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1 **Altered morpho-functional features of bones in autoimmune disease-prone**
2 **BXSB/MpJ- *Yaa* mice**

3

4 **Short title:** Bone pathology in autoimmune disease-prone mice

5

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18

19 **Abstract**

20 Bones play crucial roles in motility, electrolyte metabolism, and immunity. Clinical cases
21 have suggested bone dysfunction in several systemic autoimmune diseases. This study
22 exhibited altered bone morpho-functions in BXSB/MpJ-*Yaa* as a murine autoimmune disease
23 model. During clinical examinations, the serum Ca level was significantly higher in
24 BXSB/MpJ-*Yaa* than the healthy control BXSB/MpJ at the early stage (2-4 months), but that
25 in BXSB/MpJ-*Yaa* decreased with advancing age. Further, the increase of urinary Ca with
26 nephritis and white blood cells with mild anemia proceeded in BXSB/MpJ-*Yaa* with
27 advancing age. The thyroid and parathyroid gland morphologies and serum parathormone
28 level did not differ among strains, but the tibia was smaller in BXSB/MpJ-*Yaa* than in
29 BXSB/MpJ especially during the late stage (6 months). Histologically, osteoclasts and
30 osteoblasts showed increased and decreased tendencies, respectively, in BXSB/MpJ-*Yaa*
31 during the early stage, and osteoclasts and bone area significantly increased and decreased,
32 respectively, compared with BXSB/MpJ at later stages. The bone morphological indices were
33 affected by the expression of BXSB/MpJ-*Yaa* mutation genes and inflammatory genes in
34 BXSB/MpJ-*Yaa*. In conclusion, systemic autoimmune diseases in BXSB/MpJ-*Yaa* are
35 associated with the morpho-functional abnormalities of bones, calcium dynamics, and
36 hematopoiesis, and each factor contributes to forming the phenotypes in this disease.

37 **Keywords:** autoimmune disease, BXSB/MpJ-*Yaa*, bone, calcium, hematology, histopathology

38 **Impact statement**

39 Bone disease, such as osteoporosis and rheumatoid arthritis, increases because of the
40 progression of an aging society. Autoimmune disease are important and predisposing
41 factors for the pathogenesis of the bone disease, however, the pathological mechanism
42 is unclear. We have demonstrated that systemic autoimmune disease in BXSB/MpJ-*Yaa* is
43 closely associated with the morpho-functional abnormalities of bones including bone marrow
44 and has complicated pathology. The abnormalities are characterized by altered regulations of
45 serum calcium, anemia tendency, and hematopoiesis with increased WBCs and decreased PLs,
46 short length and low mass of long bones, imbalance in the populations of osteoclasts and
47 osteoblasts, and increased expression of candidate genes for causing and/or exacerbating their
48 phenotypes. Therefore, BXSB/MpJ-*Yaa* serves as a model to elucidate bone phenotypes in
49 systemic autoimmune disease that would be affected by the factors in the bone as well as the
50 other immune and/or mineral metabolism organs both in human and experimental medicine.

51

52 **Introduction**

53 Bones play various roles, such as assisting motor function and electrolyte metabolism of
54 circulating calcium or phosphorus. Additionally, the bone marrow (BM), a primary lymphatic
55 organ located deep in the bone, plays a crucial role in immunity. To date, the bone and BM
56 have been considered different and unrelated organs owing to the differences in their
57 development and functions. However, recently, the osteoblasts responsible for bone formation
58 were shown to participate in the production of hematopoietic stem and progenitor cells
59 (HSPCs) in mice.¹ Moreover, osteoclast precursor cells and osteoblasts expressed tumor
60 necrosis factor receptor superfamily, member 11a, nuclear factor kappa B (NFκB) activator
61 (Tnfrsf11a/RANK), and tumor necrosis factor (ligand) superfamily, member 11
62 (Tnfsf11/RANKL), respectively.² This cell to cell contact via RANK/RANKL interaction was
63 important for the differentiation of mouse osteoclast precursor cells into mature osteoclasts.²
64 Alternatively, RANKL expression was identified in T-cells. It regulates interactions between
65 T-cells and dendritic cells, maturing dendritic cells, and lymph node organogenesis in
66 humans.^{3,4}

67 The functional imbalance of osteoblasts and osteoclasts causes bone-related diseases such as
68 osteoporosis in humans, and their incidents increase because of the progression of an aging
69 society.^{5,6} Osteoporosis and osteopetrosis are associated with the hyper-activation and
70 dysfunction of osteoclasts causing the decrease and increase in bone density, respectively.^{6,7}

71 Particularly, aging and altered immune conditions are important and predisposing factors for
72 the pathogenesis of osteoporosis. Rheumatoid arthritis (RA), a bone-related autoimmune
73 disease in humans, causes chronic inflammation characterized by secondary osteoporosis and
74 synovitis, joint swelling, and destruction of cartilage and bone.^{8,9} RA patients develop motor
75 impairment with arthralgia, and bone destruction that could be induced by the actions of T
76 helper 1 (Th1)-cells, Th17-cells, B-cells, and produced cytokines, such as tumor necrosis
77 factor α (TNF- α), interleukin 1 (IL-1), and IL-6.^{8,10-13} Thus, crucial correlations between the
78 bone and immune system based on osteoimmunology, and their alternations would contribute
79 to their bidirectional pathogenesis.

