichita et al. demonstrated a pathological interaction between endogenous peroxiredoxin and TLR2 or TLR4 of macrophages in ischemic brain injury (Shichita et al., 2012). Such endogenous ligands of TLRs are called danger-associated molecular patterns (DAMPs), and are considered to be danger signal molecules that indicate the presence of tissue injury to immune cells or local intrinsic cells, thereby inducing local tissue inflammation and damage (Baccala et al., 2009; Garraud and Cognasse, 2010; Shichita et al., 2012; Mersmann et al., 2013).

Many experimental and clinical studies have indicated that TLRs expressed in intrinsic renal cells are involved in the pathogenesis of several kidney diseases (Wolfs et al., 2002; Zhang et al., 2004; Andersen-Nissen et al., 2007; Shigeoka et al., 2007; Chassin et al., 2008; Eleftheriadis et al., 2012). In particular, TLR4, TLR5, and TLR11 in tubular epithelial cells play an important role in the pathogenesis of urinary tract infections and sepsis-induced renal

failure (Zhang et al., 2004; Andersen-Nissen et al., 2007; Chassin et al., 2008).

The activation of TLR2 or TLR4 by DAMPs in tubular epithelial cells contributes to the progression of kidney ischemia-reperfusion injury and subsequent renal fibrosis (Wolfs et al., 2002; Shigeoka et al., 2007). These findings suggest that activation of the TLR signaling pathway plays a crucial role in renal tubulointerstitial injury in various pathological conditions; however, little is known regarding the involvement of TLRs in glomerular diseases.

In this Chapter, the author focused on TLR family as candidate molecules in the progression of chronic GN, and the author found marked up-regulation of *Tlr8* and its downstream cytokines in the glomerulus of BXSB-*Yaa* mice. Importantly, TLR8 protein localized to podocytes in mice and human, and the expression level of glomerular *Tlr8* mRNA significantly correlated with the progression of glomerular damage in BXSB-*Yaa* mice. Interestingly, the urinary level of *Tlr8* mRNA and the serum level of miR-21, a putative endogenous ligand of TLR8, were also significantly higher in BXSB-*Yaa* mice compared to control strain mice. These findings suggest that activated TLR8 signaling exacerbates the progression of podocyte injury in autoimmune GNs, and that TLR8 might be the novel diagnostic and therapeutic target for podocyte injury in chronic GN.

Materials and Methods

Ethics statement

All animal experimentations in Chapter 4 were performed as described in Chapter 1.

Animals

Experimental animals were handled as shown in Chapter 1. The sources of male BXSB-*Yaa* (2-4 months), male BXSB (2-4 months), female B6 (9 months), and female B6.MRL mice (9-15 months) were described in Chapter 2. Male BXSB-*Yaa* and BXSB mice were assigned to an autoimmune GN model group and a healthy control group at 2–4 months of age. The urine, serum, kidneys, and spleens were collected and processed as shown in Chapter 3.

Sample preparation

The kidneys were fixed by 4% PFA in 0.1 M PB at 4°C for histopathological analysis. PFA-fixed paraffin sections (2-µm thick) were then prepared and used for PAS-H staining, PAM-H staining, or immunofluorescence. For *in situ* hybridization, a portion of the kidneys was embedded using Tissue-Tek OCT Compound (Sakura Finetechnical; Tokyo, Japan). The splenic tissue was stored in RNAlater solution (Life Technologies, Carlsbad, CA, USA) for total RNA isolation.

Human samples

Normal kidney tissues from autopsied humans without renal disease were obtained from KAC Inc. (Tokyo, Japan). The kidney samples were applied for immunofluorescence analysis of TLR8.

Glomerular isolation

Mouse glomeruli were isolated by a bead perfusion method as described in Chapter 2 (Takemoto et al., 2002). The collected glomeruli were used for total RNA isolation.

Serological and urinary analysis

To evaluate the systemic autoimmune condition and renal function, serum levels of anti-dsDNA antibody, BUN, and Cre and the uACR levels were measured as described in Chapter 2.

In situ hybridization

cRNA probes for *Tlr8* were synthesized in the presence of DIG-labeled UTP by using a DIG RNA Labeling Kit in accordance with the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). The primer pairs for each probe are shown in Table 4-1 (product size: 758 bp). Cryosections (6 μ m) were treated with acetylation solution and digested with proteinase K. Then, the sections were incubated with a prehybridization solution and then with a hybridization buffer

containing 50% formamide, 10 mM Tris-HCl (pH 7.6), 200 mg/mL RNA, 1× Denhardt's solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll PM400; Sigma-Aldrich, St. Louis, MO, USA), 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA (pH 8.0), and sense or antisense RNA probe (final concentration, 0.2 µg/mL) for 24 h at 58°C. After washing with saline sodium citrate buffer, the sections were then incubated with 0.2% polyclonal sheep anti-DIG Fab fragments conjugated to alkaline phosphatase (1:400; Nucleic Acid Detection Kit, Roche Diagnostics) for 24 h at room temperature. The signal was detected by incubating the sections with a color substrate solution (Roche Diagnostics) containing nitroblue tetrazolium/X-phosphate in a solution composed of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl₂ in a dark room overnight at room temperature.

Immunofluorescence

In deparaffinized sections, antigen retrieval was performed in 10 mM citrate buffer at 105°C for 20 min. After washing, sections were blocked with 5% normal donkey serum for 60 min at room temperature. Then, the sections were incubated with rabbit polyclonal antibodies for TLR8 (1: 1000; Abcam, Cambridge, UK) and mouse monoclonal antibodies for synaptopodin (1: 50; Fitzgerald, MA, USA) overnight at 4°C. After washing with PBS, the sections were incubated with AlexaFluor 546-labeled donkey anti-rabbit IgG antibodies (1:500, Life Technologies) and AlexaFluor 488-labeled donkey anti-mouse IgG antibodies

(1:500, Life Technologies) for 30 min at room temperature and washed again. For nuclear staining, the sections were incubated with Hoechst 33342 (1:2000; Dojindo, Kumamoto, Japan) for 5 min and examined using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

RT-PCR and real-time PCR

To examine mRNA expression, total RNA isolation, cDNA synthesis, RT-PCR and real-time PCR analysis was performed as described in Chapter 1 and 2. The primer pairs are shown in Table 4-1.

RT- and TaqMan-based real-time PCR

MicroRNA (miRNA) expression analysis was performed as described previously (Ichii et al., 2012). Briefly, total RNA including miRNA in the glomeruli and serum were isolated using the miRNeasy kit (Qiagen). Total RNA was reverse-transcribed using miRNA-specific stemloop RT primers, RTase, RT buffer, dNTPs, and RNase inhibitor according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on the resulting cDNA using miR-21-specific TaqMan primers with specific probes (Applied Biosystems), TaqMan Universal PCR Master Mix (Applied Biosystems), and Mx3000P (Agilent Technologies).

Statistical analysis

Results were statistically analyzed as described in Chapter 1.

Results

Clinical Parameters of BXSB-Yaa Mice

For the clinical index of the systemic autoimmune condition, the serum anti-dsDNA antibody levels were significantly higher in BXSB-Yaa mice than in BXSB mice at 2 and 4 months of age (Table 4-2). Although there was no difference in the indices of renal functions including BUN and Cre between both strains at any age, uACR levels, which serve as an index of glomerular dysfunction, were significantly higher in BXSB-Yaa mice than in BXSB mice at 4 months of age (Table 4-2).

Glomerular Histopathology in BXSB-Yaa Mice

Glomerular histopathology was examined in kidney sections stained by PAS-H (Figs. 4-1a-d) or PAM-H (Figs. 4-1e-h) at 2 and 4 months of age. No GL was observed at any age in BXSB mice (Figs. 4-1 a, b, e, and f) and at 2 months in BXSB-Yaa mice (Figs. 4-1c and g). In contrast, at 4 months of age, BXSB-Yaa mice developed membranous proliferative GN, which was characterized by glomerular hypertrophy, increased numbers of mesangial cells and their matrix, thickening of the GBM, and spike-like structures on the GBM (Figs. 4-1d and h).

Glomerular Expression of TLR Family and Activation of TLR-mediated Signaling in BXSB-Yaa Mice

To determine which TLR members associated with GN pathogenesis, the author first examined the expression levels of 12 TLR family genes in the isolated glomeruli of BXSB-*Yaa* mice at 4 months of age (Fig. 4-2a). The glomerular expression levels of *Tlr1*, 2, 7, 8, 9, and 13 were significantly higher in BXSB-*Yaa* mice than in BXSB mice; in particular, the *Tlr8* expression level markedly increased (108-fold, $P<0.001$). As shown in a semi-quantitative RT-PCR analysis (Fig. 4-2b), the glomerular *Tlr8* expression level was also higher in BXSB-*Yaa* mice than in BXSB mice at 2 and 4 months of age, and the band intensity of *Tlr8* was stronger at 4 months than at 2 months in the glomeruli of BXSB-*Yaa* mice. A significant elevation of glomerular *Tlr8* expression was also demonstrated in B6.MRL mice, which comprise an autoimmune GN model that Ichii et al. previously established (Ichii et al., 2012) (Figure 4-2c). From these findings, the author focused on TLR8 in the subsequent analysis.

Figure 4-2d shows the glomerular expression levels of inflammatory mediators induced by the activation of TLRs (Eleftheriadis et al., 2012). The glomerular expression levels of inflammatory cytokines in the NF- κ b pathway including interleukin 1 beta (*Il1b*), *Il6* and tumor necrosis factor (*Tnfa*) were significantly higher in BXSB-*Yaa* mice than in BXSB mice at 4 months of age. In contrast, there was no significant difference between the strains in the expression of *Nfkb*, transforming growth factor beta (*Tgfb*), and interferon, beta 1 (*Ifnb1*).

Localization of TLR8 in Mouse and Human Kidneys

In immunofluorescence analysis, although the positive reaction of synaptopodin, a representative podocyte marker, was detected in the podocyte regions of all examined mice (Figs. 4-3a, d, and g), the reaction was weaker in BXSB-*Yaa* mice (Fig. 4-3d) than in BXSB and B6 mice (Figs. 4-3a and g) at 4 months of age. TLR8 positivity was observed along the glomerular capillary rete, especially in podocyte regions (Figs. 4-3b, e, and h). The immunoreactivity was stronger in BXSB-*Yaa* mice (Fig. 4-3e) than in the other 2 strains (Figs. 4-3b and h) at 4 months of age. TLR8 positivity co-localized with synaptopodin positivity in the glomeruli of all examined strains (Figs. 4-3c, f, and i). Although synaptopodin positivity was found decreased in BXSB-*Yaa* mice (Fig. 4-3d), the author could still detect the co-localization of synaptopodin with TLR8 (Fig. 4-3f). As observed in mice, TLR8 positivity co-localized with synaptopodin positivity in the healthy human kidney (Figs. 4-3j, k, and l).

In *in situ* hybridization analysis of *Tlr8* mRNA, no positive signal was detected in the glomeruli of BXSB mice (Fig. 4-3m). In contrast, a positive signal was localized to the podocyte region in the glomeruli of BXSB-*Yaa* mice (Fig. 4-3n).

Correlation between Podocyte Injury and Tlr8 mRNA Expression in BXSB-Yaa Mice

Correlations between indices of podocyte injury and *Tlr8* mRNA expression in isolated glomeruli were analyzed in BXSB-*Yaa* mice at 4 months of age (Table 4-3). Glomerular *Tlr8* mRNA levels positively correlated with uACR levels, a functional index of glomerular injury, in BXSB-*Yaa* mice (Spearman's test, $P < 0.01$). Further, glomerular *Tlr8* mRNA levels also negatively correlated with glomerular mRNA expression levels of podocyte functional markers, including nephrin (*Nphs1*), podocin (*Nphs2*), and synaptopodin (*Synpo*), in BXSB-*Yaa* mice. Interestingly, glomerular expression levels of TLR8-mediated cytokines including *Il1b* were significantly correlated with those of podocyte functional markers (Table 4-4).

Glomerular and Serum Levels of a Putative Endogenous Ligand of TLR8 in BXSB-Yaa Mice

Recently, it was reported that short-length RNA, especially miR-21, could be a ligand for TLR8 (Fabbri et al., 2012). The author next examined the glomerular and serum levels of miR-21, and they were significantly higher in BXSB-*Yaa* mice than in BXSB mice at 4 months of age (Fig. 4-4).

