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1 **Original research**

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

3

4 **Running title:** Diabetic kidney disease in autoimmune disease model mice

5

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22

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28

29 **Abstract**

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

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

44

45

46 **Introduction**

47 Diabetes mellitus (DM) is characterized by increased blood glucose levels (BGL) due to the hyposecretion  
48 of insulin by  $\beta$ -cell loss or dysfunction and insulin resistance (Chatterjee, et al., 2017; DiMeglio, et al., 2018). The  
49 prevalence of DM in adults aged 20–79 years is predicted to increase to 10.4% by 2040 worldwide (Ogurtsova,  
50 et al., 2017). DM often induces diabetic nephropathy (DN) This is the primary cause of chronic kidney disease  
51 (CKD) and eventually results in end-stage renal disease (Harding, et al., 2019; Olivares, et al., 2017; Qi et al.,  
52 2017). Recently, it was recognized that many patients with DM do not follow the classic DN phenotypes, such as  
53 glomerular hyperfiltration progressing to persistent albuminuria associated with hypertension and declining  
54 glomerular filtration rate (Alicic, et al., 2017), and other systemic symptoms such as autoimmune abnormalities

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

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

69 To elucidate the pathophysiology of DN, streptozotocin (STZ), which leads to oxidative stress-mediated  $\beta$ -  
70 cell death, is generally used for DM induction in healthy rodents, and previous studies have reported that STZ-  
71 injected mice show severe tubulointerstitial lesions and moderate glomerular lesions respectively (Bolzán & Bianchi,  
72 2002; Tamura, et al., 2005). Other studies have shown that spontaneous Akita or db/db mice develop glomerular  
73 lesions, which are representative of the early stages of human DN (Kitada, et al., 2016). However, they do not clearly  
74 exhibit all of the key histological features of human DN, such as severe glomerular nodular lesions and  
75 tubulointerstitial fibrosis. Importantly, altered immune conditions modify the renal phenotypes of DM animals, and  
76 glomerulosclerotic features are accelerated by immune complex-mediated glomerulopathy in STZ-injected rats  
77 (Abrass & Cohen, 1987), however, the pathological characteristics of autoimmune disease-associated DKD remain  
78 unclear.

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

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

88

89 **Materials and Methods**

90 *Experimental animals and ethics statement*

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

99

100 *DM induction and sample collection*

101 Male BXSJ and Yaa mice (12 weeks of age) received a single intraperitoneal (i.p.) injection of either STZ  
102 (300 mg/kg; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) dissolved in citrate buffer (CB; pH 4.7) or  
103 only CB. The mice were divided into four groups: 1) BXSJ injected with CB (BXSJ-CB) (n=5), and used as control  
104 mice; 2) BXSJ injected with STZ (BXSJ-STZ) (n=5), and considered as diabetic mice; 3) Yaa injected with CB  
105 (Yaa-CB) (n=8), as autoimmune disease mice; and 4) Yaa injected with STZ (Yaa-STZ) (n=8), as autoimmune  
106 disease mice accompanied with DM.

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

114

115 *Serological analysis and urinalysis*

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

125

126 *Histopathological analysis*

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

132

133 *Immunostaining*

134 Paraffin sections were deparaffinized, hydrated, and subjected to antigen retrieval (Table 1). The sections  
135 were then soaked in methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature to block internal peroxidase  
136 activity. After washing thrice in phosphate-buffered saline (PBS), the sections were incubated with blocking serum  
137 for 1 h at room temperature, followed by overnight incubation with primary antibodies at 4 °C. After washing three  
138 times with PBS, the sections were incubated with secondary antibodies for 30 min at room temperature and washed  
139 three times with PBS. For immunohistochemistry, the sections were incubated with streptavidin-conjugated  
140 horseradish peroxidase (SABPO (R) kit; Nichirei, Tokyo, Japan) for 30 min and washed three times with PBS. For