80 For the diagnosis and evaluation of bone-related diseases, blood electrolyte examination,
81 targeting Ca and phosphorus, is useful. However, their blood concentration does not
82 completely reflect bone conditions. Blood Ca concentration is regulated by various organs
83 such as bones, thyroid glands, parathyroid glands, kidneys, and intestines with regard to
84 absorption and excretion.^{14,15} During the dysfunction of Ca-regulating organs, its blood
85 concentration would be changed. Although the chronic kidney disease (CKD) patients
86 exhibited hypocalcemia and hyperphosphatemia due to impaired renal functions, these
87 imbalances are compensated by an increased activity of parathyroid hormone (PTH).
88 However, with the progression of CKD, PTH cannot control the blood Ca, which shows
89 significant decrease.¹⁶ Further, vitamin D obtained through food, produced in the skin, and

90 activated in the kidney, is related to osteogenesis by increasing blood Ca level.¹⁴ Vitamin D
91 also associates with the immune function and its abnormality. Patients with autoimmune
92 diseases, such as systemic lupus erythematosus (SLE) and RA, have low levels of vitamin D
93 in the blood, contributing to osteoporosis.¹⁷ Furthermore, the blood mineral levels show
94 complex dynamics and do not necessarily represent the state of bones in several diseases.
95 Therefore, the combination of morphological evaluations of bones and blood examinations is
96 essential to understand the pathogenesis of bone-related diseases in the patients suffering the
97 other disease.

98 To understand the effect of autoimmune diseases on bone morphology, we histopathologically
99 examined the phenotype of BXSB/MpJ-*Yaa* (BXSB-*Yaa*) mice as an autoimmune disease
100 model. As BXSB-*Yaa* carries the Y-linked autoimmune accelerator (*Yaa*) mutation on the Y
101 chromosome, the male mice develop severe autoimmune symptoms similar to SLE
102 characterized by abnormal proliferation of B-cells, auto-antibody production, and
103 splenomegaly,^{18,19} but not RA.²⁰ We have already demonstrated the pathological features of
104 nephritis and dacryoadenitis in BXSB-*Yaa*,²¹⁻²³ however, its bone morphology was unclear.
105 Therefore, we analyzed the altered bone structures, especially focusing on the population of
106 osteoblasts and osteoclasts, by comparisons between BXSB-*Yaa* and its control strain
107 BXSB/MpJ (BXSB) with the evaluation of blood mineral dynamics and autoimmune disease
108 conditions.

109 **Material and methods**

110 *Animal ethics*

111 Animal experimentation was approved by the Institutional Animal Care and Use Committee
112 of the Graduate of School of Veterinary Medicine, Hokkaido University (approval
113 No.16-0124). The animals were handled in accordance with the Guide for the Care and Use of
114 Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University
115 (approved by the Association for Assessment and Accreditation of Laboratory Animal Care
116 International). Male BXSB and BXSB-Yaa aged 2-6 months were purchased from Japan SLC,
117 Inc. (Hamamatsu, Japan) and were maintained under specific pathogen-free conditions. Blood
118 of all mice were collected from femoral arteries under deep anesthesia using the mixture of
119 medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). Finally, all
120 mice were euthanized by cervical dislocation.

121

122 *Hematological and serological analysis and urinalysis*

123 The number of white blood cells (WBCs), red blood cells (RBCs), platelets (PLs),
124 hemoglobin concentration (HC), hematocrit volume (Ht), mean corpuscular volume (MCV),
125 and mean corpuscular HC (MCHC) was measured using a XT-1800i instrument (Sysmex
126 Corporation; Kobe, Japan). Additionally, the blood smear was stained by Diff-Quik solution
127 (Sysmex Corporation) to detect reticulocytes.

128 Serum levels of anti-double strand DNA (dsDNA) antibody were measured to evaluate
129 systemic autoimmune conditions using LBIS Anti dsDNA-Mouse ELISA Kit (FUJIFILM
130 Wako Pure Chemical Corporation; Osaka, Japan) according to the manufacturer's instructions.
131 As an index of mineral metabolism of bones, the serum Ca concentration was measured using
132 a Fuji Dri-Chem 7000v instrument (FUJIFILM Medical Co., Ltd.; Tokyo, Japan).
133 Furthermore, urinary concentrations of Ca and creatinine (CRE) were determined by a Fuji
134 Dri-Chem 7000v instrument (FUJIFILM Medical Co., Ltd.) and Creatinine-test-Wako
135 (FUJIFILM Wako Pure Chemical Corporation) according to the manufacturer's instructions.
136 Serum PTH concentration was measured to evaluate parathyroid gland function using Mouse
137 PTH 1-84 ELISA kit (Quidel Corporation; San Diego, CA, USA).

138

139 ***Morphological and histological analyses of bones***

140 To evaluate the gross morphology of bones, the bone length and wet weight were measured.
141 After taking a picture of the tibia, the tibia length was measured by ImageJ.²⁴ Subsequently,
142 the ratio of tibia and fibula weight to body weight (BW) was calculated.
143 The tibia samples for histology were fixed with 4% paraformaldehyde at 4°C overnight, and
144 decalcified by 5% ethylenediaminetetraacetic acid at 4°C for 5 days. After decalcification, the
145 specimens were routinely dehydrated by ethanol and embedded into paraffin. Then, paraffin
146 sections (3 µm) were prepared and stained with hematoxylin and eosin (HE) or

147 tartrate-resistant acid phosphatase (TRAP) staining Kit (FUJIFILM Wako Pure Chemical
148 Corporation) to detect the osteoclasts.

