Original research

Altered renal pathology in an autoimmune disease mouse model after induction of diabetes mellitus

Running title: Diabetic kidney disease in autoimmune disease model mice

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Abstract

Diabetes mellitus (DM) is a predisposing factor for renal disorder progression and is referred to as diabetic kidney disease (DKD). However, there are no reports of DKD with an underlying autoimmune disorder. In this study, we compared the pathophysiological changes caused by DM induction after streptozotocin (STZ) injection in comparison with that in a control group receiving citrate buffer (CB) in the autoimmune disease model mice "BXSB/MpJ-Yaa" (Yaa) and the wild-type strain BXSB/MpJ. Both strains showed hyperglycemia after 12 weeks of STZ injection. Interestingly, the Yaa group developed membranous and proliferative glomerulonephritis, which tended to be milder glomerular lesions in the STZ group than in the CB group, as indicated by decreased mesangial area and ameliorated albuminuria. Statistically, the indices for hyperglycemia and autoimmune abnormalities were negatively and positively correlated with the histopathological parameters for mesangial matrix production and glomerular proliferative lesions, respectively. STZ treatment induced renal tubular anisonucleosis and dilations in both strains, and they were more severe in Yaa. Significantly decreased cellular infiltration was observed in the Yaa group compared to the CB group. Thus, in DKD related to autoimmune nephritis, hyperglycemia modifies its pathology by decreasing the mesangial area and interstitial inflammation and aggravating renal tubular injury.

Keywords: Autoimmune disease, Diabetes mellitus, Diabetic kidney disease, BXSB/MpJ-Yaa, Streptozotocin

Introduction

Diabetes mellitus (DM) is characterized by increased blood glucose levels (BGL) due to the hyposecretion of insulin by β-cell loss or dysfunction and insulin resistance (Chatterjee, et al., 2017; DiMeglio, et al., 2018). The prevalence of DM in adults aged 20–79 years is predicted to increase to 10.4% by 2040 worldwide (Ogurtsova, et al., 2017). DM often induces diabetic nephropathy (DN) This is the primary cause of chronic kidney disease (CKD) and eventually results in end-stage renal disease (Harding, et al., 2019; Olivares, et al., 2017; Qi et al., 2017). Recently, it was recognized that many patients with DM do not follow the classic DN phenotypes, such as glomerular hyperfiltration progressing to persistent albuminuria associated with hypertension and declining glomerular filtration rate (Alicic, et al., 2017), and other systemic symptoms such as autoimmune abnormalities

seemed to modify the representative DN phenotypes. Nowadays, the term “diabetic kidney disease (DKD)” has been used to cover both classical DN and other types of renal dysfunction in DM patients requiring the comprehensive treatments for other underlying conditions (Hirakawa, et al., 2017).

As for the relationship between immunity and DM, hyperglycemia causes dysfunction of the immune response associated with infection susceptibility by altering the complement system function and chemotaxis, locomotion, and phagocytosis of innate immunity-related cells such as neutrophils (Berbudi, et al., 2020; Jafar, et al., 2016). Furthermore, many patients with DKD have been reported to have autoimmune abnormality-related renal diseases such as membranous glomerulonephritis, membranous proliferative glomerulonephritis (MPGN), and IgA nephropathy (Jalalah, 2008; Kanodia, et al., 2017). Furthermore, the treatment of autoimmune diseases using steroids such as prednisolone could increase insulin resistance by decreasing glucose uptake and its utilization in the skeletal muscle and white adipose tissue by antagonizing the insulin response (Kuo, et al., 2015; Nerhagen, et al., 2021). Therefore, the administration of such drugs to patients with autoimmune disease-associated DKD could increase BGL. Thus, these complicated pathophysiological relationships between DM and immunity make it difficult to provide appropriate therapy.

To elucidate the pathophysiology of DN, streptozotocin (STZ), which leads to oxidative stress-mediated β-cell death, is generally used for DM induction in healthy rodents, and previous studies have reported that STZ-injected mice show severe tubulointerstitial lesions and moderate glomerular lesions respectively (Bolzán & Bianchi, 2002; Tamura, et al., 2005). Other studies have shown that spontaneous Akita or db/db mice develop glomerular lesions, which are representative of the early stages of human DN (Kitada, et al., 2016). However, they do not clearly exhibit all of the key histological features of human DN, such as severe glomerular nodular lesions and tubulointerstitial fibrosis. Importantly, altered immune conditions modify the renal phenotypes of DM animals, and glomerulosclerotic features are accelerated by immune complex-mediated glomerulopathy in STZ-injected rats (Abrass & Cohen, 1987), however, the pathological characteristics of autoimmune disease-associated DKD remain unclear.

Thus, because there are various disease-modifying factors in DKD, clarification of the pathogenesis of each type of DKD is essential for the development of therapeutic strategies. In this study, we focused on immunological

alterations as candidate modifiers of DM and/or DKD. A representative autoimmune disease mouse model, BXSB/MpJ-*Yaa* (Yaa) carrying the Y-linked autoimmune accelerator (*Yaa*) mutation on the Y chromosome, was pathologically analyzed after induction of DM with an STZ injection. Naïve Yaa generally developed MPGN with autoantibody production and splenomegaly, but DM-induced Yaa revealed altered renal histopathology characterized mainly by milder glomerular mesangial and more severe tubular lesions. This study provides novel insights into the pathogenesis of autoimmune-related DKD, which contributes to the appropriate handling of this malignant disease in humans and animals.