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

147

#### 148 *Histoplanimetry*

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

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

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

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

169

#### 170 *Quantitative polymerase chain reaction (qPCR)*

171 Total RNA from the kidney was purified using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA,  
172 USA) following the manufacturer's instructions. The purified total RNA (83.3 ng/μL) was treated as a template to  
173 synthesize complementary DNA (cDNA) using ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd.; Osaka,  
174 Japan). qPCR analysis was performed on the cDNA (20 ng/μL) using THUNDERBIRD<sup>®</sup> SYBR<sup>®</sup> qPCR Mix  
175 (Toyobo Co., Ltd.) and the following gene-specific primers (Table 2). The qPCR cycling conditions were as follows:  
176 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  
177 actin, beta (*Actb*) (Ichii, et al., 2010), and those of Yaa-CB using the delta-delta Ct method.

178

#### 179 *Statistical analysis*

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

183

184 **Results**

185 *Indices of DM and autoimmune disease*

186 For indices of DM, both BGL and BW were measured weekly following injection of either STZ or CB to  
187 BXSB and Yaa at 12 weeks of age until sampling at 24 weeks of age. As shown in Fig. 1a, all examined STZ-treated  
188 mice developed hyperglycemia (>250 mg/dL). In the BXSB-STZ group, BGL was maintained at high values (392  
189  $\pm$  48 mg/dL) throughout the observation period. The Yaa-STZ group had a relatively lower BGL (262  $\pm$  46.76  
190 mg/dL) than the BXSB-STZ group. Significant differences between BXSB-STZ and Yaa-STZ were observed at 14,  
191 16, 19, and 20 weeks of age. There was a similarity in the BGL of CB-injected mice in both the strains. Fig. 1b  
192 shows the changes in the BW. A reduction in BW was observed in the STZ-injected mice of both strains due to DM.  
193 Notably, the reduction in BW was significantly earlier in the Yaa-STZ group than in the BXSB-STZ group, and their  
194 BW became comparable at 15 weeks of age.

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

199

200 *Histopathology of the pancreas*

201 As shown in the histological examination of the H&E-stained pancreatic tissue sections at 24 weeks of age  
202 (Fig. 2a), STZ-injected mice showed a remarkable decrease in the size of pancreatic islets in both strains due to its  
203 toxicity to  $\beta$ -cells without any immune cell infiltration in or around the islets. Morphometrically, a significant  
204 reduction in the ratio of islet area to pancreatic area was observed in STZ-injected mice compared to that in the CB  
205 group without any strain-related differences (Fig. 2b). To estimate  $\beta$ -cell toxicity, immunofluorescence for  
206 glucagon<sup>+</sup>  $\alpha$ -cells and insulin<sup>+</sup>  $\beta$ -cells was performed in the pancreas (Fig. 2c). In the STZ-treated groups, insulin<sup>+</sup>  
207  $\beta$ -cells decreased, indicating sustained  $\beta$ -cell loss until 24 weeks of age after STZ injection. The ratio of the insulin<sup>+</sup>  
208 area to the glucagon<sup>+</sup> area was significantly lower in the STZ-injected mice than in the CB-injected mice without  
209 strain-related differences (Fig. 2d). Thus, our results confirmed the comparable  $\beta$ -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<sup>+</sup> 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<sup>+</sup> B-cells and  
233 CD3<sup>+</sup> 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-

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

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

244

#### 245 *Tubulointerstitial histopathology*

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

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

262 models.

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

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

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

278

#### 279 *Correlation between DM and indicators of autoimmunity or renal pathology*

280 Since the dynamics of BGL differed among individuals in Yaa-STZ, we calculated the average of BGL from  
281 12 to 24 weeks of age for each individual, and then Spearman's rank correlation test was performed to show the  
282 association between DM and each pathological parameter in all treated Yaa (Table 3). For indices of autoimmune  
283 disease development, only the serum levels of anti-dsDNA antibody showed a significant and positive correlation  
284 with the area ratio of islets and pancreas among the examined DM parameters ( $P < 0.05$ ). Among the examined  
285 renal pathological parameters, the urinary ACR ( $P < 0.05$ ), mesangial area, its ratio in the glomerulus ( $P < 0.05$ ,  $P$   
286  $< 0.01$ , respectively), and the numbers of B220<sup>+</sup> B cells and CD3<sup>+</sup> T-cells in the tubulointerstitium ( $P < 0.05$ ) were  
287 significantly and negatively correlated with BGL. The number of B220<sup>+</sup> B cells in the tubulointerstitium ( $P < 0.01$ )