149

150 *Immunohistochemistry*

151 The paraffin sections were immunostained using the methodology by a previous study.²³ The
152 antigen retrieval was applied to sections (Supplementary Table 1). Subsequently, to block
153 internal peroxidase activity, the sections were soaked in methanol containing 0.3% H₂O₂ for
154 20 minutes at 25°C. After washing three times in phosphate-buffered saline (PBS), the
155 sections were incubated with a blocking serum for 1 h at 25°C to block the non-specific sites.
156 Then, sections were incubated with primary antibodies overnight at 4°C. The sections were
157 then washed thrice in PBS and incubated with secondary antibodies for 30 minutes at 25°C
158 and washed thrice in PBS. Consequently, the sections were incubated with streptavidin
159 conjugated horseradish peroxidase (SABPO(R) kit, Nichirei; Tokyo, Japan) for 30 minutes at
160 25°C, washed three times in PBS, and the immunopositive reaction was visualized with
161 3,3'-diaminobenzidine tetrahydrochloride-H₂O₂ solution. Finally, the sections were lightly
162 stained with hematoxylin. The details of the antibody, antigen retrieval, and blocking are
163 listed in Supplementary Table 1.

164

165 *Histoplanimetry*

166 We performed the histoplanimetric analysis of tibias using histological stained sections as
167 shown in Supplementary Fig. 1. We evaluated the area ratio of bone to BM, the ratio of
168 trabecular area to tissue area (Tb.Ar/T.Ar), trabecular width (Tb.Wi), trabecular number
169 (Tb.N), trabecular separation (Tb.Sp), TRAP⁺ cells with 2 or more nuclei, as osteoclasts,
170 osteocalcin⁺ cells, osteoblasts, and osteocytes.^{25,26}

171

172 ***Quantitative polymerase chain reaction (qPCR)***

173 Total RNA from the bone including BM in a humerus was purified using the TRIzol reagent
174 (Thermo Fisher Scientific; Waltham, MA, USA) following the manufacturer's instructions.

175 The purified total RNA (83.3 ng/μl) was treated as a template to synthesize cDNA using

176 ReverTra Ace qPCR RT Master Mix (TOYOBO CO., LTD.; Osaka, Japan). Quantitative PCR

177 analysis was performed on the cDNA (20 ng/μl) using THUNDERBIRD® SYBR® qPCR

178 Mix (TOYOBO CO., LTD.) and the following gene-specific primers (Supplementary Table 2).

179 The qPCR cycling conditions were: 95°C for 1 min, (95°C for 15 s, 60°C for 45 s [40 cycles]).

180 Data were normalized by the values of actin, beta (*Actb*), and those of BXS_B at 3 months

181 using the delta-delta Ct method.

182

183 ***Statistical analysis***

184 The data were expressed as the mean ± standard error (SE) and statistically analyzed by a

185 non-parametric manner. Briefly, the significance between the two groups was analyzed by the
186 Mann-Whitney U -test ($P < 0.05$). The correlation between the two parameters was analyzed
187 using Spearman's correlation test ($P < 0.05$).

188 **Results**

189 *Autoimmune disease features found in BXS_B-Yaa*

190 We pathologically examined mice at 3 and 6 months of age (Fig. 1). The BW in BXS_B-Yaa
191 was lower than in BXS_B at both ages, and that of BXS_B significantly increased with
192 advancing age, but not in BXS_B-Yaa (Fig. 1(a)). Regarding autoimmune disease indices,
193 BXS_B-Yaa showed significantly higher values in the ratio of spleen weight to BW (S/B) and
194 the serum levels of anti-dsDNA antibody compared with BXS_B at both ages (Fig. 1(b) and
195 (c)). Further, for S/B, BXS_B-Yaa at 6 months significantly showed higher values than those at
196 3 months (Fig. 1(b)). Thus, autoimmune disease phenotypes were evident in BXS_B-Yaa and
197 deteriorated at 6 months.

198

199 *Impaired bone metabolism, renal function, and hematopoiesis in BXS_B-Yaa*

200 Considering bone metabolism indices, the serum level of Ca was significantly higher in
201 BXS_B-Yaa than in BXS_B at 2-4 months, and that of the former was significantly decreased
202 with advancing age (Fig. 1(d)), indicating the imbalanced Ca metabolism in BXS_B-Yaa. For
203 the ratio of urinary Ca to CRE level, an indicator of urinary Ca excretion, the BXS_B-Yaa at 6
204 months showed the highest value, and significant differences were observed between 3 and 6
205 months in this strain (Fig. 1(e)). Although we hypothesized secondary hyperparathyroidism in
206 BXS_B-Yaa at 6 months due to nephritis,¹⁴ neither significant age nor strain-related change

207 was observed in the serum PTH concentration (Fig. 1(f)).

208 Furthermore, we performed hematological and serological analyses, focusing on

209 hematopoiesis including, counts of WBCs, RBCs, PLs (Fig. 1(g-i)), HC, Ht, MCV, and

210 MCHC (Fig. 1(j-m)). BXSb-Yaa showed significant increase and decrease in WBCs and PLs

211 at 6 months compared with 3 months, respectively (Fig. 1(g) and (i)). Further, BXSb-Yaa at 6

212 months showed significant increase and decrease in MCV and MCHC compared with BXSb

213 at the same age, respectively (Fig. 1(l) and (m)). There was no significant age or strain-related

214 change in the other parameters. Further, at 6 months, the reticulocytes were more abundant in

215 BXSb-Yaa than in BXSb (Fig. 1(n)).

216 We also histologically examined the organs, such as kidneys, thyroid and parathyroid glands,

217 involved in the regulation of blood Ca concentration (Fig. 2(a-c)). For renal histopathology,

218 severe glomerulonephritis with immune cell infiltrations to the tubulointerstitium was

219 observed in the kidney of BXSb-Yaa at 6 months as described previously,²¹ but that was not

220 clearly observed in BXSb at both ages and BXSb-Yaa at 3 months (Fig. 2(a)). The injured

221 kidney in BXSb-Yaa at 6 months coincided with increase in urinary Ca level (as shown in Fig.