Materials and Methods

Experimental animals and ethics statement

Male BXSB/MpJ (BXSB) and Yaa mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All mice were housed at a constant temperature (22 ± 4 °C) and humidity (50 ± 20%) with a 12:12-h light-dark cycle in a specific pathogen-free facility, and they were provided free access to standard rodent chow (CLEA Rodent Diet CE-2, CLEA Japan, Inc.; TOKYO, Japan) and water accordingly. Animal experimentation was approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval No. 16-0124). All experimental animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International).

DM induction and sample collection

Male BXSB and Yaa mice (12 weeks of age) received a single intraperitoneal (i.p.) injection of either STZ (300 mg/kg; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) dissolved in citrate buffer (CB; pH 4.7) or only CB. The mice were divided into four groups: 1) BXSB injected with CB (BXSB-CB) (n=5), and used as control mice; 2) BXSB injected with STZ (BXSB-STZ) (n=5), and considered as diabetic mice; 3) Yaa injected with CB (Yaa-CB) (n=8), as autoimmune disease mice; and 4) Yaa injected with STZ (Yaa-STZ) (n=8), as autoimmune disease mice accompanied with DM.

From 11 to 24 weeks of age, body weight (BW) and BGL were measured at the end of each week. BGL was measured in the blood collected from the tail vein using a Medisafe mini GR-102 (Terumo Co. Ltd., Tokyo, Japan). At 24 weeks of age, the urine of each mouse was collected by pressure urination and preserved at -30 °C for urinalysis. Under deep anesthesia, induced using a mixture of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg), blood was collected by cutting the femoral arteries, and all mice were euthanized by cervical dislocation. The spleen, pancreas, and kidneys were collected immediately and used for further analysis. Spleen weight (SPW) was measured, and the ratio of SPW to BW (SPW/BW) was thus calculated.

Serological analysis and urinalysis

As an index of autoimmune disease development, the serum levels of anti-double-stranded DNA (dsDNA) antibody were measured to evaluate systemic autoimmune conditions using the LBIS Anti-dsDNA-Mouse ELISA Kit (Fujifilm Wako Pure Chemical Corporation) according to the manufacturer's instructions. Furthermore, to evaluate renal function, the serum levels of blood urea nitrogen (BUN) and creatinine (Cre) were measured using Fuji Drichem (Fujifilm Medical Co. Ltd., Tokyo, Japan). The urinary levels of Cre and albumin (ALB) were measured using a Urinary One-Step Creatinine Assay (Detroit R&D, Inc., Detroit, MI, USA) and LBIS Anti albumin-Mouse ELISA Kit (Fujifilm Wako Pure Chemical Corporation) according to the manufacturer's instructions. The urinary albumin-to-creatinine ratio (ACR) was also calculated. Urinary glucose, pH, and specific gravity were measured using urine test paper (Siemens Healthcare Diagnostics Co. Ltd., Tokyo, Japan).

Histopathological analysis

The tissues were immediately fixed with 4% paraformaldehyde at 4 °C. After overnight fixation, the tissues were washed, dehydrated with ascending graded ethanol, embedded in paraffin, and cut into 2-μm-thick (kidney) or 3-μm-thick (pancreas) sections respectively. Sections of the pancreas were stained with hematoxylin-eosin (HE), and the kidney sections were stained with periodic acid-Schiff hematoxylin (PAS-H), Masson's trichrome (MT), or periodic acid-methenamine silver (PAM).

Immunostaining

Paraffin sections were deparaffinized, hydrated, and subjected to antigen retrieval (Table 1). The sections were then soaked in methanol containing 0.3% H_2O_2 for 20 min at room temperature to block internal peroxidase activity. After washing thrice in phosphate-buffered saline (PBS), the sections were incubated with blocking serum for 1 h at room temperature, followed by overnight incubation with primary antibodies at 4 °C. After washing three times with PBS, the sections were incubated with secondary antibodies for 30 min at room temperature and washed three times with PBS. For immunohistochemistry, the sections were incubated with streptavidin-conjugated horseradish peroxidase (SABPO (R) kit; Nichirei, Tokyo, Japan) for 30 min and washed three times with PBS. For

visualization of the positive reactions, the sections were incubated with 10 mg of 3,3'-diaminobenzidine tetrahydrochloride in 50 mL of 0.05M Tris-HCl buffer-H_2O_2 solution. Finally, the sections were stained with haematoxylin. For immunofluorescence, the sections were incubated with fluorescent-labeled secondary antibody for 30 min (Table 1), followed by incubation with Hoechst33342 for 2 min (1:2000; Dojindo, Kumamoto, Japan). Immunofluorescence signals were examined using a fluorescence microscope (BZX-710; Keyence, Osaka, Japan). Details of the antibody, antigen retrieval, and blocking are listed in Table 1.