Detection of Tlr8 mRNA in the Urine of BXSB-Yaa Mice

The urinary *Tlr8* mRNA levels of BXSB-*Yaa* mice and control mice were determined (Fig. 4-5). The urinary *Tlr8* levels were significantly increased in BXSB-*Yaa* mice compared to BXSB mice at 4 months of age. On the other hand,

the author detected no significant difference in serum Tlr8 levels between BXSB-*Yaa* mice and BXSB mice (data not shown).

Discussion

Among glomerular cells, TLRs are expressed by mesangial cells (TLR2-4), endothelial cells (TLR2, 4, 9), and podocytes (TLR1-6, 8, 9) (Eleftheriadis et al., 2012; Gurkan et al., 2013). Pawar et al. demonstrated that the administration of poly (I:C), which is the ligand for TLR3, aggravated autoimmune GN of MRL/MpJ-*lpr* mice, whereas this ligand did not alter anti-dsDNA antibody levels and did not induce B-cell activation (Pawar et al., 2006). According to Fu et al., anti-GBM antibody-treated mice developed mild GN, but when this treatment was coupled with specific TLR ligands including peptidoglycan (TLR2), poly (I:C) (TLR3), LPS (TLR4), or flagellin (TLR5), the treated mice developed GN with greater severity associated with the activation of the NF- κ B pathway (Fu et al., 2006). Thus, pathogenic roles of TLRs and their exogenous ligands in GN have been suggested in several *in vivo* studies (Fu et al., 2006; Pawar et al., 2006; Eleftheriadis et al., 2012).

In the present study, the author demonstrated the up-regulation of TLRs including *Tlr1*, 2, 7, 8, 9, and 13, and their downstream factors (*Il1b*, *Il6*, and *Tnfa*) through the NF- κ B pathway in the glomeruli of autoimmune GN mice. From these findings, the author considered that the TLR-mediated NF- κ B pathway plays an important role in the pathogenesis of autoimmune GN. Importantly, mRNA expression of the TLR family was induced by the major cytokines such as interferon, gamma (IFN γ) and TNF α in both inflammatory cells and tissue intrinsic cells (Muzio et al., 2000; Wolfs et al., 2002). Both

experimental and clinical studies indicated that IFN γ and TNF α are up-regulated in the serum and kidneys of SLE patients and SLE-prone mice (Choubey, 2012; Osnes et al., 2013). Indeed, the author detected the upregulation of glomerular levels of *Tnfa* and *Ifng* in BXSB-*Yaa* mice compared to controls (Fig. 4-2d). Collectively, the author considered that these local cytokines increased the local *Tlr8* expression, especially in podocytes. Previous study revealed that *Tlr8* is broadly expressed on myeloid dendritic cells, monocytes, differentiated macrophages, and CD4⁺ regulatory T-cells (Peng et al., 2005). Podocytes might have differential sensitivity to *Tlr8* inducer cytokines from these immunocompetent cells, and this aspect may also contribute to glomerular overexpression in *Tlr8*. Thereby, *Tlr8* overexpression in podocyte would enhance the responsiveness for their own ligands, and these processes may finally aggravate the pathological condition of autoimmune GN.

In Chapter 3, the author found that the BXSB-type genome causes SLE-like symptoms and subsequent GN without the contribution of *Yaa*, which includes translocated genes such as *Tlr7* and *Tlr8*, and that *Yaa* accelerates the disease progression. In Chapter 4, the author observed local overexpression of *Tlr7* and *Tlr8* in BXSB-*Yaa* mouse glomeruli; expression of *Tlr8* was markedly increased. Importantly, the TLR8 mRNA and its protein were localized to podocytes in BXSB-*Yaa* mice. Furthermore, the B6.MRL lupus-prone mice also showed increased glomerular *Tlr8* expression levels with age. These results suggest that glomerular TLR8 over-expression in BXSB-*Yaa* mice was caused by the *Yaa*

mutation in addition to the autoimmunity-prone genetic background. Although Gurkan et al. recently reported that *Tlr8* mRNA was expressed in a mouse immortalized podocyte cell line (Gurkan et al., 2013), the author's present study has demonstrated for the first time that TLR8 protein is expressed by podocytes *in vivo*.

In contrast to TLR8, TLR7 was reported to be mainly expressed in infiltrating inflammatory cells, and not renal intrinsic cells, in physiological and pathological conditions (Eleftheriadis et al., 2012). In addition to TLR8, podocytes have been reported to express several other members of the TLR family as described above, and recent studies have indicated a pathological correlation between the TLR-mediated NF- κ B pathway in podocytes and their injury *in vitro* (Banas et al., 2008; Machida et al., 2010). Banas et al. revealed that TLR4 in podocytes interacts with the innate immune system to mediate glomerular injury (Banas et al., 2008). Moreover, Machida et al. suggested that expression of TLR9 in podocytes is associated with glomerular disease *in vivo* (Machida et al., 2010). Because of their unique localization in the glomerulus, podocytes are continuously exposed to various plasma solutes containing TLR ligands such as PAMPs and DAMPs. Therefore, podocytes may contribute to renal immunosurveillance by the TLR-mediated immune system.

TLR8 is localized in the endosomal membrane and capable of recognizing single-stranded RNA and short double-stranded RNA from microbial organisms, leading to the production of a variety of NF- κ B-mediated cytokines (Kawai and

Akira, 2010). Several studies have indicated that TLR8 also recognizes endogenous miRNAs (Kawai and Akira, 2010). Although previous studies have demonstrated that secreted miRNAs, which are generally secreted within exosomes, can regulate gene expression in the recipient cells via canonical binding to their target mRNAs (Bartel, 2009), Fabbri et al. showed that exosomal miR-21 and miR-29a can function as ligands for TLR8 (Fabbri et al., 2012). SLE patients show elevated serum levels of miRNAs including miR-21 (Ceribelli et al., 2011; Ahearn et al., 2012). Furthermore, Pan et al. identified that miR-21 was overexpressed in CD4⁺ T-cells from patients with lupus and MRL/MpJ-*lpr* mice (Pan et al., 2010). In the present study, the author demonstrated the serum and glomerular over-expression of miR-21 in autoimmune GN models. From these findings, the author considered that an NF-κB-mediated pathway initiated by the interaction between TLR8 and endogenous ligands including miR-21 correlated with the pathogenesis of autoimmune GN.

The author also found that the glomerular expression levels of *Tlr8* were significantly correlated with those of podocyte functional markers and uACRs. These results suggest that the TLR8-mediated pathway closely correlated with podocyte injury. It has been reported that IL-1β, which is one of the most important cytokines in the NF-κB pathway, is involved in kidney injury, and that in the glomerulus it is mainly produced in podocytes (Niemir et al., 1997; Iyer et al., 2009). Furthermore, recent studies have revealed that various inflammatory

factors including IL-1 β induce podocyte injury by reducing the production of podocyte functional markers, especially nephrin (Timoshanko et al., 2004; Takano et al., 2007). Actually, the author found the strong significant correlation between glomerular expression levels of TLR8-mediated cytokines including *Il1b* and those of podocyte functional markers. Further, the author revealed the T-cells as well as B-cells infiltration to BXSB-*Yaa* mouse glomerulus with disease progression (Chapter 2). From these findings, the author considered that factors downstream of TLR8 such as IL-1 β directly contribute to podocyte injury and promote the glomerular recruitment of leukocytes in autoimmune GN pathogenesis.

Furthermore, the urinary expression level of *Tlr8* mRNA was significantly higher in BXSB-*Yaa* mice than in control mice. Recently, it has been suggested that glomerular injury in proteinuric renal diseases is strongly associated with the effacement of podocytes caused by disruption of FPs and/or the SD, and the mRNA of these cells is detected in the urine of patients with renal disease (Wickman et al., 2013). The author observed TLR8 expression in human and murine podocytes. Therefore, altered urinary levels of *Tlr8* mRNA may indicate podocyte injury in autoimmune GN.

In conclusion, the author showed that the TLR family, especially the TLR8-mediated pathway, correlates with podocyte injury in murine autoimmune GN, suggesting that the TLR8 molecule is a novel therapeutic and diagnostic target for mouse and human glomerular diseases.

Summary

Chapter 3 elucidated the pathological interactions between the immune-associated genes on Chr. 1 and the genetic locus on Chr. Y in the GN pathogenesis of BXSB-*Yaa* mice. Although the GN candidate genes on Chr.1 were reported in several studies, those on *Yaa* locus were still unknown. The TLR family coded on *Yaa* locus serves as a pathogen sensor and participates in the local autoimmune response. In this Chapter, the author found a correlation between glomerular injury and TLR expression by analyzing BXSB-*Yaa* mice.

In isolated glomeruli, mRNA expression of several TLRs was significantly higher in BXSB-*Yaa* mice than in healthy control BXSB mice; in particular, expression of *Tlr8* and its downstream cytokines was markedly increased. In mouse kidneys, TLR8 protein and mRNA were localized to podocytes, and TLR8 protein expression in the glomerulus was higher in BXSB-*Yaa* mice than in BXSB mice. In healthy human kidney, TLR8 protein also specifically localized to podocytes. In BXSB-*Yaa* mice, the glomerular levels of *Tlr8* mRNA were negatively correlated with glomerular levels of podocyte functional markers (*Nphs1*, *Nphs2*, and *Synpo*) and positively with urinary albumin levels. Furthermore, the glomerular and serum levels of miR-21, a putative microRNA ligand of TLR8, were significantly higher in BXSB-*Yaa* mice than in BXSB mice. The urinary levels of *Tlr8* mRNA were also significantly higher in BXSB-*Yaa* mice than in BXSB mice.

In conclusion, the author selected TLR8 as the candidate molecule in the

progression of CKD and suggested the overactivation of TLR8 closely correlates with the progression of podocyte injury in chronic GN.

Tables and Figures

Table 4-1. Summary of specific gene primers

Genes (accession no.)	Primer sequence (5'-3') F: forward, R: reverse	Product size (bp)	Application
<i>Tlr1</i> (NM_030682)	F: GTGAATGCAGTTGGTGAAGAAC R: ATGGCCATAGACATTCTCTGAG	125	Real-time PCR
<i>Tlr2</i> (NM_011905)	F: GAGCATCCGAATTGCATCA R: CACATGACAGAGACTCCTGAGC	163	Real-time PCR
<i>Tlr3</i> (NM_126166)	F: GATACAGGGATTGCACCCATA R: GCATTGGTTTGTGGAAGACAC	122	Real-time PCR
<i>Tlr4</i> (NM_021297)	F: TTCAGAACTTCAGTGGCTGGA R: CTGGATAGGGTTTCTCTGTCAGT	115	Real-time PCR
<i>Tlr5</i> (NM_016928)	F: ATGCCAGACACATCTGTGAGA R: ATCCTGCCGTCTGAAGAACA	177	Real-time PCR
<i>Tlr6</i> (NM_011604)	F: ATGGTACCGTCAGTGCTGGA R: TCTGTCTTGGCTCATGTTGC	104	Real-time PCR
<i>Tlr7</i> (NM_133211)	F: TGACTCTCTTCTCCTCCACCAG R: TCTGTGCAGTCCACGATCAC	198	Real-time PCR
<i>Tlr8</i> (NM_133212)	F: GTTATGTTGGCTGCTCTGGTTCAC R: TCACTCTCTTCAAGGTGGTAGC	203	Real-time PCR
<i>Tlr8</i> (NM_133212)	F: ATGGAAAACATGCCCCCTCAGTC R: GGACAGTTTCCACTCAGATCTAG	758	<i>in situ</i> hybridization
<i>Tlr9</i> (NM_031178)	F: GAATCCTCCATCTCCCAACA R: GGGTACAGACTTCAGGAACAGC	181	Real-time PCR
<i>Tlr11</i> (NM_205819)	F: CACCATTGTGGAGGGAAGAG R: TCAGAATGAGGAGAACAGAGCA	158	Real-time PCR
<i>Tlr12</i> (NM_205823)	F: GAACTTCTGCCTGCTCTGGA R: AACACGCAGAGTGTGGTACG	146	Real-time PCR
<i>Tlr13</i> (NM_205820.1)	F: TCCTCTGTTGCATGATGTCTG R: CTGTCTTAGGCATCCAGGTTACA	182	Real-time PCR
<i>Nphs1</i> (NM_019459)	F: ACCTGTATGACGAGGTGGAGAG R: TCGTGAAGAGTCTCACACCAG	218	Real-time PCR
<i>Nphs2</i> (NM_130456)	F: AAGGTTGATCTCCGTCTCCAG R: TTCCATGCGGTAGTAGCAGAC	105	Real-time PCR
<i>Synpo</i> (NM_177340.2)	F: CATCGGACCTTCTTCCTGTG R: TCGGAGTCTGTGGGTGAG	90	Real-time PCR
<i>Il1b</i> (NM008361)	F: AAGGAGAACCAAGCAACGAC R: AACTCTGCAGACTCAAACCTCCAC	208	Real-time PCR
<i>Il6</i> (NM031168)	F: TGTATGAACAACGATGATGCAC R: TGGTACTCCAGAAGACCAGAGG	137	Real-time PCR
<i>Tgfb</i> (NM011577)	F: AGCCTGGACACACAGTACAGC R: CGACCCACGTAGTAGACGATG	125	Real-time PCR
<i>Tnfa</i> (NM013693)	F: CGAGTGACAAGCCTGTAGCC R: GAGAACCTGGGAGTAGACAAGG	167	Real-time PCR
<i>Ifnb1</i> (NM010510.1)	F: CAGCTCCAAGAAAGGACGAAC R: GGCAGTGTAACCTTTCTGTCAT	138	Real-time PCR
<i>Ifng</i> (NM008337.3)	F: CCTTTGGACCCTCTGACTTG R: TTCCACATCTATGCCACTTGAG	201	Real-time PCR
<i>Actb</i> (NM007393)	F: TGTTACCAACTGGGACGACA R: GGGGTGTTGAAGGTCTCAA	165	Real-time PCR