222 1(e)). In thyroid glands, histopathological changes such as dilation of follicle or inflammation

223 were not observed in all examined mice (Fig. 2(b)). Further, the localization and the number

224 of immune-positive parafollicular cells for calcitonin, which decreases blood Ca level by

225 inhibiting bone resorption, did not remarkably differ. Parathyroid glands were similarly

226 observed among all examined mice, and were surrounded by the connective tissues in thyroid
227 glands (Fig. 2(c)). Similar to thyroid glands, no histopathological change including
228 inflammation was observed within the parathyroid glands in all mice. Further, the localization
229 and the number of immune-positive principal cells for PTH, increasing bone resorption, did
230 also not remarkably differ.

231

232 *Altered bone morphology in BXSB-Yaa*

233 Macroscopically, during observation periods, the tibia of BXSB-Yaa were smaller, and BM
234 was whiter compared to BXSB at 6 months (Fig. 3(a)) as associating with the increased
235 WBCs and anemic phenotypes of BXSB-Yaa (Fig. 1(g), (l) and (n)). The tibia length and the
236 weight of tibia and fibula significantly increased in BXSB with advancing age, but an
237 age-related significant increase in BXSB-Yaa was only observed in the tibia length (Fig. 3(b)
238 and (c)). Importantly, BXSB-Yaa exhibited significantly shorter and lighter tibia compared to
239 BXSB at 6 months (Fig. 3(b) and (c)). Further, the ratio of tibia and fibula weight to BW in
240 BXSB-Yaa at 6 months was significantly lower than in the same strain at 3 months or BXSB
241 at 6 months (Fig. 3(d)).

242 Regarding the histopathological analysis of the tibias, we observed entire bone sections and
243 identified drastic morphological changes around the epiphysis in BXSB-Yaa. Briefly, although
244 no remarkable strain difference was observed at 3 months, the reduction of trabeculas and

245 thinning of compact bone was observed in BXS_B-Yaa at 6 months (Fig. 3(e)). In the area ratio
246 of bone to BM, an indicator of bone thickness, BXS_B-Yaa showed significantly lower values
247 compared with BXS_B at 6 months (Fig. 3(f)). Further, to evaluate trabecular bones, we
248 examined Tb.Ar/T.Ar, Tb.Wi, Tb.N, and Tb.Sp, according to a parallel plate model (Fig.
249 3(g-j)).^{25,26} Regarding the values of Tb.Ar/T.Ar, Tb.Wi, and Tb.N, BXS_B-Yaa at 6 months of
250 age showed significantly lower values compared with the same strain at 3 months or BXS_B at
251 6 months of age, and Tb.Wi in BXS_B-Yaa at 3 months was also significantly lower than
252 BXS_B at the same age (Fig.3 (g-j)). Additionally, BXS_B-Yaa at 6 months of age showed
253 significantly higher values in Tb.Sp compared with BXS_B-Yaa at 3 months and BXS_B at 6
254 months of age (Fig.3 (j)). However, no significant difference was observed in the density of
255 osteocytes between the strains at both examined ages (Fig. 3(k)).

256

257 *Altered number of osteogenesis-associated cells in BXS_B-Yaa*

258 To evaluate osteoclasts, we observed the cells positive for TRAP, the enzyme produced by
259 osteoclasts.²⁷ TRAP⁺ osteoclasts were mainly localized to the surface of the medullary cavity,
260 and were more abundant near the epiphyseal cartilages in both strains at both ages (Fig. 4(a)).
261 However, the enzyme activity of TRAP seemed to be stronger in BXS_B-Yaa at 6 months
262 compared with other mice. These findings were confirmed by histoplanimetry, displaying that
263 the number of TRAP⁺ osteoclasts was higher in BXS_B-Yaa than in BXS_B at both ages, and

264 significant difference was observed at 6 months (Fig. 4(c)).

265 Subsequently, we also evaluated osteoblasts by immunohistochemistry targeting the

266 osteocalcin produced by osteoblasts, which is generally regarded as a marker of bone

267 formation.²⁸ Similar to TRAP⁺ osteoclasts, the osteocalcin⁺ cells lined the surface of the

268 medullary cavity (Fig. 4(b)). There were no age or strain-related differences in the numerical

269 values of osteoblasts. However, BXSB-Yaa tended to display lower values compared to

270 BXSB at 3 months of age, and subsequently tended to increase with advancing age, in the

271 osteocalcin⁺ osteoblasts (Fig. 4(d)).

272

273 ***mRNA expression of Yaa locus genes and inflammatory cytokines in BXSB-Yaa***

274 In order to identify the factors associated with autoimmune disease and bone abnormalities,

275 the mRNA expression of the humerus including BM was examined by qPCR. Firstly, we

276 examined the genes on the *Yaa* locus (Fig. 5), since BXSB-Yaa had the duplicated 15 genes

277 on Y chromosome because of translocation of X chromosome.¹⁹ The genes, such as Rab9,

278 member of the RAS oncogene family (*Rab9*), tyrosin β 4 X chromosome (*Tmsb4x*),

279 phosphoribosyl pyrophosphate synthetase 2 (*Prps2*), toll-like receptor 7 (*Tlr7*), and *Tlr8*, were

280 higher in BXSB-Yaa compared with BXSB at 3 and 6 months of ages without age-related

281 changes. Thus, the expression of the genes seemed to be increased by the influence of *Yaa*

282 mutation. Further, BXSB-Yaa significantly showed higher expression in MSL complex