288 was significantly correlated with urinary glucose levels. The mesangial area ratio ( $P < 0.05$ ) and the number of  
289 B220<sup>+</sup> B cells and CD3<sup>+</sup> T-cells in the tubulointerstitium ( $P < 0.01$ ,  $P < 0.05$ , respectively) showed a significant  
290 positive correlation with the area ratio of islets to pancreas, whereas the glomerular mRNA expression of *Neph2* ( $P$   
291  $< 0.05$ ) showed a significant negative correlation. The glomerular mRNA expression of *Neph2* and the ratio of the  
292 tubular lumen area to the cortex area were significantly and negatively correlated with the area ratio of insulin<sup>+</sup> cells  
293 to glucagon<sup>+</sup> cells ( $P < 0.05$ ). The number of B220<sup>+</sup> B cells and CD3<sup>+</sup> T-cells in the tubulointerstitium was  
294 significantly and positively correlated with the ratio of the latter ( $P < 0.01$ ,  $P < 0.05$ , respectively).

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

298

299 **Discussion**

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

303 At 12 weeks after injection of STZ in both studied strains, there was no dramatic change in the indices of  
304 autoimmunity, SPW/BW, and serum levels of autoantibody. Furthermore, there was no correlation between these  
305 indices and the BGL in Yaa, although previous reports revealed that DM could have some impact on the immune  
306 system, such as dysfunction of the innate immune system cells (Berbudi, et al., 2020; Jafar, et al., 2016) and  
307 suppression of humoral and cellular immunity (Muller, et al., 2011; Gaulton, et al., 1985). In addition, STZ-injected  
308 mice show lymphopenia in the early stage of diabetes due to hyperglycemia through changes in the regulatory T  
309 (Treg) population or generation of advanced glycation end products (AGEs) (Rubinstein, et al., 2008; Zhen, et al.,  
310 2012). The positive correlations between serum levels of autoantibodies and pancreatic islet damage in Yaa might  
311 partially reflect the relationship between autoimmunity and DM. Furthermore, for BGL, Yaa-STZ showed a  
312 relatively lower BGL than BXSB-STZ during the observation period. STZ injection induced pancreatic islet  
313 shrinkage by oxidative damage-mediated  $\beta$ -cell loss in both the strains (Bolzán & Bianchi, 2002), suggesting that  
314 this lower BGL in Yaa-STZ might be related to causes other than  $\beta$ -cell damage by STZ. BGL was also affected by  
315 the general condition of the patients, such as absorption defects of glucose or metabolic abnormalities due to poor  
316 appetite or chronic inflammation in the intestine. Indeed, Yaa-STZ significantly reduced BW compared with BXSB-  
317 STZ during the age of 12–14 weeks after STZ injection, indicating the deterioration of the general condition. Direct  
318 toxicity of STZ to immune cells had also been suggested in the past literature (Muller, et al., 2011). In this study,  
319 we concluded that DM or STZ had a weak effect on the progression of systemic autoimmune disease in the Yaa  
320 strain, despite the relatively high BGL ( $262 \pm 46$  mg/dL) compared with that in normal conditions.