Table 4-2. Clinical parameters of BXSB and BXSB-*Yaa* mice

		dsDNA ($\mu\text{g/mL}$)	BUN (mg/dL)	Cre (mg/dL)	uACR ($\mu\text{g/mg}$)
BXSB	2 month	148.49	17.73	1.56	60.20
		± 16.07	± 0.13	± 0.17	± 9.04
	4 month	135.43	29.80	0.56	47.35
		± 11.22	± 3.50	± 0.03	± 7.40
BXSB- <i>Yaa</i>	2 month	535.15	20.20	2.32	58.72
		$\pm 203.72^*$	± 0.28	± 1.03	± 9.08
	4 month	890.15	38.93	1.30	439.93
		$\pm 95.44^*$	± 9.92	± 0.10	$\pm 253.78^*$

Values are the mean $\pm$ S.E. dsDNA, double-strand DNA antibody level; BUN, serum blood urea nitrogen level; Cre, serum creatinine level; uACR, urinary albumin creatinine ratio. * Significantly different from BXSB mice at the same age (Mann-Whitney *U*-test, $P < 0.05$); $n \geq 3$.

Table 4-3. Relationship between glomerular *Tlr8* expression levels and podocyte injury indices

Value / Parameter	uACR	Glomerular <i>Nphs1</i> expression	Glomerular <i>Nphs2</i> expression	Glomerular <i>Synpo</i> expression
Spearman's rank correlation coefficient	0.847	-0.800	-0.738	-0.801
<i>P</i> value	<0.01	<0.01	<0.01	<0.01

BXSB-*Yaa* mice 4 months. uACR: urinary albumin creatinine Ratio. mRNA expression of podocyte functional markers quantified by real-time PCR. $n \geq 5$.

Table 4-4. Relationship between glomerular TLR8-mediated cytokine expression levels and podocyte functional marker expression levels

cytokine/podocyte marker	<i>Il1b</i>	<i>Il6</i>	<i>Tnfa</i>
<i>Nphs1</i>	-0.756**	-0.503*	-0.574*
<i>Nphs2</i>	-0.635**	-0.429	-0.515*
<i>Synpo</i>	-0.768**	-0.674**	-0.691**

Values are the Spearman's rank correlation coefficients. * and **, significantly correlated (Spearman's rank-correlation test, * $P < 0.05$. ** $P < 0.01$); $n \geq 8$.

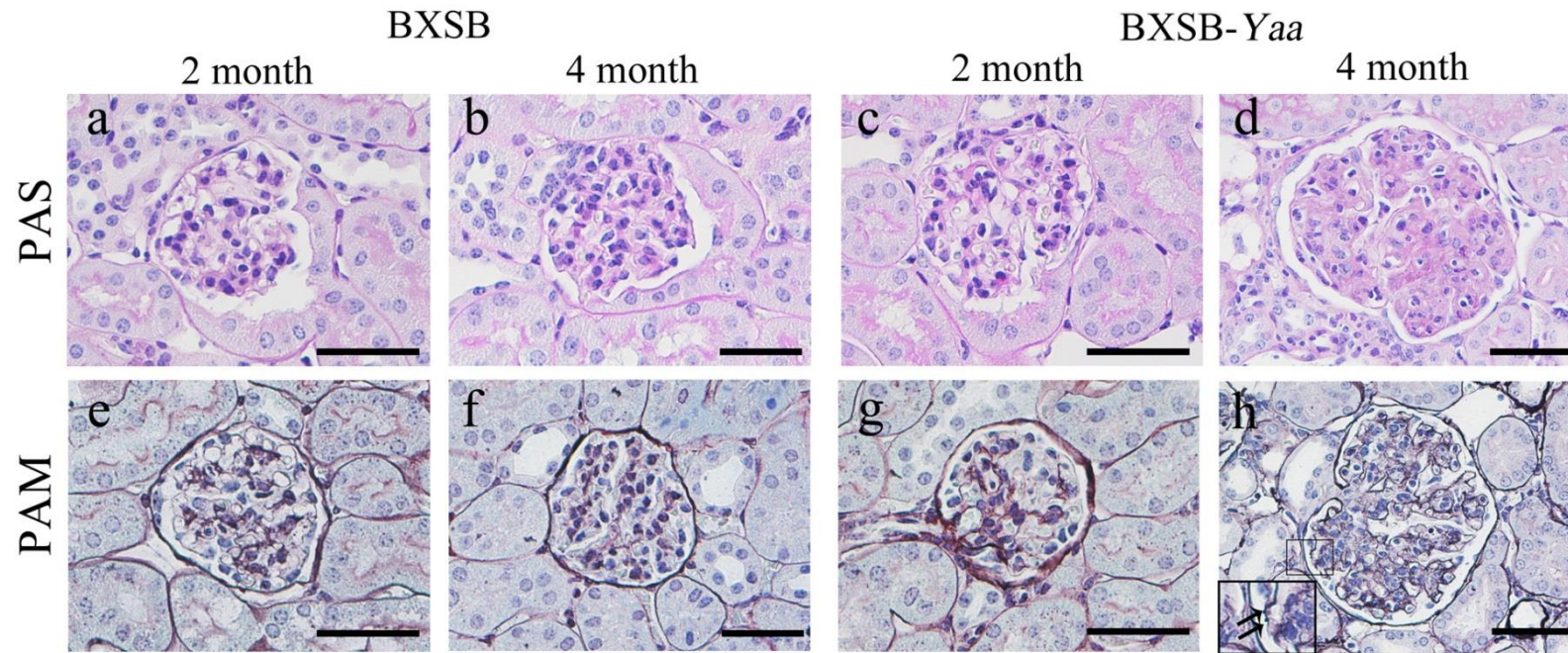


Figure 4-1. Glomerular histopathology of BXSB-Yaa mice

(a-d) Histopathology of glomeruli in PAS-H-stained sections of BXSB and BXSB-Yaa mice. In BXSB mice (a and b), there is no histological difference between the ages of 2 and 4 months. In BXSB-Yaa mice (c and d), mesangial matrix expansion and mesangial cell proliferation are clearly observed at 4 months relative to 2 months.

(e-h) Histopathology of glomeruli in PAM-H-stained sections of BXSB and BXSB-Yaa mice. In BXSB mice (e and f), there is no histological difference between 2 and 4 months. In BXSB-Yaa mice (g and h), glomerular hypertrophy, wrinkling of the GBM, and spike-like structure of the GBM (inset, arrows) are clearly observed at 4 months (h). Bars = 50 μ m.

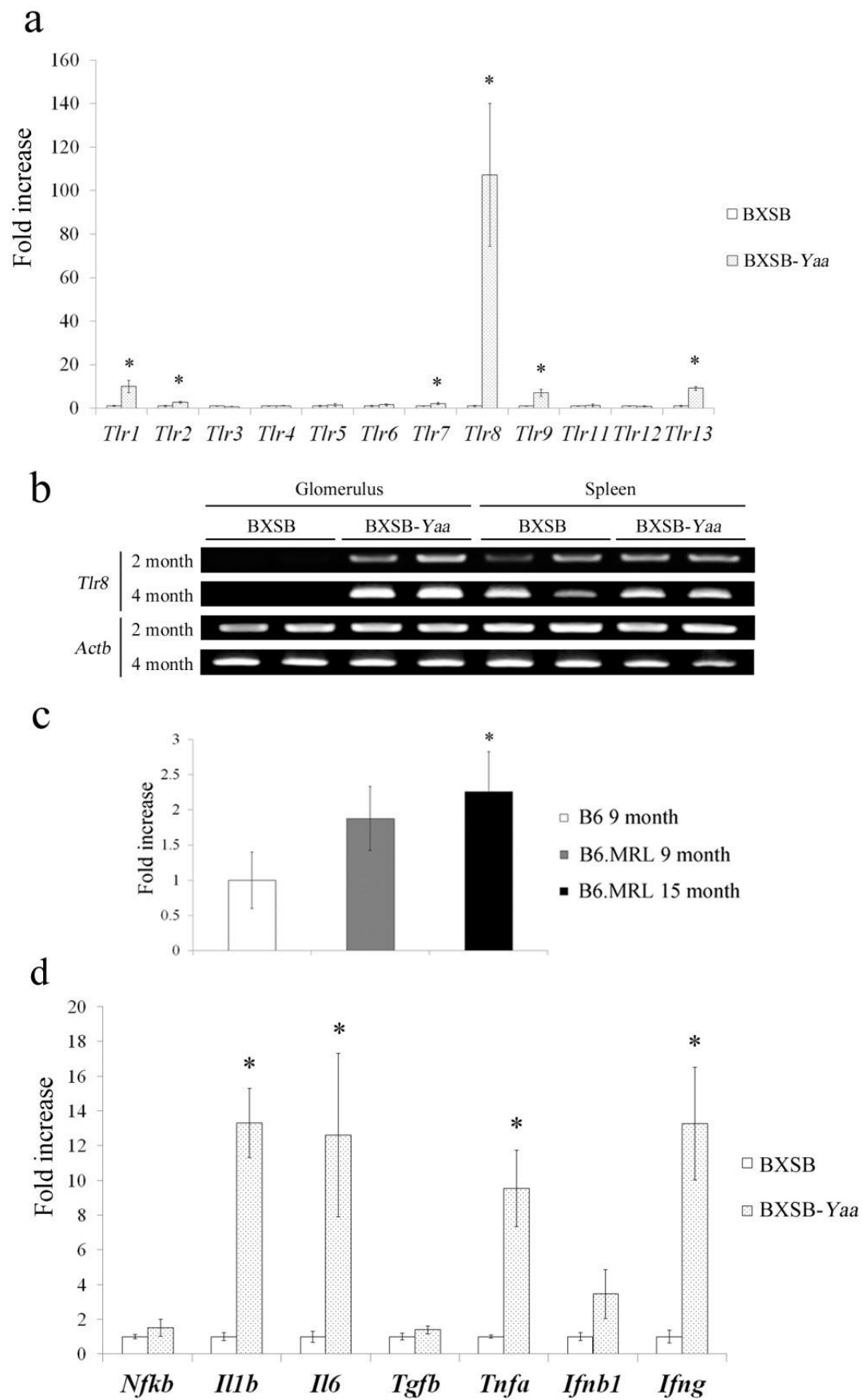


Figure 4-2. mRNA expression of the TLR family and its downstream factors in the glomeruli of BXSB-Yaa and B6.MRL mice.

(a) Relative mRNA expression of TLR family genes in isolated glomeruli from BXSB-Yaa and BXSB mice at 4 months. The expression levels were normalized using expression of *Actb*. Values are the mean $\pm$ S.E. *, significantly different from control BXSB mice (Mann-Whitney *U*-test, $P < 0.05$); $n \geq 4$.

(b) RT-PCR analysis for *Tlr8* and *Actb* mRNA expression in the glomerulus and spleen of BXSB and BXSB-Yaa mice at 4 months of age. $n = 2$.