283 subunit 3 (*Msl3*) and Rho GTPase activating protein 6 (*Arhgap6*) compared with BXSB at 6
284 months and BXSB-Yaa at 3 months. An age-related significant increase was observed in the
285 expression of amelogenin X-linked (*Amelx*) in BXSB-Yaa, and this strain at 6 months also
286 significantly showed higher expression in Midline 1 (*Mid1*) than BXSB at the same age.
287 Consequently, the mRNA levels of inflammatory cytokines associating with bone resorption,
288 such as *Il1a*, *Il1b*, *Il6*, *Il8*, and *Tnf*, were measured (Fig. 5).^{10-12,29} The expression of *Il1a* and
289 *Tnf* in BXSB-Yaa at 6 months was significantly higher than in BXSB at 6 months and in
290 BXSB-Yaa at 3 months. Furthermore, the mRNA levels of *Il1b* in both strains at 6 months
291 were higher than that at 3 months, and the increase of BXSB-Yaa was more significant.

292

293 ***Correlation between altered bone morphology and autoimmune disease in BXSB-Yaa***

294 Correlation between the indices of bone morphology and autoimmune disease is shown in
295 Table 1. The ratio of tibia and fibula weight to BW in all mice showed significant and
296 negative correlations with S/B and mRNA expression of *Tlr8* and *Tlr7* in bones; and that in
297 BXSB-Yaa showed significant and negative correlations with *Arhgap6* expression in bones.
298 The area ratio of bone to BM showed significant and negative correlations with serum
299 anti-dsDNA antibody levels and mRNA expression of *Rab9*, *Tmsb4x*, *Prps2*, *Msl3*, *Arhgap6*,
300 and *Mid1* in bones; and that in BXSB-Yaa showed significant and negative correlations with
301 *Tmsb4x*, *Prps2*, and *Msl3* expression in bones. Tb.Ar/T.Ar showed significant and negative

302 correlations with S/B, serum anti-dsDNA antibody levels, and mRNA expression of *Tmsb4x*,
303 *Tlr7*, *Tlr8*, *Prps2*, *Msl3*, *Arhgap6*, and *Il1b* in bones, and that in BXSB-Yaa showed
304 significant and negative correlations with S/B and *Il1a* and *Il1b* expression in bones. Tb.Wi
305 showed significant and negative correlations with S/B, serum anti-dsDNA antibody levels,
306 and the mRNA expression of *Rab9*, transcription elongation factor A (SII) N-terminal and
307 central domain (*Tceanc*), *Tmsb4x*, *Tlr7*, *Tlr8*, *Prps2*, holocytochrome c synthase (*Hccs*), *Il1a*,
308 and *Il1b* in bones, and that of BXSB-Yaa showed significant and negative correlations with
309 oral-facial digital syndrome 1 (*Ofd1*), *Rab9*, *Tmsb4x*, *Il1a*, and *Il6* expression. The number of
310 TRAP⁺ osteoclasts showed significant and positive correlations with S/B, serum anti-dsDNA
311 antibody levels, and mRNA expression of *Tlr8*, *Msl3*, *Arhgap6*, and *Mid1* in bones; and that
312 in BXSB-Yaa showed significant and positive correlations with *Rab9* expression in bones.
313 The numerical values of osteocalcin⁺ osteoblast area showed significant and positive
314 correlations with mRNA expression of *Amelx* in bones, and that in BXSB-Yaa showed
315 significant and negative correlations with the *Tlr7* expression in bones.

316 **Discussion**

317 We clarified that the autoimmune disease-prone BXSB-Yaa had higher level of serum Ca at
318 2-4 months. Ca serum level is an indicator of bone metabolism, and hypercalcemia is
319 predominantly caused by osteoclast activation as found in human osteoporosis with advancing
320 age or menopause.^{30,31} In fact, BXSB-Yaa showed an increase in the number of TRAP⁺
321 osteoclasts from 3 months. Further, at 6 months, the serum level and urinary excretion of Ca
322 in BXSB-Yaa significantly decreased and increased, respectively, with the progression of
323 autoimmune disease phenotypes including nephritis. Although the renal tubules play a crucial
324 role in the Ca reabsorption from primitive urine, the Ca could not be reabsorbed through renal
325 tubules in aged BXSB-Yaa because of renal injury. Notably, secondary renal
326 hyperparathyroidism was observed to develop to compensate the increased urinary loss of Ca
327 in the CKD patients.¹⁶ However, there was no change in the serum PTH level as well as the
328 morphologies of thyroid and parathyroid glands in BXSB-Yaa during the examined periods.
329 Importantly, in aged BXSB-Yaa, the tibia length and the weight of tibia and fibula were
330 observed to significantly decrease compared with BXSB. Therefore, altered Ca dynamics in
331 BXSB-Yaa suggested that their osteoclast number was increased and activated via a
332 PTH-independent mechanism as a direct consequence of autoimmune disease or *Yaa*
333 mutations to bone metabolism.
334

335 Previous studies have reported the development of hemolytic anemia, one of the causes of
336 osteoporosis, in mice carrying *Yaa* mutations.³² Here, we also revealed that aged BXSB-Yaa
337 showed higher and lower values in MCV and MCHC, respectively compared to BXSB.
338 However, number of RBCs and Ht was comparable between both strains. A high value of
339 MCV signifies the production of large and immature RBCs including reticulocytes in
340 response to anemia.³³ MCHC indicated the hemoglobin concentration in RBC; generally, a
341 reduced MCHC was followed by a decrease in the MCV as found in microcytic hypochromic
342 anemia because of iron deficiency.³³ Alternatively, the altered patterns of MCV and MCHC in
343 BXSB-Yaa were not typical cases of anemia. Importantly, we also found significantly
344 increased numbers of WBCs and decreased PLs in aged BXSB-Yaa. Therefore, these data
345 reflect that the altered hematopoiesis, such as abnormal lymphocyte production as shown in
346 the previous study about human multiple myeloma,³⁴ might also affect the values of
347 hemoanalysis and BM morphology in BXSB-Yaa.