Histoplanimetry

To compare the area ratio of islets to pancreas among different groups, HE-stained pancreatic sections (8 sections per 20-μm thickness of each mouse) were converted to virtual slides using Nano Zoomer 2.0 RS (Hamamatsu Photonics Co., Ltd.; Hamamatsu, Japan). NDP. view2 (Hamamatsu Photonics Co., Ltd.) and Image J (NIH; Bethesda, Maryland, USA) were used to measure the cross-sectional area of the islets and pancreas, respectively. Then, the percentages of islet areas against the pancreatic areas were calculated and compared among the studied groups.

In PAS-stained kidney sections, more than 30 glomeruli were randomly selected from each mouse kidney section within different groups that were subjected to the following measurements using BZ-X Analyzer software (Keyence): the glomerular PAS^+ area (as an index for the mesangial area), total number of glomerular nuclei, and glomerular size. Furthermore, more than 20 fields of renal cortex/mouse kidney sections within different groups were randomly captured under a high-power field (×400), and this was used to calculate the ratio of the tubular lumen area to the renal cortex area within each field, as well as to measure the nuclear size of tubular epithelium cells.

In immunohistochemistry, the number of $B220^+$ B-cells or $CD3^+$ T-cells observed in the digital images of glomeruli (>30 cells) and tubulointerstitium (>20 renal cortex areas at high-power field "×400"), and the number of interleukin 1 family, member 6 [also known as interleukin-36 alpha (IL-36α)]$^+$ tubules (3 sections per 50 μm thickness of each mouse), indicating damaged renal tubules (Ichii, et al., 2010), were counted manually in each mouse.

For immunofluorescence, the area ratios of insulin$^+$ cells to glucagon$^+$ cells were calculated using a BZ-X Analyzer (Keyence; 8 sections per 20 μm thickness of each mouse).

Quantitative polymerase chain reaction (qPCR)

Total RNA from the kidney was purified using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The purified total RNA (83.3 ng/μL) was treated as a template to synthesize complementary DNA (cDNA) using ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd.; Osaka, Japan). qPCR analysis was performed on the cDNA (20 ng/μL) using THUNDERBIRD® SYBR® qPCR Mix (Toyobo Co., Ltd.) and the following gene-specific primers (Table 2). The qPCR cycling conditions were as follows: 95 °C for 1 min, followed by 95 °C for 15 s and 60 °C for 45 s (40 cycles). Data were normalized by the values of actin, beta (*Actb*) (Ichii, et al., 2010), and those of Yaa-CB using the delta-delta Ct method.

Statistical analysis

The results are expressed as mean ± standard error (SE). The Mann-Whitney *U* test was used for analysis between the two groups. Furthermore, Spearman's correlation test was performed to analyze the correlation between the two parameters. In all analyses, a P-value < 0.05 was regarded as a significant difference.

Results

Indices of DM and autoimmune disease

For indices of DM, both BGL and BW were measured weekly following injection of either STZ or CB to BXSB and Yaa at 12 weeks of age until sampling at 24 weeks of age. As shown in Fig. 1a, all examined STZ-treated mice developed hyperglycemia (>250 mg/dL). In the BXSB-STZ group, BGL was maintained at high values (392 ± 48 mg/dL) throughout the observation period. The Yaa-STZ group had a relatively lower BGL (262 ± 46.76 mg/dL) than the BXSB-STZ group. Significant differences between BXSB-STZ and Yaa-STZ were observed at 14, 16, 19, and 20 weeks of age. There was a similarity in the BGL of CB-injected mice in both the strains. Fig. 1b shows the changes in the BW. A reduction in BW was observed in the STZ-injected mice of both strains due to DM. Notably, the reduction in BW was significantly earlier in the Yaa-STZ group than in the BXSB-STZ group, and their BW became comparable at 15 weeks of age.

Figure 1c-e shows the autoimmune disease indices, including the ratio of SPW/BW, SPW, and serum levels of anti-dsDNA antibody at 24 weeks of age. Yaa showed significantly higher values than BXSB in the CB- and STZ-treated groups, but no significant strain difference was observed in either of the groups. These data indicated that DM had less effect on systemic autoimmunity in both BXSB and Yaa strains.

Histopathology of the pancreas

As shown in the histological examination of the H&E-stained pancreatic tissue sections at 24 weeks of age (Fig. 2a), STZ-injected mice showed a remarkable decrease in the size of pancreatic islets in both strains due to its toxicity to β-cells without any immune cell infiltration in or around the islets. Morphometrically, a significant reduction in the ratio of islet area to pancreatic area was observed in STZ-injected mice compared to that in the CB group without any strain-related differences (Fig. 2b). To estimate β-cell toxicity, immunofluorescence for glucagon$^+$ α-cells and insulin$^+$ β-cells was performed in the pancreas (Fig. 2c). In the STZ-treated groups, insulin$^+$ β-cells decreased, indicating sustained β-cell loss until 24 weeks of age after STZ injection. The ratio of the insulin$^+$ area to the glucagon$^+$ area was significantly lower in the STZ-injected mice than in the CB-injected mice without strain-related differences (Fig. 2d). Thus, our results confirmed the comparable β-cell damage induced by STZ

210 treatment in both the strains.