(c) Relative mRNA expression of *Tlr8* in isolated glomeruli from B6.MRL and control mice. The expression levels were normalized using *Actb*. Values are the mean $\pm$ S.E. *, significantly different from the control C57BL/6 mice (Mann-Whitney *U*-test, $P < 0.05$); $n \geq 5$

(d) Relative mRNA expression of *Nfkb*, *Il1b*, *Il6*, *Tgfb*, *Tnfa*, *Ifnb1*, and *Ifng* in isolated glomeruli from BXSB-Yaa and BXSB mice at 4 months of age. The expression levels were normalized using *Actb*. The values are the mean $\pm$ S.E. *, significantly different from the control BXSB-Yaa mice (Mann-Whitney *U*-test, $P < 0.05$); $n \geq 4$.

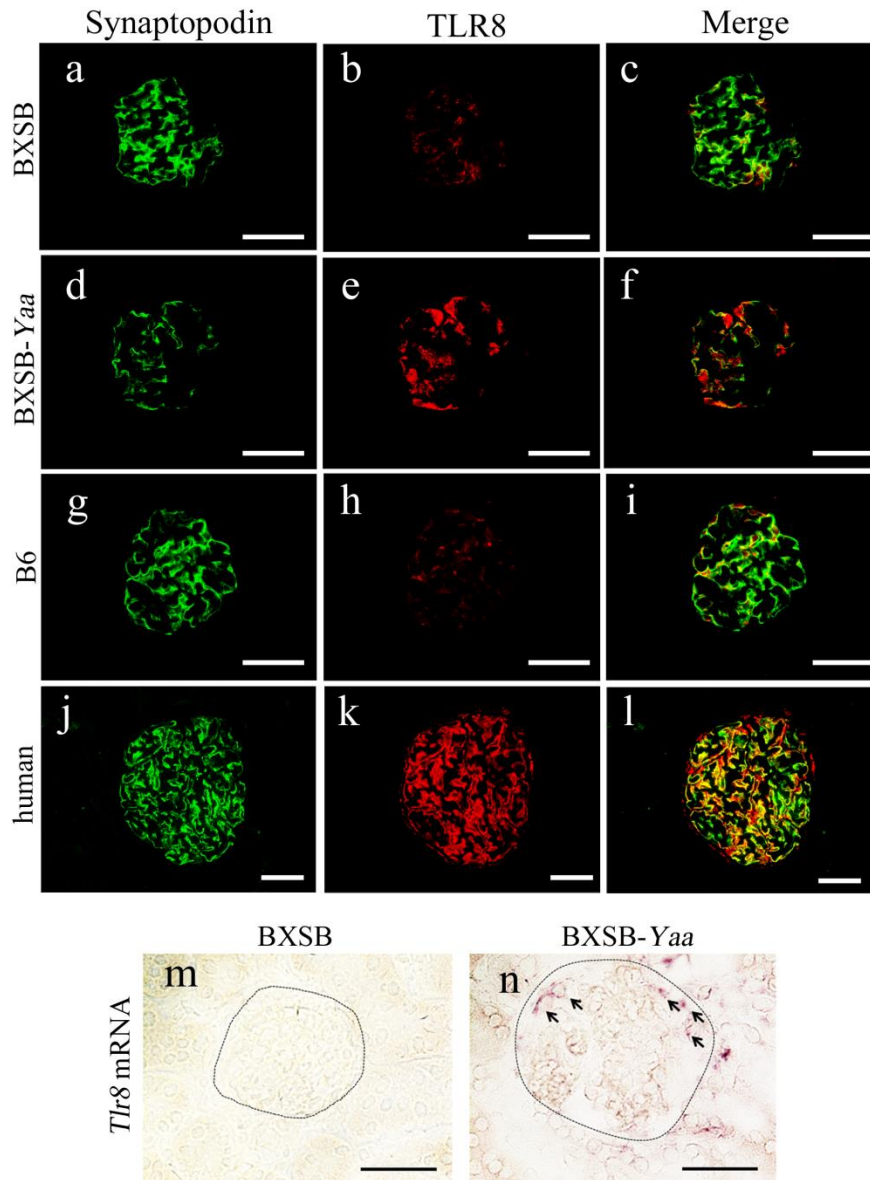


Figure 4-3. Localization of TLR8 protein and mRNA in the kidneys of mice and humans.

(a-l) Immunofluorescence of synaptopodin (a, d, g, and j), TLR8 (b, e, h, and k), and merged image (c, f, i, and l) in glomeruli of BXSB (a-c), BXSB-Yaa (d-f), and B6 (g-i) mice and humans (j-l). Synaptopodin immunoreactivity (green) is co-localized with that of TLR8 (red) (c, f, i, and l). TLR8 positivity in BXSB-Yaa mice (e) is stronger than that in BXSB (b) and in B6 (h) mice. In the human glomerulus (j-l), TLR8 immunoreactivity is co-localized with synaptopodin immunoreactivity, similar to the mouse glomerulus.

(m and n) *In situ* hybridization for *Tlr8* mRNA. Positive reactions are observed in the glomeruli of BXSB-Yaa mice, especially in podocyte regions (n, arrow). In contrast, no positive reaction is observed in the BXSB mice glomerulus (m). Bars = 50 μ m.

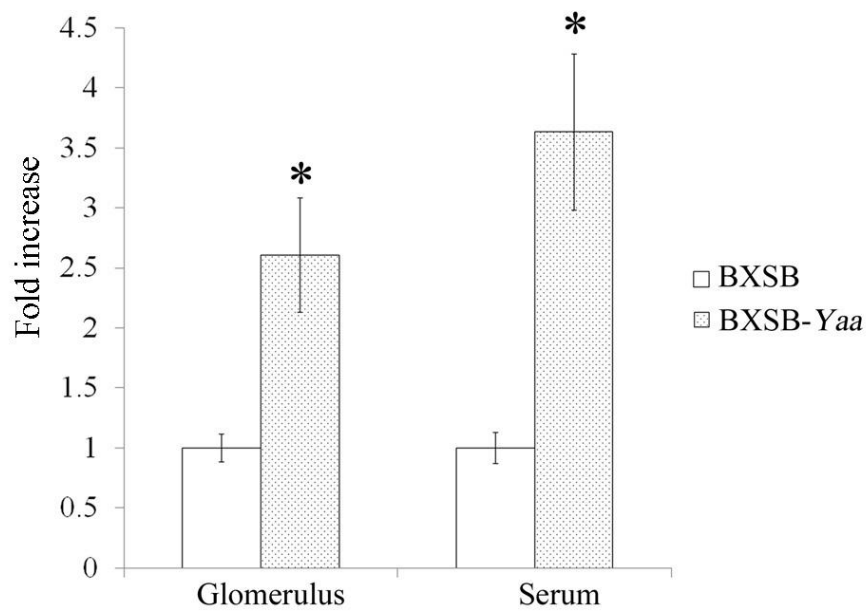


Figure 4-4. Glomerular and serum levels of miR-21.

The relative glomerular and serum expression levels of miR-21 in BXSB and BXSB-*Yaa* mice at 4 months of age. Values are the mean $\pm$ S.E. Data are presented as the fold increase vs. BXSB mice in the same samples. *, significantly different from the control BXSB mice (Mann-Whitney *U*-test, $P < 0.05$); $n = 3$.

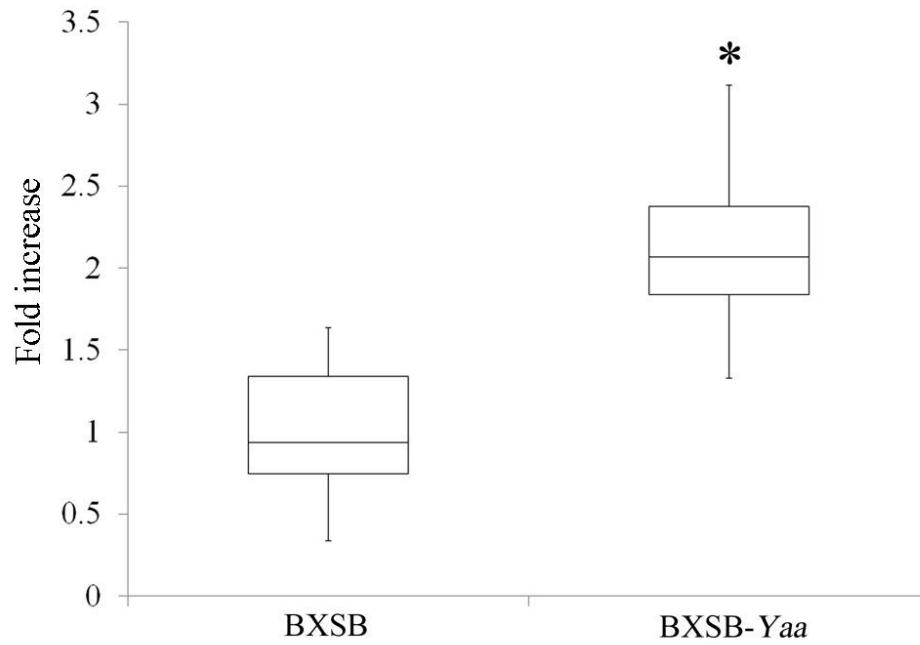


Figure 4-5. Urinary levels of *Tlr8* mRNA.

Box plots of the relative *Tlr8* mRNA level in urine from BXSB and BXSB-Yaa mice. Values are the mean $\pm$ S.E. Data are presented as a fold increase vs. BXSB mice. *, significantly different from the control BXSB mice (Welch's t-test, $P < 0.05$); $n \geq 4$.

Conclusion

Recently, the growing number of elderly individuals has led to the worldwide increase in both humans and animals with chronic kidney disease (CKD). Since progressive CKD causes an increased risk of cardiovascular diseases as well as end-stage renal disease, the control of CKD is one of the most important tasks for human and animal health. The most suitable strategy for CKD control is the establishment of novel methods to provide medical care at an early stage, because kidney is non-regenerative organ. Hence, the final goal of current CKD studies is the elucidation of molecular pathogenesis to develop novel diagnostic and therapeutic methods. In this thesis, the author investigated the molecular pathogenesis of chronic glomerulonephritis (GN), which is one of the major CKD primary diseases, using autoimmune GN murine models, to clarify the pathological features, genetic basis, and the diagnostic or therapeutic targets of CKD.

In Chapter 1, the author investigated the renal pathology and urine of BXSB/MpJ-*Yaa* (BXSB-*Yaa*) mice, a representative model for autoimmune GN due to mutated *Yaa* locus on chromosome (Chr.) Y. BXSB-*Yaa* mice developed glomerular lesions (GLs) and tubulointerstitial lesions (TILs) characterized by the expansion of mesangial matrix, proliferation of mesangial cells, dilated tubules by urinary casts, and perivascular cell infiltration. In BXSB-*Yaa* mouse urine, the cell numbers as well as the mRNA of cell specific markers for podocytes, distal tubules (DTs), and collecting ducts (CDs) increased with disease progression. Further, injured DTs and CDs produced C3 mRNA and protein, and its mRNA was detected

BXSB-*Yaa* mouse urine at high rates. Briefly, in CKD condition, injured epithelial cells of the glomerulus, DT, and CD were dropped into the urine.

Recent studies have indicated that CKD often begins with GLs including blood urine barrier (BUB) disruption, leading to subsequent TILs mediated by ultrafiltration of several proteins. The author next focused on the injuries of podocyte, a glomerular epithelial cell regulating BUB integrity, as an early pathological event of GL in CKD progression. In addition to BXSB-*Yaa* mice, B6.MRL-(*D1Mit202-D1Mit403*) (B6.MRL) mice, which carry autoimmune GN susceptibility locus in the telomeric region of Chr.1, were investigated as autoimmune models. Both GN models developed membranous proliferative GN with albuminuria and elevated serum anti-double strand DNA antibody (anti-dsDNA) levels. Ultrastructural analysis revealed the abnormal ultrastructural morphology of podocytes such as foot process effacement and microvillus-like structure in both models. Further, the prominent decreases of mRNA and protein levels of podocyte functional factors were observed in the glomerulus of both models, and they negatively correlated with urinary albumin ratios (uACRs). These results strongly emphasized the clinicopathological importance of podocyte injuries with altered function and morphology in CKD.