348

349 BXSB-Yaa tended to show an increase in osteoclasts and decrease in osteoblasts in the tibia at
350 3 months. These altered populations to bone resorption patterns would be associated with the
351 increased serum Ca level in BXSB-Yaa. Further, a sustained increase in osteoclast number
352 from 3 to 6 months would be critical for the abnormal remodeling of bones, as characterized
353 by significant reduction in bone length, weight, and trabeculas at 6 months. Importantly, the

354 osteocyte number was not altered, however, the bone area was reduced in the tibia of
355 BXSB-Yaa during periods of observation, indicating total decrease of osteocytes, important
356 cells for mineral metabolism.³⁵ Along with PTH, inflammatory cytokines including IL-1, IL-6,
357 and TNF- α can activate osteoclasts as found in the patients with RA and osteoporosis.¹⁰⁻¹²
358 However, these cytokines were not significantly correlated with the indices for bone
359 morphology and osteoclast numbers in the present study. Notably, in BXSB-Yaa, the
360 numerical values of osteoblasts was rescued at 6 months of age. However, the functional
361 maturation was not clear in the increased numerical values of osteoblasts in BXSB-Yaa. Thus,
362 the hyperimmune status in BM would be induced by autoimmune abnormality, and it might
363 indirectly affect the function and/or population of cells-associating bone remodeling in
364 BXSB-Yaa. Importantly, similar pathological alterations characterized by the loss of bone
365 tissue due to imbalance between osteoclasts and osteoblasts were observed in the osteoporosis
366 patients.⁶ The patients of autoimmune disease also develop osteoporosis, for example, RA in
367 humans causes secondary osteoporosis due to inflammatory cytokines, medicine, or
368 immobilization.³⁶ Therefore, BXSB-Yaa would be a suitable model to analyze the altered
369 bone remodeling due to autoimmune abnormalities.

370

371 The causative factors of autoimmune disorder in BXSB-Yaa were encoded on the *Yaa* locus.¹⁹

372 We examined the expression of all 15 protein-coding genes on this locus to discuss their

373 pathological contributions of autoimmunity and bone morphology. At 3 months, *Rab9*,
374 *Tmsb4x*, *Prps2*, *Tlr7*, and *Tlr8* were highly expressed in BXS_B-Yaa bones. There was no
375 relation between *Rab9* and autoimmunity. However, this gene was associated with osteoclast
376 function to secrete lysosomal enzymes in rats.³⁷ *Tmsb4x* is a diagnostic indicator of
377 osteoporosis or a regulator of the differentiation of hematopoietic cells in humans,^{38,39} and
378 these genes were also significantly correlated with the indices of bone area in BXS_B-Yaa.
379 *Prps2* also significantly correlated with the indices of bone area in BXS_B-Yaa, and the
380 variants of *Prps2* genes were associated with the development of SLE in humans,⁴⁰ however,
381 there is no report on the bone. Furthermore, the associations of TLR7 or 8 and autoimmune
382 abnormalities were well analyzed in BXS_B-Yaa,^{22,41} and the roles of candidate genes in
383 developing SLE-like symptoms including nephritis were suggested. TLR7 was associated
384 with RA by contributing to produce inflammatory cytokines, such as IL-1, IL-6, and TNF- α ,
385 from macrophages in mice.^{42,43} TLR8 may have a role in human RA, however, the detailed
386 function of murine TLR8 is unclear.⁴⁴ Therefore, these genes would be candidates to develop
387 the autoimmune disease and/or following bone abnormalities.

388 The expression of *Msl3*, *Arhgap6*, *Amelx* and *Mid1* on *Yaa* locus significantly increased with
389 the progression of the autoimmune disease. However, there is no evidence between *Msl3* or
390 *Mid1* and autoimmunity or bones, but *Mid1* seemed to be associated with activating the innate
391 immune pathway in allergic asthma.⁴⁵ Alternatively, human *Arhgap6*, activating the Ras

392 homolog gene family, member A (RhoA),⁴⁶ and *Amelx* in mice seemed to act as the mediator
393 of osteoblasts and osteoclasts- genesis, respectively.^{47,48} Therefore, these genes would
394 deteriorate autoimmune disease and/or bone abnormalities as exacerbating factors.
395 There were neither age nor strain-related differences in the expression of *Ofd1*, *Tceanc*, and
396 *Hccs*. However, the expression of these genes had significant and negative correlation with
397 Tb.Wi. Additionally, *Ofd1* seemed to be associated with endochondral skeletal development,⁴⁹
398 although there is no report of the evidence between *Tceanc* or *Hccs* and autoimmunity or bone
399 study. Thus, these genes might also contribute to bone abnormality.

400

401 We have demonstrated that systemic autoimmune disease in BXSb-Yaa is closely associated
402 with the morpho-functional abnormalities of bones including BM. The abnormalities are
403 characterized by altered regulations of serum Ca, anemia tendency, and hematopoiesis with
404 increased WBCs and decreased PLs, short length and low mass of long bones, imbalance in
405 the populations of osteoclasts and osteoblasts, and increased expression of candidate genes for
406 causing and/or exacerbating their phenotypes. Therefore, as shown in BXSb-Yaa, we
407 concluded that the bone phenotypes in systemic autoimmune disease would elaborately be
408 affected by the factors in the bone as well as the other immune and/or mineral metabolism
409 organs.