211

212 *Renal function*

213 Serological analysis (Figs. 3a, 3b) showed the serum BUN but Cre in BXSB tended to be increased by STZ
214 injection, but the difference was not significant. Furthermore, the CB-treated groups (but not STZ-treated groups)
215 in Yaa showed significantly higher serum Cre levels than BXSB (Fig. 3b).

216 For urinalysis (Figs. 3c-3e), no significant difference was observed in urine pH (Fig. 3c) and specific gravity
217 (Fig. 3d) among all the groups. In contrast, STZ-treated mice showed a significant increase in urine sugar in both
218 the strains, but no significant strain difference was observed in either group (Fig. 3e).

219

220 *Glomerular pathology*

221 Glomerular lesions were observed with PAS-H, MT, and PAM staining (Fig. 4a). Both treatment groups in Yaa
222 showed increased glomerular size and cell numbers compared with BXSB. In PAS-H staining of both treatment
223 groups, Yaa revealed the development of glomerular proliferative lesions, including increased mesangial cells and
224 matrix. MT-stained kidney sections of both treatment groups in Yaa revealed an increased aniline blue^{+} area
225 indicating the sclerotic area; however, they did not show nodular glomerular sclerosis. In addition, PAM staining
226 clearly revealed spike-like structures or double-contoured features in the glomerular basement membrane of Yaa
227 glomeruli in both treatment groups.

228 As shown in Fig. 4b, all examined parameters (including glomerular size, nuclear number, mesangial area, and
229 area ratio) in the glomerulus showed a significant increase in Yaa compared with BXSB in both CB- and STZ-
230 treated groups. Interestingly, the ratio of mesangial area in the glomerulus of Yaa significantly decreased by STZ
231 injection.

232 Fig. 5a shows the immunohistochemical staining results for inflammatory cells, including B220^{+} B-cells and
233 CD3^{+} T-cells. Increased infiltration of such cells into the glomerulus was observed in both the Yaa treatment groups.
234 As shown in Fig. 5b, the number of these immune cell infiltrations into the glomerulus significantly increased in
235 Yaa compared to that in BXSB; however, there was no significant difference among treatments between the CB-

and STZ-treated groups. Thus, STZ injection altered MPGN features by decreasing the mesangial area ratio in Yaa mice, rather than having a remarkable effect on glomerular inflammation.

As for glomerular function (Fig. 5c), Yaa showed significantly increased urinary ACR compared with that in BXSB in both the CB- and STZ-treatment groups, and a decreased tendency of such value was observed in Yaa-STZ compared to Yaa-CB (P =0.092), without a significant difference. The mRNA expression of nephrosis 2 (*Neph2*) and synaptopodin (*Synpo)*, crucial podocyte functional molecules for the maintenance of the glomerular filtration barrier, Yaa-STZ showed a higher tendency for the mean mRNA expression of both genes compared with Yaa-CB, but the difference was not significant (P = 0.0728 for *Neph2, P* = 0.4175 for *Synpo*) (Fig. 5d).

Tubulointerstitial histopathology

Fig. 6a shows the histological features of the renal cortex in both STZ-and CB-injected (BXSB and Yaa) mice groups at 24 weeks of age. In PAS-H-stained kidney sections, the nuclei of proximal and distal tubular epithelial cells in the STZ-administered groups of both strains showed remarkable nuclear abnormalities, including anisokaryosis and intranuclear vacuolization. Furthermore, in Yaa, urinary casts were frequently observed in the renal tubular lumens, and prominent dilation of renal tubules was observed compared to BXSB, especially in Yaa-STZ. Intranuclear acclimatization was also remarkable in Yaa-STZ compared to Yaa-CB. The MT-stained kidney sections showed an increase in the aniline blue^{+} area in the ~~glomerulus and~~ tubulointerstitium of Yaa, indicating sclerotic lesions and tubulointerstitial fibrosis in the renal cortex, respectively (Fig. 6b).

The results of the quantitative analysis of nuclear size variation in renal tubular epithelial cells are shown in Fig. 6c. BXSB-STZ showed anisokaryosis, and the cell appearance % of cells with a nuclear size over 21 μm^2 or less than 10 μm^2 increased in both proximal and distal tubules compared with BXSB-CB. However, the cell appearance % of cells with a nuclear size over 21 μm^2 was higher in Yaa than in BXSB, and this value significantly increased in the distal tubules of Yaa-STZ when compared with that in Yaa-CB. Additionally, a significant increase in the area of the renal tubular lumen was observed in Yaa compared to that in BXSB in both the CB- and STZ-treated groups, and the Yaa-STZ group showed a significantly higher value than the Yaa-CB group (Fig. 6d). Thus, these data indicate that STZ treatment exacerbates tubulointerstitial lesions, especially in autoimmune disease

models.