In Chapter 3, the author explored the genetic factors affecting CKD pathogenesis, especially focusing on GL of BXSB-*Yaa* mice. Surprisingly, female BXSB/MpJ (BXSB) mice showed the increase of serum anti-dsDNA antibodies as well as membranous proliferative GN with aging in despite of *Yaa* absence. On the

other hand, male BXSB mice developed neither autoimmune symptom nor GN. From the comprehensive gene expression analysis, the author found that the expression of autoimmune GN candidate genes on the telomeric region of Chr.1, such as *Fcgr3* and *Ifi202b*, drastically upregulated in the glomerulus of male BXSB-*Yaa* mice compared to male BXSB mice. Their renal expression was also significantly upregulated in female BXSB mice. These results suggest that sex-differences in immunity might affect GN pathogenesis of BXSB strain, and that *Yaa* contributes the progression of CKD by enhance the expression of autoimmune GN candidate genes localizing to the telomeric region of Chr.1 on BXSB-type genome.

Based on the data of Chapter 3, the Chapter 4 focused on Toll-like receptor (TLR) family coded on *Yaa* locus to identify the early diagnostic and therapeutic targets for CKD. Recent studies have revealed that the TLR family plays a crucial role in local autoimmune response. The exhaustive expression analysis for TLR family genes showed the *Tlr8* overexpression in glomerulus of BXSB-*Yaa* and B6.MRL mice. TLR8 protein and mRNA localized in podocytes of mouse as well as human kidney. The isolated-glomerular *Tlr8* expression were significantly correlated with those in podocyte functional markers negatively and uACR positively in BXSB-*Yaa* mice. Further, the glomerular and serum levels of miR-21, a putative miRNA ligand of TLR8, were significantly higher in BXSB-*Yaa* mice than in BXSB mice. The urinary levels of *Tlr8* mRNA were also significantly higher in BXSB-*Yaa* mice than in BXSB mice. These

results strongly suggest that the overactivation of TLR8 contributes to progression of podocyte injury in GN. Altered levels of urinary *Tlr8* mRNA may thus reflect podocyte injury, and TLR8 would be novel target of diagnosis and therapy for CKD.

In conclusion, this thesis clarified the molecular pathogenesis of CKD by using murine model for chronic GN, and emphasized the podocyte injuries as an early sign of CKD progression. Importantly, TLR8 was identified as the novel early diagnostic and therapeutic targets for CKD, especially for the podocyte injury in chronic GN. The author strongly believes that these findings lead early diagnosis and novel therapy of CKD in the fields of medicine as well as veterinary medicine.

References

1. Abe, K., Li, K., Sacks, S.H., and Sheerin, N.S. 2004. The membrane attack complex, C5b-9, up regulates collagen gene expression in renal tubular epithelial cells. *Clin. Exp. Immunol.*, **136**: 60-66.
2. Ahearn, J.M., Liu, C.C., Kao, A.H., and Manzi, S. 2012. Biomarkers for systemic lupus erythematosus. *Transl. Res.*, **159**: 326-342.
3. Andersen-Nissen, E., Hawn, T.R., Smith, K.D., Nachman, A., Lampano, A.E., Uematsu, S., Akira, S., and Aderem, A. 2007. Cutting edge: Tlr5-/- mice are more susceptible to Escherichia coli urinary tract infection. *J. Immunol.*, **178**: 4717-4720.
4. Andrews, B.S., Eisenberg, R.A., Theofilopoulos, A.N., Izui, S., Wilson, C.B., McConahey, P.J., Murphy, E.D., Roths, J.B., and Dixon, F.J. 1978. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J. Exp. Med.*, **148**: 1198-1215.
5. Asanuma, K., Kim, K., Oh, J., Giardino, L., Chabanis, S., Faul, C., Reiser, J., and Mundel, P. 2005. Synaptopodin regulates the actin-bundling activity of alpha-actinin in an isoform-specific manner. *J. Clin. Invest.*, **115**: 1188-1198.
6. Asanuma, K., and Mundel, P. 2003. The role of podocytes in glomerular pathobiology. *Clin. Exp. Nephrol.*, **7**: 255-259.
7. Asanuma, K., Yanagida-Asanuma, E., Faul, C., Tomino, Y., Kim, K., and Mundel, P. 2006. Synaptopodin orchestrates actin organization and cell motility via regulation of RhoA signalling. *Nat. Cell Biol.*, **8**: 485-491.
8. Asanuma, K., Yanagida-Asanuma, E., Takagi, M., Kodama, F., and Tomino, Y. 2007. The role of podocytes in proteinuria. *Nephrology (Carlton)*, **12 Suppl 3**: S15-20.
9. Baccala, R., Gonzalez-Quintial, R., Lawson, B.R., Stern, M.E., Kono, D.H.,

- Beutler, B., and Theofilopoulos, A.N. 2009. Sensors of the innate immune system: their mode of action. *Nat. Rev. Rheumatol.*, **5**: 448-456.
10. Banas, M.C., Banas, B., Hudkins, K.L., Wietecha, T.A., Iyoda, M., Bock, E., Hauser, P., Pippin, J.W., Shankland, S.J., Smith, K.D., Stoelcker, B., Liu, G., Grone, H.J., Kramer, B.K., and Alpers, C.E. 2008. TLR4 links podocytes with the innate immune system to mediate glomerular injury. *J. Am. Soc. Nephrol.*, **19**: 704-713.
 11. Barksby, H.E., Lea, S.R., Preshaw, P.M., and Taylor, J.J. 2007. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. *Clin. Exp. Immunol.*, **149**: 217-225.
 12. Bartel, D.P. 2009. MicroRNAs: target recognition and regulatory functions. *Cell*, **136**: 215-233.
 13. Bartlett, P.C., Van Buren, J.W., Bartlett, A.D., and Zhou, C. 2010. Case-control study of risk factors associated with feline and canine chronic kidney disease. *Vet. Med. Int.*, **2010**.
 14. Bekar, K.W., Owen, T., Dunn, R., Ichikawa, T., Wang, W., Wang, R., Barnard, J., Brady, S., Nevarez, S., Goldman, B.I., Kehry, M., and Anolik, J.H. 2010. Prolonged effects of short-term anti-CD20 B cell depletion therapy in murine systemic lupus erythematosus. *Arthritis Rheum.*, **62**: 2443-2457.
 15. Bouman, A., Heineman, M.J., and Faas, M.M. 2005. Sex hormones and the immune response in humans. *Hum. Reprod. Update*, **11**: 411-423.
 16. Brooimans, R.A., Stegmann, A.P., van Dorp, W.T., van der Ark, A.A., van der Woude, F.J., van Es, L.A., and Daha, M.R. 1991. Interleukin 2 mediates stimulation of complement C3 biosynthesis in human proximal tubular epithelial cells. *J. Clin. Invest.*, **88**: 379-384.
 17. Catania, J.M., Chen, G., and Parrish, A.R. 2007. Role of matrix

- metalloproteinases in renal pathophysiologies. *Am. J. Physiol. Renal Physiol.*, **292**: F905-911.
18. Ceribelli, A., Yao, B., Dominguez-Gutierrez, P.R., Nahid, M.A., Satoh, M., and Chan, E.K. 2011. MicroRNAs in systemic rheumatic diseases. *Arthritis Res. Ther.*, **13**: 229.
 19. Chan, O.T., Hannum, L.G., Haberman, A.M., Madaio, M.P., and Shlomchik, M.J. 1999. A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J. Exp. Med.*, **189**: 1639-1648.
 20. Chassin, C., Vimont, S., Cluzeaud, F., Bens, M., Goujon, J.M., Fernandez, B., Hertig, A., Rondeau, E., Arlet, G., Hornef, M.W., and Vandewalle, A. 2008. TLR4 facilitates translocation of bacteria across renal collecting duct cells. *J. Am. Soc. Nephrol.*, **19**: 2364-2374.
 21. Chen, J., Panchanathan, R., and Choubey, D. 2008. Stimulation of T cells up-regulates expression of Ifi202, an interferon-inducible lupus susceptibility gene, through activation of JNK/c-Jun pathway. *Immunol. Lett.*, **118**: 13-20.
 22. Choubey, D. 2012. Interferon-inducible Ifi200-family genes as modifiers of lupus susceptibility. *Immunol. Lett.*, **147**: 10-17.
 23. Dalla Vestra, M., Masiero, A., Roiter, A.M., Saller, A., Crepaldi, G., and Fioretto, P. 2003. Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. *Diabetes*, **52**: 1031-1035.
 24. David, S., Biancone, L., Caserta, C., Bussolati, B., Cambi, V., and Camussi, G. 1997. Alternative pathway complement activation induces proinflammatory activity in human proximal tubular epithelial cells. *Nephrol. Dial. Transplant.*, **12**: 51-56.
 25. Diez-Sampedro, A., Lenz, O., and Fornoni, A. 2011. Podocytopathy in

- diabetes: a metabolic and endocrine disorder. *Am. J. Kidney Dis.*, **58**: 637-646.
26. Durvasula, R.V., and Shankland, S.J. 2006. Podocyte injury and targeting therapy: an update. *Curr. Opin. Nephrol. Hypertens.*, **15**: 1-7.
 27. Eleftheriadis, T., Pissas, G., Liakopoulos, V., Stefanidis, I., and Lawson, B.R. 2012. Toll-like receptors and their role in renal pathologies. *Inflamm Allergy Drug Targets*, **11**: 464-477.
 28. Espeli, M., Bokers, S., Giannico, G., Dickinson, H.A., Bardsley, V., Fogo, A.B., and Smith, K.G. 2011. Local renal autoantibody production in lupus nephritis. *J. Am. Soc. Nephrol.*, **22**: 296-305.
 29. Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., Lovat, F., Fadda, P., Mao, C., Nuovo, G.J., Zanesi, N., Crawford, M., Ozer, G.H., Wernicke, D., Alder, H., Caligiuri, M.A., Nana-Sinkam, P., Perrotti, D., and Croce, C.M. 2012. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc. Natl. Acad. Sci. U. S. A.*, **109**: E2110-2116.
 30. Fassett, R.G., Venuthurupalli, S.K., Gobe, G.C., Coombes, J.S., Cooper, M.A., and Hoy, W.E. 2011. Biomarkers in chronic kidney disease: a review. *Kidney Int.*, **80**: 806-821.
 31. Fillatreau, S. 2013. Cytokine-producing B cells as regulators of pathogenic and protective immune responses. *Ann. Rheum. Dis.*, **72 Suppl 2**: ii80-84.
 32. Finco, D.R., and Duncan, J.R. 1976. Evaluation of blood urea nitrogen and serum creatinine concentrations as indicators of renal dysfunction: a study of 111 cases and a review of related literature. *J. Am. Vet. Med. Assoc.*, **168**: 593-601.
 33. Floege, J., Radeke, H.R., and Johnson, R.J. 1994. Glomerular cells in vitro versus the glomerulus in vivo. *Kidney Int.*, **45**: 360-368.

34. Fu, Y., Xie, C., Chen, J., Zhu, J., Zhou, H., Thomas, J., Zhou, X.J., and Mohan, C. 2006. Innate stimuli accentuate end-organ damage by nephrotoxic antibodies via Fc receptor and TLR stimulation and IL-1/TNF-alpha production. *J. Immunol.*, **176**: 632-639.
35. Fujii, T., Hamano, Y., Ueda, S., Akikusa, B., Yamasaki, S., Ogawa, M., Saisho, H., Verbeek, J.S., Taki, S., and Saito, T. 2003. Predominant role of FcgammaRIII in the induction of accelerated nephrotoxic glomerulonephritis. *Kidney Int.*, **64**: 1406-1416.
36. Fujimoto, M. 2010. Regulatory B cells in skin and connective tissue diseases. *J. Dermatol. Sci.*, **60**: 1-7.
37. Galkina, E., and Ley, K. 2006. Leukocyte recruitment and vascular injury in diabetic nephropathy. *J. Am. Soc. Nephrol.*, **17**: 368-377.
38. Garraud, O., and Cognasse, F. 2010. Platelet Toll-like receptor expression: the link between "danger" ligands and inflammation. *Inflamm. Allergy Drug Targets*, **9**: 322-333.
39. Gurkan, S., Cabinian, A., Lopez, V., Bhaumik, M., Chang, J.M., Rabson, A.B., and Mundel, P. 2013. Inhibition of type I interferon signalling prevents TLR ligand-mediated proteinuria. *J. Pathol.*, **231**: 248-256.
40. Hara, M., Yanagihara, T., Hirayama, Y., Ogasawara, S., Kurosawa, H., Sekine, S., and Kihara, I. 2010. Podocyte membrane vesicles in urine originate from tip vesiculation of podocyte microvilli. *Hum. Pathol.*, **41**: 1265-1275.
41. Hara, M., Yanagihara, T., and Kihara, I. 2001. Urinary podocytes in primary focal segmental glomerulosclerosis. *Nephron*, **89**: 342-347.
42. Hara, M., Yanagihara, T., and Kihara, I. 2007. Cumulative excretion of urinary podocytes reflects disease progression in IgA nephropathy and Schonlein-Henoch purpura nephritis. *Clin. J. Am. Soc. Nephrol.*, **2**: 231-238.