410

411 **Author contributions statement**

412 T.N., O.I. and Y.K. designed and performed the experiments, and T.N., M.A.M., Y.O. S.O.K.
413 and E.Y.H.A. provided the samples and analyzed the data. All authors were involved in
414 writing the paper and have approved the final manuscript.

415

416 **Declaration of conflicting interests**

417 This manuscript has not been published or presented elsewhere in part or in entirety and is not
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421

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424

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- 575

576 **Figure legends**

577 **Figure 1. Autoimmune disease-associated phenotypes, serological and hematological**
578 **analysis, and urinalysis in mice.** (a) Body weight (BW). (b) The ratio of spleen weight to
579 BW (S/B). (c) The serum levels of anti-double strand DNA (dsDNA) antibody. (d) Serum
580 calcium (Ca) level. (e) The ratio of urinary Ca to creatinine (CRE) level. (f) Serum
581 parathyroid hormone (PTH) level. (g) The number of white blood cells (WBCs). (h) The
582 number of red blood cells (RBCs). (i) The number of platelets (PLs). (j) Hematocrit
583 concentration (HC). (k) Hematocrit volume (Ht). (l) Mean corpuscular volume (MCV). (m)
584 Mean corpuscular HC (MCHC). (n) Blood smear. Some reticulocytes (arrows) are observed in
585 the smear of BXSB-Yaa at 6 months. Diff-Quik staining. Bars = 50 μ m. BXSB: BXSB/MpJ.
586 BXSB-Yaa: BXSB/MpJ-Yaa. The numbers of samples used in the studies are as follows: n =
587 8-18 (a and b), n = 5-10 (c), n = 9-16 (d), n = 5 (e), n = 8-18 (f), and n = 4-9 (g-m). Each bar
588 represents the mean \pm SE. *: Significance with the other strain at the same age
589 (Mann-Whitney *U*-test, **P* < 0.05, ***P* < 0.01). †: Significance with the same strain at other
590 age (Mann-Whitney *U*-test, †*P* < 0.05, ††*P* < 0.01).

591

592 **Figure 2. Histology of thyroid and parathyroid glands in mice.** (a) Renal histology. Severe
593 glomerulonephritis (asterisks) and cell infiltrations into the tubulointerstitium (arrow) are
594 observed in the kidneys of BXSB-Yaa at 6 months. PAS staining. (b) Thyroid glands.
595 Immunohistochemistry for calcitonin. Neither age nor strain- related changes are observed in
596 the organ. (c) Parathyroid glands. Immunohistochemistry for parathyroid hormone. Neither
597 age nor strain-related changes are observed in each organ. BXSB: BXSB/MpJ. BXSB-Yaa:
598 BXSB/MpJ-Yaa. The number of samples used in the studies is as follows: n = 4 (a), n = 5-12
599 (b), and n = 4-8 (c). Bars = 100 μ m.

600

601 **Figure 3. Morphological differences of bones in mice.** (a) Gross morphology of bones. At 6

602 months, the tibia and fibula of BXS_B/MpJ-*Yaa* (BXS_B-*Yaa*) is macroscopically smaller, and
603 its bone marrow (BM) is whiter compared with BXS_B/MpJ (BXS_B). Bars = 10 mm. (b) Tibia
604 length. (c) Tibia and fibula weight. (d) The ratio of tibia and fibula weight to body weight
605 (BW). (e) Tibia histology. The reduction of trabeculas and thinning of compact bone are
606 observed in BXS_B-*Yaa* at 6 months. Neither age nor strain-related differences in the number
607 of osteocytes (arrows) are observed. HE staining. Bars = 100 μm. (f) The area ratio of bone to
608 bone marrow (BM). (g) The ratio of trabecular area to tissue area (Tb.Ar/T.Ar). (h) Trabecular
609 width (Tb.Wi). (i) Trabecular number (Tb.N). (j) Trabecular separation (Tb.Sp). (k) The
610 density of osteocytes. BXS_B: BXS_B/MpJ. BXS_B-*Yaa*: BXS_B/MpJ-*Yaa*. The number of
611 samples used in the studies is as follows: n = 4-14. Each bar represents the mean ± SE. *:
612 significance with the other strain at same age (Mann-Whitney *U*-test, **P* < 0.05, ***P* < 0.01).
613 †: significance with the same strain at other age (Mann-Whitney *U*-test, †*P* < 0.05, ††*P* <
614 0.01).

615

616 **Figure 4. Altered number of osteoclasts and osteoblasts in mouse bones.** (a) Tibia
617 histology stained by tartrate-resistant acid phosphatase (TRAP) for osteoclast detection.
618 TRAP⁺ osteoclasts (arrows) in all mice are mainly localized to the surface of the medullary
619 cavity, especially near the epiphyseal cartilages, and seem to elicit a stronger reaction in
620 BXS_B-*Yaa* at 6 months compared with the other mice. (b) Tibia histology stained by
621 immunohistochemistry of osteocalcin for osteoblasts. The numerical values of osteocalcin⁺
622 osteoblasts (arrow heads), also localized to surface of medullary, seems to be lesser in
623 BXS_B-*Yaa* compared with BXS_B at 3 months. (c) The number of positive cells for TRAP. (d)
624 The numerical values of positive cells for osteocalcin. BXS_B: BXS_B/MpJ. BXS_B-*Yaa*:
625 BXS_B/MpJ-*Yaa*. Bars = 100 μm. The number of samples used in the studies is as follows: n =
626 5-12. Each bar represents the mean ± SE. *: significance with the other strain at the same age
627 (Mann-Whitney *U*-test, *P* < 0.05).