Next, we examined the inflammatory features in the tubulointerstitium at 24 weeks of age. Immunohistochemical staining of IL-36α, a marker of damaged renal tubules, was performed accordingly (Fig. 7a). IL-36α$^+$ tubules were abundantly observed in Yaa. Morphometrical measurements revealed a significant increase in the number of IL-36α$^+$ tubules in BXSB following STZ injection compared to that in the CB group. Moreover, both treatment groups in Yaa showed significantly more positive tubules than BXSB, but there was no significant difference among the Yaa-treated groups (Fig. 7b).

Next, the mRNA expression of inflammatory cytokines in the kidney was evaluated by qPCR (Fig. 7c). No significant differences were observed in the mRNA expression levels of interleukin 1 alpha (*Il1a*), interleukin 1 beta (*Il1b*), interleukin 6 (*Il6*), tumor necrosis factor (*Tnf*), and transforming growth factor beta 1 (*Tgfb1*), an important regulator of fibrosis in the kidney.

Immunohistochemical staining revealed immune cell infiltration (B220$^+$ B-cells and CD3$^+$ T-cells) into the tubule interstitial tissue in Yaa (Fig. 7d). Interestingly, STZ treatment seemed to be decreased in Yaa-STZ compared with that in Yaa-CB. As shown in Fig. 7e, both Yaa treated groups showed significantly higher interstitial immune cell infiltration than BXSB. Moreover, the Yaa-STZ group showed significantly decreased interstitial immune cell infiltration compared to that in the Yaa-CB group.

Correlation between DM and indicators of autoimmunity or renal pathology

Since the dynamics of BGL differed among individuals in Yaa-STZ, we calculated the average of BGL from 12 to 24 weeks of age for each individual, and then Spearman's rank correlation test was performed to show the association between DM and each pathological parameter in all treated Yaa (Table 3). For indices of autoimmune disease development, only the serum levels of anti-dsDNA antibody showed a significant and positive correlation with the area ratio of islets and pancreas among the examined DM parameters ($P < 0.05$). Among the examined renal pathological parameters, the urinary ACR ($P <0.05$), mesangial area, its ratio in the glomerulus ($P < 0.05$, $P < 0.01$, respectively), and the numbers of B220$^+$ B cells and CD3$^+$ T-cells in the tubulointerstitium ($P < 0.05$) were significantly and negatively correlated with BGL. The number of B220$^+$ B cells in the tubulointerstitium ($P < 0.01$)

was significantly correlated with urinary glucose levels. The mesangial area ratio ($P < 0.05$) and the number of $B220^+$ B cells and $CD3^+$ T-cells in the tubulointerstitium ($P < 0.01$, $P < 0.05$, respectively) showed a significant positive correlation with the area ratio of islets to pancreas, whereas the glomerular mRNA expression of *Neph2* ($P < 0.05$) showed a significant negative correlation. The glomerular mRNA expression of *Neph2* and the ratio of the tubular lumen area to the cortex area were significantly and negatively correlated with the area ratio of $insulin^+$ cells to $glucagon^+$ cells ($P < 0.05$). The number of $B220^+$ B cells and $CD3^+$ T-cells in the tubulointerstitium was significantly and positively correlated with the ratio of the latter ($P < 0.01$, $P < 0.05$, respectively).

As for the correlation between indices of autoimmunity and renal pathology in Yaa (Table 4), the size and nuclear number of glomeruli were significantly and positively correlated with the serum levels of anti-dsDNA antibody ($P < 0.05$).

Discussion

In the current study, to evaluate the immunological alterations as a candidate modifier of DM and/or DKD, we pathologically examined autoimmune disease-prone Yaa after induction of DM by STZ injection and compared its phenotype with that of wild-type BXSB.

At 12 weeks after injection of STZ in both studied strains, there was no dramatic change in the indices of autoimmunity, SPW/BW, and serum levels of autoantibody. Furthermore, there was no correlation between these indices and the BGL in Yaa, although previous reports revealed that DM could have some impact on the immune system, such as dysfunction of the innate immune system cells (Berbudi, et al., 2020; Jafar, et al., 2016) and suppression of humoral and cellular immunity (Muller, et al., 2011; Gaulton, et al., 1985). In addition, STZ-injected mice show lymphopenia in the early stage of diabetes due to hyperglycemia through changes in the regulatory T (Treg) population or generation of advanced glycation end products (AGEs) (Rubinstein, et al., 2008; Zhen, et al., 2012). The positive correlations between serum levels of autoantibodies and pancreatic islet damage in Yaa might partially reflect the relationship between autoimmunity and DM. Furthermore, for BGL, Yaa-STZ showed a relatively lower BGL than BXSB-STZ during the observation period. STZ injection induced pancreatic islet shrinkage by oxidative damage-mediated β-cell loss in both the strains (Bolzán & Bianchi, 2002), suggesting that this lower BGL in Yaa-STZ might be related to causes other than β-cell damage by STZ. BGL was also affected by the general condition of the patients, such as absorption defects of glucose or metabolic abnormalities due to poor appetite or chronic inflammation in the intestine. Indeed, Yaa-STZ significantly reduced BW compared with BXSB-STZ during the age of 12–14 weeks after STZ injection, indicating the deterioration of the general condition. Direct toxicity of STZ to immune cells had also been suggested in the past literature (Muller, et al., 2011). In this study, we concluded that DM or STZ had a weak effect on the progression of systemic autoimmune disease in the Yaa strain, despite the relatively high BGL (262 ± 46 mg/dL) compared with that in normal conditions.