43. Hara, M., Yanagihara, T., Kihara, I., Higashi, K., Fujimoto, K., and Kajita, T. 2005. Apical cell membranes are shed into urine from injured podocytes: a novel phenomenon of podocyte injury. *J. Am. Soc. Nephrol.*, **16**: 408-416.
44. Hara, M., Yanagihara, T., Takada, T., Itoh, M., Matsuno, M., Yamamoto, T., and Kihara, I. 1998. Urinary excretion of podocytes reflects disease activity in children with glomerulonephritis. *Am. J. Nephrol.*, **18**: 35-41.
45. Harris, D.P., Goodrich, S., Gerth, A.J., Peng, S.L., and Lund, F.E. 2005. Regulation of IFN-gamma production by B effector 1 cells: essential roles for T-bet and the IFN-gamma receptor. *J. Immunol.*, **174**: 6781-6790.
46. Haywood, M.E., Rose, S.J., Horswell, S., Lees, M.J., Fu, G., Walport, M.J., and Morley, B.J. 2006. Overlapping BXSB congenic intervals, in combination with microarray gene expression, reveal novel lupus candidate genes. *Genes Immun.*, **7**: 250-263.
47. Henry, T., and Mohan, C. 2005. Systemic lupus erythematosus--recent clues from congenic strains. *Arch. Immunol. Ther. Exp. (Warsz.)*, **53**: 207-212.
48. Hiroki, J., Shimokawa, H., Higashi, M., Morikawa, K., Kandabashi, T., Kawamura, N., Kubota, T., Ichiki, T., Amano, M., Kaibuchi, K., and Takeshita, A. 2004. Inflammatory stimuli upregulate Rho-kinase in human coronary vascular smooth muscle cells. *J. Mol. Cell. Cardiol.*, **37**: 537-546.
49. Ichii, O., Kamikawa, A., Otsuka, S., Hashimoto, Y., Sasaki, N., Endoh, D., and Kon, Y. 2010a. Overexpression of interferon-activated gene 202 (Ifi202) correlates with the progression of autoimmune glomerulonephritis associated with the MRL chromosome 1. *Lupus*, **19**: 897-905.
50. Ichii, O., Konno, A., Sasaki, N., Endoh, D., Hashimoto, Y., and Kon, Y. 2008a. Altered balance of inhibitory and active Fc gamma receptors in murine autoimmune glomerulonephritis. *Kidney Int.*, **74**: 339-347.

51. Ichii, O., Konno, A., Sasaki, N., Endoh, D., Hashimoto, Y., and Kon, Y. 2008b. Autoimmune glomerulonephritis induced in congenic mouse strain carrying telomeric region of chromosome 1 derived from MRL/MpJ. *Histol. Histopathol.*, **23**: 411-422.
52. Ichii, O., Konno, A., Sasaki, N., Endoh, D., Hashimoto, Y., and Kon, Y. 2009. Onset of autoimmune glomerulonephritis derived from the telomeric region of MRL-chromosome 1 is associated with the male sex hormone in mice. *Lupus*, **18**: 491-500.
53. Ichii, O., Otsuka, S., Sasaki, N., Namiki, Y., Hashimoto, Y., and Kon, Y. 2012. Altered expression of microRNA miR-146a correlates with the development of chronic renal inflammation. *Kidney Int.*, **81**: 280-292.
54. Ichii, O., Otsuka, S., Sasaki, N., Yabuki, A., Ohta, H., Takiguchi, M., Hashimoto, Y., Endoh, D., and Kon, Y. 2010b. Local overexpression of interleukin-1 family, member 6 relates to the development of tubulointerstitial lesions. *Lab. Invest.*, **90**: 459-475.
55. Ichii, O., Yabuki, A., Sasaki, N., Otsuka, S., Ohta, H., Yamasaki, M., Takiguchi, M., Namiki, Y., Hashimoto, Y., Endoh, D., and Kon, Y. 2011. Pathological correlations between podocyte injuries and renal functions in canine and feline chronic kidney diseases. *Histol. Histopathol.*, **26**: 1243-1255.
56. Ichimura, T., Bonventre, J.V., Bailly, V., Wei, H., Hession, C.A., Cate, R.L., and Sanicola, M. 1998. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *J. Biol. Chem.*, **273**: 4135-4142.
57. Ichimura, T., Hung, C.C., Yang, S.A., Stevens, J.L., and Bonventre, J.V. 2004. Kidney injury molecule-1: a tissue and urinary biomarker for

- nephrotoxicant-induced renal injury. *Am. J. Physiol. Renal Physiol.*, **286**: F552-563.
58. Inokuchi, S., Shirato, I., Kobayashi, N., Koide, H., Tomino, Y., and Sakai, T. 1996. Re-evaluation of foot process effacement in acute puromycin aminonucleoside nephrosis. *Kidney Int.*, **50**: 1278-1287.
 59. Iwano, M., Plieth, D., Danoff, T.M., Xue, C., Okada, H., and Neilson, E.G. 2002. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J. Clin. Invest.*, **110**: 341-350.
 60. Iyer, S.S., Pulskens, W.P., Sadler, J.J., Butter, L.M., Teske, G.J., Ulland, T.K., Eisenbarth, S.C., Florquin, S., Flavell, R.A., Leemans, J.C., and Sutterwala, F.S. 2009. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc. Natl. Acad. Sci. U. S. A.*, **106**: 20388-20393.
 61. Izui, S., Higaki, M., Morrow, D., and Merino, R. 1988. The Y chromosome from autoimmune BXSB/MpJ mice induces a lupus-like syndrome in (NZW x C57BL/6)F1 male mice, but not in C57BL/6 male mice. *Eur. J. Immunol.*, **18**: 911-915.
 62. Jiang, Y., Hirose, S., Sanokawa-Akakura, R., Abe, M., Mi, X., Li, N., Miura, Y., Shirai, J., Zhang, D., Hamano, Y., and Shirai, T. 1999. Genetically determined aberrant down-regulation of FcγRIIB1 in germinal center B cells associated with hyper-IgG and IgG autoantibodies in murine systemic lupus erythematosus. *Int. Immunol.*, **11**: 1685-1691.
 63. Kanda, T., Wakino, S., Homma, K., Yoshioka, K., Tatematsu, S., Hasegawa, K., Takamatsu, I., Sugano, N., Hayashi, K., and Saruta, T. 2006. Rho-kinase as a molecular target for insulin resistance and hypertension. *FASEB J.*, **20**: 169-171.
 64. Kawai, T., and Akira, S. 2010. The role of pattern-recognition receptors in

- innate immunity: update on Toll-like receptors. *Nat. Immunol.*, **11**: 373-384.
65. Kawano, S., Lin, Q., Amano, H., Kaneko, T., Nishikawa, K., Tsurui, H., Tada, N., Nishimura, H., Takai, T., Shirai, T., Takasaki, Y., and Hirose, S. 2013. Phenotype conversion from rheumatoid arthritis to systemic lupus erythematosus by introduction of Yaa mutation into FcgammaRIIB-deficient C57BL/6 mice. *Eur. J. Immunol.*, **43**: 770-778.
 66. Kim, Y.H., Goyal, M., Kurnit, D., Wharram, B., Wiggins, J., Holzman, L., Kershaw, D., and Wiggins, R. 2001. Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat. *Kidney Int.*, **60**: 957-968.
 67. Kovesdy, C.P., and Kalantar-Zadeh, K. 2009. Review article: Biomarkers of clinical outcomes in advanced chronic kidney disease. *Nephrology (Carlton)*, **14**: 408-415.
 68. Kretzler, M. 2005. Role of podocytes in focal sclerosis: defining the point of no return. *J. Am. Soc. Nephrol.*, **16**: 2830-2832.
 69. Kriz, W., Gretz, N., and Lemley, K.V. 1998. Progression of glomerular diseases: is the podocyte the culprit? *Kidney Int.*, **54**: 687-697.
 70. Kriz, W., and LeHir, M. 2005. Pathways to nephron loss starting from glomerular diseases-insights from animal models. *Kidney Int.*, **67**: 404-419.
 71. Lavozy, C., Rodriguez-Diez, R., Benito-Martin, A., Rayego-Mateos, S., Rodriguez-Diez, R.R., Alique, M., Ortiz, A., Mezzano, S., Egido, J., and Ruiz-Ortega, M. 2012. Angiotensin II contributes to renal fibrosis independently of Notch pathway activation. *PLoS One*, **7**: e40490.
 72. Lech, M., and Anders, H.J. 2013. The pathogenesis of lupus nephritis. *J. Am. Soc. Nephrol.*, **24**: 1357-1366.
 73. Lemley, K.V., Lafayette, R.A., Safai, M., Derby, G., Blouch, K., Squarer, A.,

- and Myers, B.D. 2002. Podocytopenia and disease severity in IgA nephropathy. *Kidney Int.*, **61**: 1475-1485.
74. Li, L., Nukala, S., Du, Y., Han, J., Liu, K., Hutcheson, J., Pathak, S., Li, Q., and Mohan, C. 2012. Murine lupus strains differentially model unique facets of human lupus serology. *Clin. Exp. Immunol.*, **168**: 178-185.
 75. Ludlow, L.E., Purton, L.E., Klarmann, K., Gough, D.J., Hii, L.L., Trapani, J.A., Keller, J.R., Clarke, C.J., and Johnstone, R.W. 2008. The role of p202 in regulating hematopoietic cell proliferation and differentiation. *J. Interferon Cytokine Res.*, **28**: 5-11.
 76. Lysaght, M.J. 2002. Maintenance dialysis population dynamics: current trends and long-term implications. *J. Am. Soc. Nephrol.*, **13 Suppl 1**: S37-40.
 77. Machida, H., Ito, S., Hirose, T., Takeshita, F., Oshiro, H., Nakamura, T., Mori, M., Inayama, Y., Yan, K., Kobayashi, N., and Yokota, S. 2010. Expression of Toll-like receptor 9 in renal podocytes in childhood-onset active and inactive lupus nephritis. *Nephrol. Dial. Transplant.*, **25**: 2530-2537.
 78. Matsusaka, T., Xin, J., Niwa, S., Kobayashi, K., Akatsuka, A., Hashizume, H., Wang, Q.C., Pastan, I., Fogo, A.B., and Ichikawa, I. 2005. Genetic engineering of glomerular sclerosis in the mouse via control of onset and severity of podocyte-specific injury. *J. Am. Soc. Nephrol.*, **16**: 1013-1023.
 79. Merino, R., Fossati, L., and Izui, S. 1992. The lupus-prone BXSB strain: the Yaa gene model of systemic lupus erythematosus. *Springer Semin. Immunopathol.*, **14**: 141-157.
 80. Mersmann, J., Iskandar, F., Latsch, K., Habeck, K., Sprunck, V., Zimmermann, R., Schumann, R.R., Zacharowski, K., and Koch, A. 2013. Attenuation of myocardial injury by HMGB1 blockade during ischemia/reperfusion is toll-like receptor 2-dependent. *Mediators Inflamm.*, **2013**: 174168.

81. Meyer-Schwesinger, C., Dehde, S., Sachs, M., Mathey, S., Arefi, K., Gatzemeier, S., Balabanov, S., Becker, J.U., Thaiss, F., and Meyer, T.N. 2012. Rho-kinase inhibition prevents proteinuria in immune-complex-mediated antipodocyte nephritis. *Am. J. Physiol. Renal Physiol.*, **303**: F1015-1025.
82. Moser, K., Kalies, K., Szyska, M., Humrich, J.Y., Amann, K., and Manz, R.A. 2012. CXCR3 promotes the production of IgG1 autoantibodies but is not essential for the development of lupus nephritis in NZB/NZW mice. *Arthritis Rheum.*, **64**: 1237-1246.
83. Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L.P., Allavena, P., and Mantovani, A. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J. Immunol.*, **164**: 5998-6004.
84. Nakai, S., Wada, A., Kitaoka, T., Shinzato, T., Nagura, Y., Kikuchi, K., Masakane, I., Shinoda, T., Yamazaki, C., Sakai, R., Marubayashi, S., Morita, O., Iseki, K., Usami, T., Kimata, N., Suzuki, K., Tabei, K., Fushimi, K., Miwa, N., Yauchi, M., Wakai, K., and Akiba, T. 2006. An overview of regular dialysis treatment in Japan (as of 31 December 2004). *Ther. Apher. Dial.*, **10**: 476-497.
85. Nakamura, T., Ushiyama, C., Suzuki, S., Hara, M., Shimada, N., Ebihara, I., and Koide, H. 2000. Urinary excretion of podocytes in patients with diabetic nephropathy. *Nephrol. Dial. Transplant.*, **15**: 1379-1383.
86. Nakatsue, T., Koike, H., Han, G.D., Suzuki, K., Miyauchi, N., Yuan, H., Salant, D.J., Gejyo, F., Shimizu, F., and Kawachi, H. 2005. Nephrin and podocin dissociate at the onset of proteinuria in experimental membranous nephropathy. *Kidney Int.*, **67**: 2239-2253.