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

325 identified in several mouse strains, including C57BL/6, which is one of the prototype strains in BXSB and Yaa  
326 (Sasaki, et al., 2016; Tamura, et al., 2005). Therefore, hyperglycemia for more than 12 weeks might be needed to  
327 induce representative glomerular features, as found in human DN just as in BXSB and Yaa. Furthermore, Yaa  
328 developed MPGN characterized by glomerular hypertrophy, increased glomerular cell number, mesangial expansion,  
329 and glomerular sclerosis, as previously reported (Masum, et al., 2018). Characteristically, Yaa-STZ tended to show  
330 milder glomerular lesions than Yaa-CB, as indicated by decreased mesangial area and ameliorated tendency in ACR  
331 and podocyte marker expression. In MPGN, glomerular lesions, especially mesangial lesions, are formed through  
332 immunological stimulations, such as *in situ* deposition of immune globulin, immune-complex, or complement (Sethi  
333 & Fervenza, 2012). In contrast, the increased proportion of peripheral Tregs by STZ and hyperglycemia might alter  
334 local immunity in the kidneys (Muller, et al., 2011; Zhen, et al., 2012). In general, ECM protein production by  
335 mesangial cells was increased under high-glucose conditions *in vitro* by various mechanisms that may involve the  
336 polyol pathway, activated protein kinase C, and increased TGF- $\beta$  (Ayo, et al., 1991; Ayo, et al., 1990; Derylo, et al.,  
337 1998; Wolf, et al., 1992). However, *in vivo*, the activity of mesangial matrix production would be changed by the  
338 complex pathological crosstalk between local autoimmunity and hyperglycemia, as found in the glomerulus of Yaa-  
339 STZ.

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

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

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

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

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

382

### 383 **Conclusions**

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

388

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391

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485

486 **Figure legends**

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

496

497 **Fig 2. Histopathological features of pancreas in the streptozotocin (STZ)- and citrate buffer (CB)-treated**  
498 **(BXSb and Yaa) mice groups at 24 weeks of age. (a)** Hematoxylin and eosin-stained pancreatic tissue sections.  
499 **(b)** Ratio of islet area to pancreatic area. **(c)** Double immunofluorescence staining of glucagon<sup>+</sup>  $\alpha$ -cells (green) or  
500 insulin<sup>+</sup>  $\beta$ -cells (red), and Hoechst nuclear staining (Blue). **(d)** Ratio of the insulin-positive area to the glucagon-  
501 positive area. Values are presented as mean  $\pm$  SE. {n = 5 (BXSb-CB), 5 (BXSb-STZ), 8 (Yaa-CB), 8 (Yaa-STZ)},  
502 analyzed using the Mann–Whitney *U*-test. Statistically significant differences between treatments in the same strain  
503 (<sup>††</sup>*P* < 0.01). Bars = 50  $\mu$ m.

504

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

511

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

523

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

534

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

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

548

549 **Fig 7. Analysis of the degree of inflammation in the tubulointerstitial tissue of streptozotocin (STZ)- and**  
550 **citrate buffer (CB)-injected (BXSB and Yaa) mice groups at 24 weeks of age. (a)** Immunohistochemical  
551 staining for IL-36α in the kidney sections. IL-36α<sup>+</sup> tubules are more in Yaa than in BXSB in both the STZ- and  
552 CB-injected groups. **(b)** Graph showing the number of IL-36α<sup>+</sup> tubules in the kidney. **(c)** Graph showing the  
553 relative mRNA expression level of inflammatory cytokines in the kidney of both Yaa-treated groups. **(d)**  
554 Immunohistochemical staining for B220<sup>+</sup> B- and CD3<sup>+</sup> T-cells in the tubulointerstitium. Yaa shows increased cell  
555 infiltration in both the CB- and STZ-injected groups. Arrowheads indicate positive cells. **(e)** Graph showing the  
556 number of B220<sup>+</sup> or CD3<sup>+</sup> cells in the tubulointerstitium. BXSB-CB: CB-injected BXSB. BXSB-STZ: STZ-  
557 injected BXSB. Yaa-CB: Yaa-injected BXSB. Yaa-STZ: Yaa-injected STZ. Values are presented as mean ± SE. {n  
558 = 5 (BXSB-CB), 5 (BXSB-STZ), 8 (Yaa-CB), ≥7 (Yaa-STZ)}, analyzed using the Mann–Whitney *U*-test.  
559 Significant differences in the strain in the same treatment (\*\**P* < 0.01). Significant differences between treatments  
560 in the same strain (†*P* < 0.05). Bars = 50 μm.