Our examination of glomerular lesion progression following STZ injection in both mouse strains revealed that it did not show representative glomerular lesions similar to those reported in human DN, especially nodular glomerular sclerosis (Tervaert, et al., 2010). This result emphasizes the species-specific histopathological differences related to the susceptibility to glomerular damage in DN, and the resistant loci for glomerular sclerosis

identified in several mouse strains, including C57BL/6, which is one of the prototype strains in BXSB and Yaa (Sasaki, et al., 2016; Tamura, et al., 2005). Therefore, hyperglycemia for more than 12 weeks might be needed to induce representative glomerular features, as found in human DN just as in BXSB and Yaa. Furthermore, Yaa developed MPGN characterized by glomerular hypertrophy, increased glomerular cell number, mesangial expansion, and glomerular sclerosis, as previously reported (Masum, et al., 2018). Characteristically, Yaa-STZ tended to show milder glomerular lesions than Yaa-CB, as indicated by decreased mesangial area and ameliorated tendency in ACR and podocyte marker expression. In MPGN, glomerular lesions, especially mesangial lesions, are formed through immunological stimulations, such as *in situ* deposition of immune globulin, immune-complex, or complement (Sethi & Fervenza, 2012). In contrast, the increased proportion of peripheral Tregs by STZ and hyperglycemia might alter local immunity in the kidneys (Muller, et al., 2011; Zhen, et al., 2012). In general, ECM protein production by mesangial cells was increased under high-glucose conditions *in vitro* by various mechanisms that may involve the polyol pathway, activated protein kinase C, and increased TGF-β (Ayo, et al., 1991; Ayo, et al., 1990; Derylo, et al., 1998; Wolf, et al., 1992). However, *in vivo*, the activity of mesangial matrix production would be changed by the complex pathological crosstalk between local autoimmunity and hyperglycemia, as found in the glomerulus of Yaa-STZ.

In this study, the BXSB-STZ group showed an increase in the number of IL-36α^+ damaged renal tubules, and this number of both treatment groups in Yaa was higher than that of both treatment groups of the BXSB strain. In addition, STZ treatment induced anisonucleosis and tubular dilation in the proximal and distal tubules of BXSB, and its tendency was more severe in Yaa. Renal tubular injury is caused not only by glomerular injury, which results in albuminuria and impaired microvascular perfusion, as found in Yaa, but also by direct and excessive exposure to glucose. Briefly, increasing filtered glucose from the glomerulus leads to osmotic diuresis, that produces mechanical pressure and increases its tubular transport load because almost all substances in primitive urine are reabsorbed by the renal tubules (Nespoux & Vallon, 2018). Cultured tubular epithelial cells respond to high glucose levels with cell hypertrophy, altered collagen synthesis, and cytokine secretion, such as TGF-β (Phillips, et al., 1997; Ziyadeh, et al., 1990). Importantly, osmotic nephrosis, characterized by morphological changes in the proximal tubules, is reported to be exacerbated in human patients and in experimental models of kidney injury (Jensen et al., 2013;

Matsushita et al., 2018; Dickenmann et al., 2018). Our results revealed that Yaa-CB showed dilated lumen of renal tubules and Yaa-STZ progressed its pathology with the increase of abnormal nuclei in tubular epithelium, although no markedly altered serological and urinary parameters were observed, such as BUN, Cre, urinary pH, specific gravity, and ACR in the STZ-injected groups of both studied strains as compared to those receiving CB. BXSB-STZ also showed increases in abnormal nuclei of tubular cells and IL-36α^{+} tubules, and a tendency of dilated tubular lumen compared to BXSX-CB, which suggested that hyperglycemia could directly affect tubular morphology. Thus, we suggest that the significant increase in tubular dilation in Yaa-STZ was the result of exacerbated tubular injury observed in Yaa-CB due to hyperglycemia.

As for inflammatory pathology, an increase in infiltrated CD3^{+} T cells but not CD20^{+} B-cells into the tubulointerstitium was reported in STZ-induced diabetic C57BL/6 mice (Moon, et al., 2012), which meant that T-cells might play a predominant role in the pathogenesis of inflammation in the tubulointerstitium in STZ-induced diabetic mice. In contrast, our results showed no increase in CD3^{+} T cells in the tubulointerstitium of BXSB-STZ, and the number of these cells in Yaa-STZ decreased compared to that in the CB-treated group. Furthermore, similar immunological changes have been observed in B220^{+} B-cells. Yaa has been reported to accumulate autoreactive B-cells due to gene mutations (Pistkun, et al., 2006), which leads to tubulointerstitial inflammation. In addition, Yaa-STZ showed negative correlations between the numbers of T- and B-cells in the tubulointerstitium with blood glucose levels. This suppression of local cell infiltration due to hyperglycemia has also been observed in autoimmune pancreatitis model mice (Muller-Graff, et al., 2018). Thus, although the inhibitory mechanism of inflammation in Yaa-STZ remained unclear in this study, we concluded that systemic and/or local immune suppression by hyperglycemia modifies tubulointerstitial cell infiltration in Yaa.