87. Nguyen, C., Limaye, N., and Wakeland, E.K. 2002. Susceptibility genes in the pathogenesis of murine lupus. *Arthritis Res.*, **4 Suppl 3**: S255-263.
88. Niemir, Z.I., Stein, H., Dworacki, G., Mundel, P., Koehl, N., Koch, B., Autschbach, F., Andrassy, K., Ritz, E., Waldherr, R., and Otto, H.F. 1997. Podocytes are the major source of IL-1 alpha and IL-1 beta in human glomerulonephritides. *Kidney Int.*, **52**: 393-403.
89. Niranjana, T., Bielecki, B., Gruenewald, A., Ponda, M.P., Kopp, J.B., Thomas, D.B., and Susztak, K. 2008. The Notch pathway in podocytes plays a role in the development of glomerular disease. *Nat. Med.*, **14**: 290-298.
90. Nowling, T.K., and Gilkeson, G.S. 2011. Mechanisms of tissue injury in lupus nephritis. *Arthritis Res. Ther.*, **13**: 250.
91. Okamoto, A., Fujio, K., Tsuno, N.H., Takahashi, K., and Yamamoto, K. 2012. Kidney-infiltrating CD4⁺ T-cell clones promote nephritis in lupus-prone mice. *Kidney Int.*, **82**: 969-979.
92. Oke, V., and Wahren-Herlenius, M. 2013. Cutaneous lupus erythematosus: clinical aspects and molecular pathogenesis. *J. Intern. Med.*, **273**: 544-554.
93. Osnes, L.T., Nakken, B., Bodolay, E., and Szodoray, P. 2013. Assessment of intracellular cytokines and regulatory cells in patients with autoimmune diseases and primary immunodeficiencies - novel tool for diagnostics and patient follow-up. *Autoimmun. Rev.*, **12**: 967-971.
94. Pagtalunan, M.E., Miller, P.L., Jumping-Eagle, S., Nelson, R.G., Myers, B.D., Rennke, H.G., Coplon, N.S., Sun, L., and Meyer, T.W. 1997. Podocyte loss and progressive glomerular injury in type II diabetes. *J. Clin. Invest.*, **99**: 342-348.
95. Pan, W., Zhu, S., Yuan, M., Cui, H., Wang, L., Luo, X., Li, J., Zhou, H., Tang, Y., and Shen, N. 2010. MicroRNA-21 and microRNA-148a contribute to DNA

- hypomethylation in lupus CD4⁺ T cells by directly and indirectly targeting DNA methyltransferase 1. *J. Immunol.*, **184**: 6773-6781.
96. Pawar, R.D., Patole, P.S., Ellwart, A., Lech, M., Segerer, S., Schlondorff, D., and Anders, H.J. 2006. Ligands to nucleic acid-specific toll-like receptors and the onset of lupus nephritis. *J. Am. Soc. Nephrol.*, **17**: 3365-3373.
 97. Peinado, H., Olmeda, D., and Cano, A. 2007. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat. Rev. Cancer*, **7**: 415-428.
 98. Peng, G., Guo, Z., Kiniwa, Y., Voo, K.S., Peng, W., Fu, T., Wang, D.Y., Li, Y., Wang, H.Y., and Wang, R.F. 2005. Toll-like receptor 8-mediated reversal of CD4⁺ regulatory T cell function. *Science*, **309**: 1380-1384.
 99. Pritchard, N.R., Cutler, A.J., Uribe, S., Chadban, S.J., Morley, B.J., and Smith, K.G. 2000. Autoimmune-prone mice share a promoter haplotype associated with reduced expression and function of the Fc receptor FcγRII. *Curr. Biol.*, **10**: 227-230.
 100. Radeke, H.H., Janssen-Graalfs, I., Sowa, E.N., Chouchakova, N., Skokowa, J., Loscher, F., Schmidt, R.E., Heeringa, P., and Gessner, J.E. 2002. Opposite regulation of type II and III receptors for immunoglobulin G in mouse glomerular mesangial cells and in the induction of anti-glomerular basement membrane (GBM) nephritis. *J. Biol. Chem.*, **277**: 27535-27544.
 101. Ramanujam, M., Bethunaickan, R., Huang, W., Tao, H., Madaio, M.P., and Davidson, A. 2010. Selective blockade of BAFF for the prevention and treatment of systemic lupus erythematosus nephritis in NZM2410 mice. *Arthritis Rheum.*, **62**: 1457-1468.
 102. Rao, M., Wong, C., Kanetsky, P., Girndt, M., Stenvinkel, P., Reilly, M., and Raj, D.S. 2007. Cytokine gene polymorphism and progression of renal and

- cardiovascular diseases. *Kidney Int.*, **72**: 549-556.
103. Reiser, J., von Gersdorff, G., Simons, M., Schwarz, K., Faul, C., Giardino, L., Heider, T., Loos, M., and Mundel, P. 2002. Novel concepts in understanding and management of glomerular proteinuria. *Nephrol. Dial. Transplant.*, **17**: 951-955.
 104. Reth, M. 1989. Antigen receptor tail clue. *Nature*, **338**: 383-384.
 105. Ryan, G.B., and Karnovsky, M.J. 1975. An ultrastructural study of the mechanisms of proteinuria in aminonucleoside nephrosis. *Kidney Int.*, **8**: 219-232.
 106. Sacks, S.H., Zhou, W., and Sheerin, N.S. 1996. Complement synthesis in the injured kidney: does it have a role in immune complex glomerulonephritis? *J. Am. Soc. Nephrol.*, **7**: 2314-2319.
 107. Sarnak, M.J., Levey, A.S., Schoolwerth, A.C., Coresh, J., Culleton, B., Hamm, L.L., McCullough, P.A., Kasiske, B.L., Kelepouris, E., Klag, M.J., Parfrey, P., Pfeffer, M., Raij, L., Spinosa, D.J., and Wilson, P.W. 2003. Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Hypertension*, **42**: 1050-1065.
 108. Sato, Y., Wharram, B.L., Lee, S.K., Wickman, L., Goyal, M., Venkatareddy, M., Chang, J.W., Wiggins, J.E., Lienczewski, C., Kretzler, M., and Wiggins, R.C. 2009. Urine podocyte mRNAs mark progression of renal disease. *J. Am. Soc. Nephrol.*, **20**: 1041-1052.
 109. Schiffer, M., Bitzer, M., Roberts, I.S., Kopp, J.B., ten Dijke, P., Mundel, P., and Bottinger, E.P. 2001. Apoptosis in podocytes induced by TGF-beta and Smad7. *J. Clin. Invest.*, **108**: 807-816.

110. Schocklmann, H.O., Lang, S., and Sterzel, R.B. 1999. Regulation of mesangial cell proliferation. *Kidney Int.*, **56**: 1199-1207.
111. Seeling, M., Hillenhoff, U., David, J.P., Schett, G., Tuckermann, J., Lux, A., and Nimmerjahn, F. 2013. Inflammatory monocytes and Fcγ receptor IV on osteoclasts are critical for bone destruction during inflammatory arthritis in mice. *Proc. Natl. Acad. Sci. U. S. A.*, **110**: 10729-10734.
112. Sheerin, N.S., Risley, P., Abe, K., Tang, Z., Wong, W., Lin, T., and Sacks, S.H. 2008. Synthesis of complement protein C3 in the kidney is an important mediator of local tissue injury. *FASEB J.*, **22**: 1065-1072.
113. Shibata, S., Nagase, M., and Fujita, T. 2006. Fluvastatin ameliorates podocyte injury in proteinuric rats via modulation of excessive Rho signaling. *J. Am. Soc. Nephrol.*, **17**: 754-764.
114. Shichita, T., Hasegawa, E., Kimura, A., Morita, R., Sakaguchi, R., Takada, I., Sekiya, T., Ooboshi, H., Kitazono, T., Yanagawa, T., Ishii, T., Takahashi, H., Mori, S., Nishibori, M., Kuroda, K., Akira, S., Miyake, K., and Yoshimura, A. 2012. Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. *Nat. Med.*, **18**: 911-917.
115. Shigeoka, A.A., Holscher, T.D., King, A.J., Hall, F.W., Kiosses, W.B., Tobias, P.S., Mackman, N., and McKay, D.B. 2007. TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. *J. Immunol.*, **178**: 6252-6258.
116. Shirato, I. 2002. Podocyte process effacement in vivo. *Microsc. Res. Tech.*, **57**: 241-246.
117. Shirato, I., Sakai, T., Kimura, K., Tomino, Y., and Kriz, W. 1996. Cytoskeletal changes in podocytes associated with foot process effacement in Masugi nephritis. *Am. J. Pathol.*, **148**: 1283-1296.

118. Skoberne, A., Konieczny, A., and Schiffer, M. 2009. Glomerular epithelial cells in the urine: what has to be done to make them worthwhile? *Am. J. Physiol. Renal Physiol.*, **296**: F230-241.
119. Smyth, L.J., Duffy, S., Maxwell, A.P., and McKnight, A.J. 2014. Genetic and epigenetic factors influencing chronic kidney disease. *Am. J. Physiol. Renal Physiol.*, **307**: F757-f776.
120. Subramanian, S., Tus, K., Li, Q.Z., Wang, A., Tian, X.H., Zhou, J., Liang, C., Bartov, G., McDaniel, L.D., Zhou, X.J., Schultz, R.A., and Wakeland, E.K. 2006. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. *Proc. Natl. Acad. Sci. U. S. A.*, **103**: 9970-9975.
121. Szeto, C.C., Lai, K.B., Chow, K.M., Szeto, C.Y., Yip, T.W., Woo, K.S., Li, P.K., and Lai, F.M. 2005. Messenger RNA expression of glomerular podocyte markers in the urinary sediment of acquired proteinuric diseases. *Clin. Chim. Acta*, **361**: 182-190.
122. Takai, T. 2005. Fc receptors and their role in immune regulation and autoimmunity. *J. Clin. Immunol.*, **25**: 1-18.
123. Takano, Y., Yamauchi, K., Hayakawa, K., Hiramatsu, N., Kasai, A., Okamura, M., Yokouchi, M., Shitamura, A., Yao, J., and Kitamura, M. 2007. Transcriptional suppression of nephrin in podocytes by macrophages: roles of inflammatory cytokines and involvement of the PI3K/Akt pathway. *FEBS Lett.*, **581**: 421-426.
124. Takemoto, M., Asker, N., Gerhardt, H., Lundkvist, A., Johansson, B.R., Saito, Y., and Betsholtz, C. 2002. A new method for large scale isolation of kidney glomeruli from mice. *Am. J. Pathol.*, **161**: 799-805.
125. Tanaka, M., Asada, M., Higashi, A.Y., Nakamura, J., Oguchi, A., Tomita, M., Yamada, S., Asada, N., Takase, M., Okuda, T., Kawachi, H., Economides,

- A.N., Robertson, E., Takahashi, S., Sakurai, T., Goldschmeding, R., Muso, E., Fukatsu, A., Kita, T., and Yanagita, M. 2010. Loss of the BMP antagonist USAG-1 ameliorates disease in a mouse model of the progressive hereditary kidney disease Alport syndrome. *J. Clin. Invest.*, **120**: 768-777.
126. Tanaka, M., Endo, S., Okuda, T., Economides, A.N., Valenzuela, D.M., Murphy, A.J., Robertson, E., Sakurai, T., Fukatsu, A., Yancopoulos, G.D., Kita, T., and Yanagita, M. 2008. Expression of BMP-7 and USAG-1 (a BMP antagonist) in kidney development and injury. *Kidney Int.*, **73**: 181-191.
127. Tang, Z., Lu, B., Hatch, E., Sacks, S.H., and Sheerin, N.S. 2009. C3a mediates epithelial-to-mesenchymal transition in proteinuric nephropathy. *J. Am. Soc. Nephrol.*, **20**: 593-603.
128. Timoshanko, J.R., Kitching, A.R., Iwakura, Y., Holdsworth, S.R., and Tipping, P.G. 2004. Leukocyte-derived interleukin-1beta interacts with renal interleukin-1 receptor I to promote renal tumor necrosis factor and glomerular injury in murine crescentic glomerulonephritis. *Am. J. Pathol.*, **164**: 1967-1977.
129. Tonelli, M., Wiebe, N., Culeton, B., House, A., Rabbat, C., Fok, M., McAlister, F., and Garg, A.X. 2006. Chronic kidney disease and mortality risk: a systematic review. *J. Am. Soc. Nephrol.*, **17**: 2034-2047.
130. Tsokos, G.C. 2011. Systemic lupus erythematosus. *N. Engl. J. Med.*, **365**: 2110-2121.
131. Uchida, S. 2011. Differential diagnosis of chronic kidney disease (CKD): by primary diseases. *Jpn. Med. Assoc. J.*, **54**: 22-26.
132. Ueki, A., Sai, T., Oka, H., Tabata, M., Hosokawa, K., and Mochizuki, Y. 1987. Biosynthesis and secretion of the third component of complement by human endothelial cells in vitro. *Immunology*, **61**: 11-14.