Thus, our study emphasized that the increased susceptibility of the tubules to injuries, such as drugs, endotoxins, and osmotic nephrosis, should be noted in the clinical treatment of patients with kidney diseases complicated with DM, especially those related to autoimmune abnormalities, but not via cellular inflammation (Harding, et al., 2019; Perazella, 2019; Zhang, et al., 2019). In this study, however, there was a limitation - we could not entirely exclude the possibility that STZ directly affects the kidney pathology in examined mice because the STZ molecule is structurally similar to glucose, which could also accumulate in the tubular cells of the kidney via

GLUT2 glucose transporter (Lenzen, 2008). Future studies are hence, needed to consider the direct effect of STZ on the pathogenesis of autoimmune kidney disease with the induction of DM independent of STZ. Furthermore, it should be clarified whether the decreased mesangial area in Yaa-STZ reflects the alteration due to pathological exacerbation (active phase of proliferating mesangial cells) or amelioration (suppression of mesangial-matrix expansion) by long-term observation for more than 12 weeks.

Conclusions

In conclusion, in DKD related to autoimmune nephritis, hyperglycemia modifies its pathology by decreasing the mesangial area and aggravating tubulointerstitial lesions, which were mainly observed as injury in renal tubular epithelial cells. This study provides novel insights into the pathogenesis of autoimmune-related DKD, which contributes to the appropriate handling to prevent exacerbations of this disease condition in zoobiquity.

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Figure legends

Fig 1. Indices of diabetes mellitus and autoimmunity. (a) Blood glucose levels. **(b)** Body weight (BW). These parameters were measured at the end of each week when the mice were aged 11-24 weeks. **(c)** Ratio of spleen weight to BW. **(d)** Spleen weight. **(e)** Serum levels of anti-double-stranded DNA (dsDNA) antibody. The values in the graphs (**c- e)** were measured at the age of 24 weeks. Citrate buffer (CB) or streptozotocin (STZ) was injected as a single dose at the age of 12 weeks. BXSB-CB: CB-injected BXSB. BXSB-STZ: STZ-injected BXSB. Yaa-CB: Yaa-injected BXSB. Yaa-STZ: Yaa-injected STZ. Values are presented as mean ± SE. {n = 5 (BXSB-CB), 5 (BXSB-STZ), 8 (Yaa-CB), 8 (Yaa-STZ)}, analyzed using the Mann–Whitney *U*-test. Statistically significant differences between BXSB-CB vs. Yaa-CB (**P* <0.05, ***P* <0.01); BXSB-STZ vs. Yaa-STZ ($^{\dagger}P$ <0.05); BXSB-CB vs. BXSB-STZ ($^{\ddagger}P$ <0.05); Yaa-CB vs. Yaa-STZ ($^{\S}P$ <0.05, $^{\S\S}P$ <0.01).

Fig 2. Histopathological features of pancreas in the streptozotocin (STZ)- and citrate buffer (CB)-treated (BXSB and Yaa) mice groups at 24 weeks of age. (a) Hematoxylin and eosin-stained pancreatic tissue sections. **(b)** Ratio of islet area to pancreatic area. **(c)** Double immunofluorescence staining of glucagon^{+} α-cells (green) or insulin^{+} β-cells (red), and Hoechst nuclear staining (Blue). **(d)** Ratio of the insulin-positive area to the glucagon-positive area. Values are presented as mean ± SE. {n = 5 (BXSB-CB), 5 (BXSB-STZ), 8 (Yaa-CB), 8 (Yaa-STZ)}, analyzed using the Mann–Whitney *U*-test. Statistically significant differences between treatments in the same strain ($^{\dagger\dagger}P$ <0.01). Bars = 50 μm.

Fig 3. Renal function (serological analysis and urinalysis) in the streptozotocin (STZ)- and citrate buffer (CB)-treated (BXSB and Yaa) mice groups at 24 weeks of age. (a) Serum blood urea nitrogen (BUN). **(b)** Serum creatinine (Cre). **(c)** Urine pH. **(d)** Specific gravity of urine. **(e)** Urine glucose. Values are presented as mean ± SE. {n = 5 (BXSB-CB), 5 (BXSB-STZ), 8 (Yaa-CB), ≥7 (Yaa-STZ)}, analyzed using the Mann–Whitney *U*-test. Significant differences in the strain after the same treatment (**P* <0.05). Significant differences between treatments in the same strain ($^{\dagger}P$ <0.05, $^{\dagger\dagger}P$ <0.01).

Fig 4. Histopathological features of glomerular lesions in the streptozotocin (STZ)- and citrate buffer (CB)-treated (BXSB and Yaa) mice groups at 24 weeks of age. (a) Glomerular features in Periodic Acid-Schiff hematoxylin (PAS-H), Masson's trichrome (MT), and periodic acid methenamine silver (PAM)-stained kidney sections. Yaa shows an increase in glomerular size, and the nuclear number, with expansion of the mesangial area as shown in PAS-H-stained section (left column), and severe glomerular sclerosis as shown in MT-stained section (middle column) when compared with BXSB. In PAM-stained kidney sections (right column), spike-like structures or double-contoured features in the glomerular basement membrane are observed in Yaa (inset). **(b)** Graphs showing histoplanimetry for glomerular size, nuclear number, mesangial area, and its ratio in the glomerulus among different groups. Values are presented as mean ± SE. {n = 5 (BXSB-CB), 5 (BXSB-STZ), 8 (Yaa-CB), 8 (Yaa-STZ)}, analyzed using the Mann–Whitney *U*-test. Significant differences in the strain in the same treatment, $*P < 0.05$. Significant differences between treatments in the same strain, $^{\dagger}P < 0.05$. Bars = 25 μm.

Fig 5. Infiltration of glomerular immune cells, glomerular function, and podocyte function molecules as markers in the kidney of streptozotocin (STZ)- and citrate buffer (CB)-treated (BXSB and **Yaa) mice at 24 weeks of age. (a)** Immunohistochemical staining for $B220^{+}$ B- and $CD3^{+}$ T-cells in the kidney. The glomerulus of Yaa shows increased cell infiltration in both treatment groups. Arrowheads indicate positive cells. **(b)** Graph showing the number of $B220^{+}$ or $CD3^{+}$ cells in the glomerulus. **(c)** Graph showing the ratio of urine albumin to creatinine (ACR). **(d)** Graph showing the relative mRNA expression level of *Neph2* and *Synpo* in the kidney. BXSB-CB: CB-injected BXSB. BXSB-STZ: STZ-injected BXSB. Yaa-CB: Yaa-injected BXSB. Yaa-STZ: Yaa-injected STZ. Values are presented as mean ± SE. {n = 5 (BXSB-CB), 5 (BXSB-STZ), 8 (Yaa-CB), ≥7 (Yaa-STZ)}, analyzed using the Mann–Whitney *U*-test. Significant differences in the strain in the same treatment ($**P < 0.01$). Bars = 50 μm.

Fig 6. Histopathological features of tubulointerstitial lesion in the streptozotocin (STZ)- and citrate buffer (CB)-injected (BXSB and Yaa) mice at 24 weeks of age. (a) Tubulointerstitial features in Periodic Acid-Schiff hematoxylin (PAS-H)-stained kidney sections in different groups. The nuclei of tubular epithelial cells in STZ-

injected mice show anisokaryosis (black arrowheads), and intranuclear vacuolization (red arrowheads), which is remarkably increased in STZ-injected mice compared with that in CB-injected mice. Urinary casts (arrow) are frequently observed in the renal tubular lumens, and the dilations of renal tubules are prominent in Yaa compared with those in BXSB. **(b)** Fibrotic features observed on Masson's trichrome (MT)-stained kidney sections in different groups. An increase in the aniline blue^{+} area in the glomerulus and tubulointerstitium is observed in Yaa compared with that in BXSB. **(c)** Graph showing the ratio of nuclear size of epithelial cells in proximal and distal tubules. **(d)** Graph showing the ratio of the luminal area to the renal cortex area. Values are presented as mean ± SE. {n = 5 (BXSB-CB), 5 (BXSB-STZ), 8 (Yaa-CB), 8 (Yaa-STZ)}, analyzed using the Mann–Whitney *U*-test. Significant differences in the strain in the same treatment (*P<0.05). Significant differences between treatments in the same strain ($^{\dagger}P$ <0.05). Bars = 50 μm.

Fig 7. Analysis of the degree of inflammation in the tubulointerstitial tissue of streptozotocin (STZ)- and citrate buffer (CB)-injected (BXSB and Yaa) mice groups at 24 weeks of age. (a) Immunohistochemical staining for IL-36α in the kidney sections. IL-36α^{+} tubules are more in Yaa than in BXSB in both the STZ- and CB-injected groups. **(b)** Graph showing the number of IL-36α^{+} tubules in the kidney. **(c)** Graph showing the relative mRNA expression level of inflammatory cytokines in the kidney of both Yaa-treated groups. **(d)** Immunohistochemical staining for B220^{+} B- and CD3^{+} T-cells in the tubulointerstitium. Yaa shows increased cell infiltration in both the CB- and STZ-injected groups. Arrowheads indicate positive cells. **(e)** Graph showing the number of B220^{+} or CD3^{+} cells in the tubulointerstitium. BXSB-CB: CB-injected BXSB. BXSB-STZ: STZ-injected BXSB. Yaa-CB: Yaa-injected BXSB. Yaa-STZ: Yaa-injected STZ. Values are presented as mean ± SE. {n = 5 (BXSB-CB), 5 (BXSB-STZ), 8 (Yaa-CB), ≥7 (Yaa-STZ)}, analyzed using the Mann–Whitney *U*-test. Significant differences in the strain in the same treatment (**P <0.01). Significant differences between treatments in the same strain ($^{\dagger}P$ <0.05). Bars = 50 μm.