133. Waldman, M., and Appel, G.B. 2006. Update on the treatment of lupus nephritis. *Kidney Int.*, **70**: 1403-1412.
134. Wang, G., Lai, F.M., Lai, K.B., Chow, K.M., Kwan, B.C., Li, P.K., and Szeto, C.C. 2008. Urinary messenger RNA expression of podocyte-associated molecules in patients with diabetic nephropathy treated by angiotensin-converting enzyme inhibitor and angiotensin receptor blocker. *Eur. J. Endocrinol.*, **158**: 317-322.
135. Wang, G., Lai, F.M., Tam, L.S., Li, K.M., Lai, K.B., Chow, K.M., Li, K.T., and Szeto, C.C. 2007. Messenger RNA expression of podocyte-associated molecules in urinary sediment of patients with lupus nephritis. *J. Rheumatol.*, **34**: 2358-2364.
136. Wharram, B.L., Goyal, M., Wiggins, J.E., Sanden, S.K., Hussain, S., Filipiak, W.E., Saunders, T.L., Dysko, R.C., Kohno, K., Holzman, L.B., and Wiggins, R.C. 2005. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *J. Am. Soc. Nephrol.*, **16**: 2941-2952.
137. White, K.E., Bilous, R.W., Marshall, S.M., El Nahas, M., Remuzzi, G., Piras, G., De Cosmo, S., and Viberti, G. 2002. Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes*, **51**: 3083-3089.
138. Wickman, L., Afshinnia, F., Wang, S.Q., Yang, Y., Wang, F., Chowdhury, M., Graham, D., Hawkins, J., Nishizono, R., Tanzer, M., Wiggins, J., Escobar, G.A., Rovin, B., Song, P., Gipson, D., Kershaw, D., and Wiggins, R.C. 2013. Urine podocyte mRNAs, proteinuria, and progression in human glomerular diseases. *J. Am. Soc. Nephrol.*, **24**: 2081-2095.
139. Wiggins, R.C. 2007. The spectrum of podocytopathies: a unifying view of glomerular diseases. *Kidney Int.*, **71**: 1205-1214.

140. Wolfs, T.G., Buurman, W.A., van Schadewijk, A., de Vries, B., Daemen, M.A., Hiemstra, P.S., and van 't Veer, C. 2002. In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. *J. Immunol.*, **168**: 1286-1293.
141. Xin, H., Pramanik, R., and Choubey, D. 2003. Retinoblastoma (Rb) protein upregulates expression of the Ifi202 gene encoding an interferon-inducible negative regulator of cell growth. *Oncogene*, **22**: 4775-4785.
142. Xiu, Y., Nakamura, K., Abe, M., Li, N., Wen, X.S., Jiang, Y., Zhang, D., Tsurui, H., Matsuoka, S., Hamano, Y., Fujii, H., Ono, M., Takai, T., Shimokawa, T., Ra, C., Shirai, T., and Hirose, S. 2002. Transcriptional regulation of Fcgr2b gene by polymorphic promoter region and its contribution to humoral immune responses. *J. Immunol.*, **169**: 4340-4346.
143. Yanagita, M. 2006. Modulator of bone morphogenetic protein activity in the progression of kidney diseases. *Kidney Int.*, **70**: 989-993.
144. Yanagita, M., Okuda, T., Endo, S., Tanaka, M., Takahashi, K., Sugiyama, F., Kunita, S., Takahashi, S., Fukatsu, A., Yanagisawa, M., Kita, T., and Sakurai, T. 2006. Uterine sensitization-associated gene-1 (USAG-1), a novel BMP antagonist expressed in the kidney, accelerates tubular injury. *J. Clin. Invest.*, **116**: 70-79.
145. Yi, F., dos Santos, E.A., Xia, M., Chen, Q.Z., Li, P.L., and Li, N. 2007. Podocyte injury and glomerulosclerosis in hyperhomocysteinemic rats. *Am. J. Nephrol.*, **27**: 262-268.
146. Zhang, C., Hu, J.J., Xia, M., Boini, K.M., Brimson, C., and Li, P.L. 2010. Redox signaling via lipid raft clustering in homocysteine-induced injury of podocytes. *Biochim. Biophys. Acta*, **1803**: 482-491.
147. Zhang, D., Zhang, G., Hayden, M.S., Greenblatt, M.B., Bussey, C., Flavell,

- R.A., and Ghosh, S. 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science*, **303**: 1522-1526.
148. Zhou, X.J., Lv, J.C., Bu, D.F., Yu, L., Yang, Y.R., Zhao, J., Cui, Z., Yang, R., Zhao, M.H., and Zhang, H. 2010. Copy number variation of FCGR3A rather than FCGR3B and FCGR2B is associated with susceptibility to anti-GBM disease. *Int. Immunol.*, **22**: 45-51.
149. Zimmerman, M., Yang, D., Hu, X., Liu, F., Singh, N., Browning, D., Ganapathy, V., Chandler, P., Choubey, D., Abrams, S.I., and Liu, K. 2010. IFN-gamma upregulates survivin and Ifi202 expression to induce survival and proliferation of tumor-specific T cells. *PLoS One*, **5**: e14076.

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Conclusion in Japanese

自己免疫性糸球体腎炎モデルの分子病態に関する研究

— 慢性腎臓病の早期進行サインとしての足細胞傷害 —

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近年、高度医療化に伴う寿命の延長を背景に、ヒトおよび動物の慢性腎臓病 (CKD) が世界的に急増している。CKD の進行は末期腎不全を引き起こし、心血管系疾患による死亡リスクを高めることから、その制御は医学・獣医学双方の課題である。腎臓は非再生性の臓器であるため、CKD では早期診断と治療が重要である。故に、現代の CKD 研究の最終目標は、その詳細な病理機序の解明と、それに基づく新たな診断ならびに治療法の開発である。そこで筆者は、CKD の主たる一次疾患である慢性糸球体腎炎 (GN) に着目し、本研究では自己免疫性 GN モデルマウスを用い、CKD へと導く GN の分子病理、遺伝学的因子、および増悪因子を精査した。

第一章において、筆者は疾患モデルとして BXSB/MpJ-*Yaa* (BXSB-*Yaa*) マウスを用い、腎臓および尿の異常を精査した。本マウスは Y 染色体上に変異遺伝子座 (*Yaa*) を有し、自己免疫性 GN を発症する。BXSB-*Yaa* マウスは糸球体総核数の増加とメサンギウム基質の増生を特徴とする膜性増殖性 GN を発症し、その尿細管間質には尿円柱による尿細管の拡張および囲管性細胞浸

潤が認められた。尿中細胞診の結果、BXSB-*Yaa* マウスの尿中細胞数は病態進行と共に増加し、尿を用いた RT-PCR 法で糸球体足細胞、遠位尿細管 (DT) 上皮細胞、および集合管 (CD) 上皮細胞マーカーの mRNA が検出された。さらに、傷害を受けた DT および CD は C3 を産生しており、C3 mRNA は BXSB-*Yaa* マウスの尿中で高率に検出された。以上より、CKD 進行において傷害を受けた腎臓の上皮細胞は尿中に脱落することが明らかとなった。

多くの CKD の病態では、まず血液尿関門 (BUB) 破綻を含む糸球体病変 (GL) が現れ、次いで漏出蛋白が尿細管間質病変 (TIL) を引き起こし、最終的に末期腎不全へと移行する。そこで筆者は、CKD の早期進行サインとして足細胞傷害に着目し、その分子病理を精査した。足細胞は BUB を制御する糸球体の上皮細胞として機能的に重要である。本章では、BXSB-*Yaa* マウスに加えて、第一染色体テロメア領域に自己免疫性 GN 原因遺伝子座を持つ B6.MRL-(*D1Mit202-D1Mit403*) (B6.MRL) マウスを自己免疫性 GN モデルとして用いた。両マウスは明らかな膜性増殖性 GN を呈し、血中抗 dsDNA 抗体濃度および尿中アルブミンクレアチニン比 (uACR) は各対照群のそれらに比べて有意に高かった。電顕観察では、足細胞領域に足突起の癒合、および不整な微絨毛様構造が観察された。両マウスにおける各足細胞機能因子の蛋白および糸球体内 mRNA 発現量は対照群のそれらに比べて低く、特に後者は uACR と負の相関を示した。これらの結果は、CKD 進行における足細胞の形態機能の重要性を示しており、その異常は臨床病理学的に悪性の病理変化であることを強調した。

第三章において、筆者は GL 形成に関与する遺伝的因子を解析した。*Yaa*

原因遺伝子座を持たない雌 BXSB/MpJ (BXSB) マウスを経時的に観察したところ、加齢と共に血中抗 dsDNA 抗体濃度の増加、脾/体重量の増加、および膜性増殖性 GN の発症を示した。一方、観察期間を通じて雄 BXSB マウスは自己免疫傾向および GN 発症を示さなかった。糸球体の遺伝子発現を雄 BXSB-*Yaa* と BXSB マウスで網羅的に比較したところ、*Fcgr3* および *Ifi202b* の発現量が前者で顕著に増加していた。これらの遺伝子は第一染色体テロメア領域に位置する自己免疫性 GN の原因遺伝子候補である。また、雌 BXSB マウス腎臓においても、*Fcgr3* および *Ifi202b* の発現量は対照群のそれらよりも高く、加齢と共に増加した。以上の結果より、BXSB 系統における CKD 進行は自己免疫応答における性差、ならびに *Yaa* 遺伝子座による第一染色体テロメア領域由来 GN 原因遺伝子の発現増強作用の影響を強く受けることが示唆された。

第三章の結果は、GN 進行を制御する遺伝子が *Yaa* 遺伝子座上に存在することを示唆する。第四章では、*Yaa* 遺伝子座上の Toll-like receptor (TLR) ファミリーに着目し、CKD 診断ならびに治療における分子標的としての可能性を検証した。近年、TLR ファミリーは局所の自己免疫応答を制御する抗原センサーとして注目されている。BXSB-*Yaa* および B6.MRL マウス糸球体における TLR ファミリーの遺伝子発現を網羅的に解析した結果、*Tlr8* とその下流因子の発現量が対照群よりも有意に高かった。マウス腎臓において TLR8 蛋白・mRNA は糸球体足細胞特異的に局在し、ヒトでも TLR8 蛋白は糸球体足細胞に局在した。BXSB-*Yaa* マウスの糸球体において、*Tlr8* 発現量は uACR と正の相関を、足細胞機能因子の発現量と負の相関を示した。さらに、TLR8 の内因性

リガンドである miR-21 の量は、BXSB-Yaa マウスの糸球体および血清で対照群のそれらよりも多かった。また、尿中 *Tlr8* mRNA は BXSB-Yaa マウスで対照群よりも高レベルに検出された。これらの結果は、TLR8 シグナルの過度の活性化は CKD の足細胞傷害に深く関与し、TLR8 は CKD の新たな診断ならびに治療法開発の標的分子となる可能性を示した。

結論として、筆者は慢性 GN モデルマウスを用いて CKD の分子病態を明らかにし、足細胞傷害が CKD の早期進行サインになることを示した。TLR8 は CKD、特に慢性 GN における足細胞傷害の早期診断ならびに治療法の開発に有用な標的分子になる。本研究で得られた結果は、医学および獣医学領域における CKD 制圧に寄与する重要な基礎的知見となる。