628

629 **Figure 5. mRNA expression of *Yaa* locus genes and inflammatory cytokines in the bones**

630 **of mice.** The genes on *Yaa* locus, such as *Rab9*, *Tmsb4x*, *Tlr8*, *Tlr7*, and *Prps2*, are higher in

631 BXSB-*Yaa* compared with BXSB at both ages. Additionally, BXSB-*Yaa* at 6 months also

632 significantly shows higher expression in *Msl3* and *Arhgap6* compared with BXSB and

633 BXSB-*Yaa* at 3 months, respectively. Age-related significant increase is observed in the

634 expression of *Amelx* in BXSB-*Yaa*, and this strain at 6 months also significantly shows higher

635 expression in *Mid1* than BXSB. The mRNA levels of inflammatory cytokines, such as *Il1a*

636 and *Tnf*, in BXSB-*Yaa* at 6 months are significantly higher than in BXSB at the same age and

637 in BXSB-*Yaa* at 3 months. Further, an age-related significant increase is observed in the

638 mRNA levels of *Il1b* in both strains. BXSB: BXSB/MpJ. BXSB-*Yaa*: BXSB/MpJ-*Yaa*. The

639 number of samples used in the studies is as follows: $n = 5-15$. Each bar represents the mean \pm

640 SE. *: significance with the other strain at the same age (Mann-Whitney *U*-test, $*P < 0.05$,

641 $**P < 0.01$). †: significance with the same strain at other ages (Mann-Whitney *U*-test, $\dagger P <$

642 0.05 , $\dagger\dagger P < 0.01$).

643

Figure 1

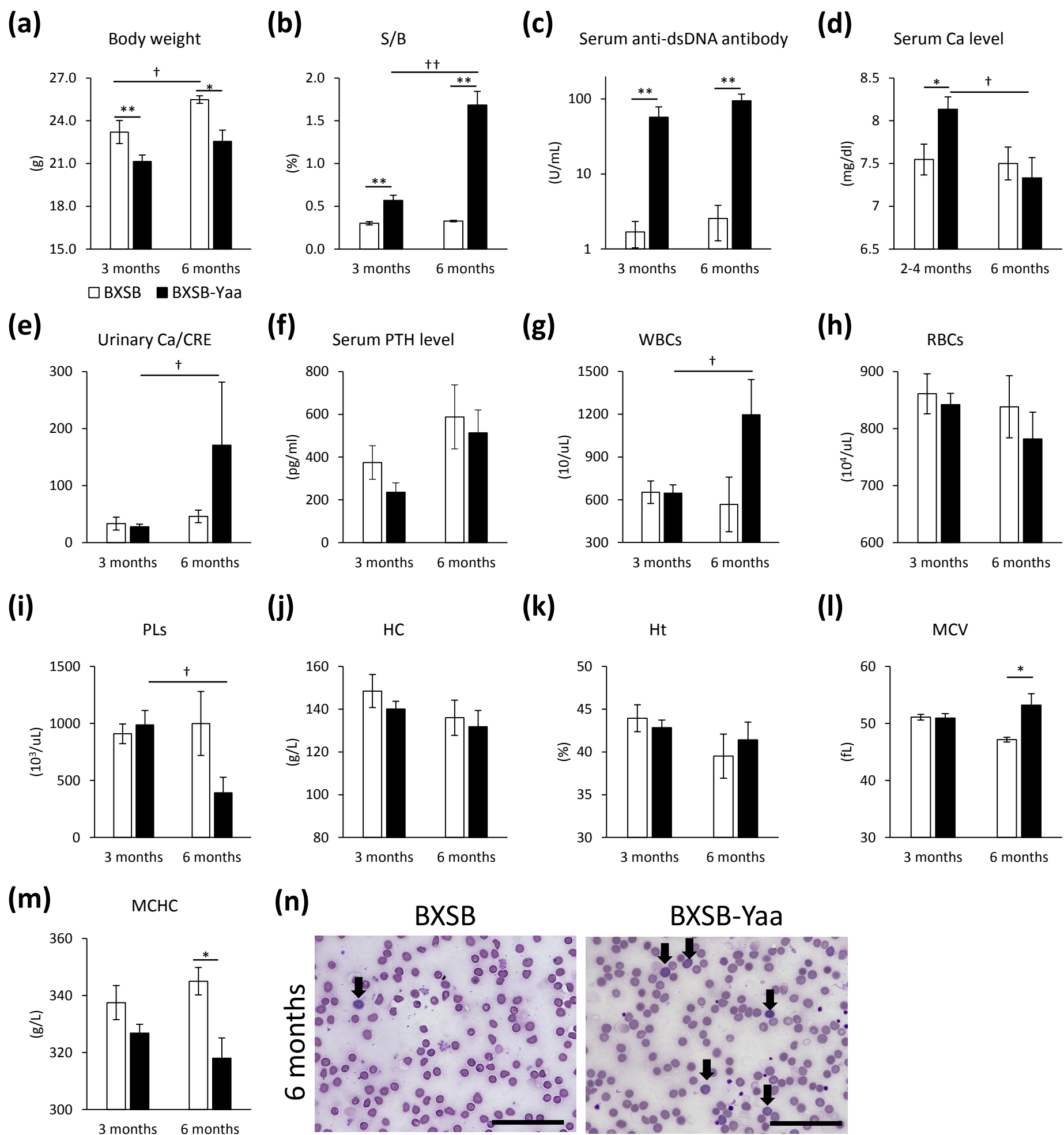


Figure 2

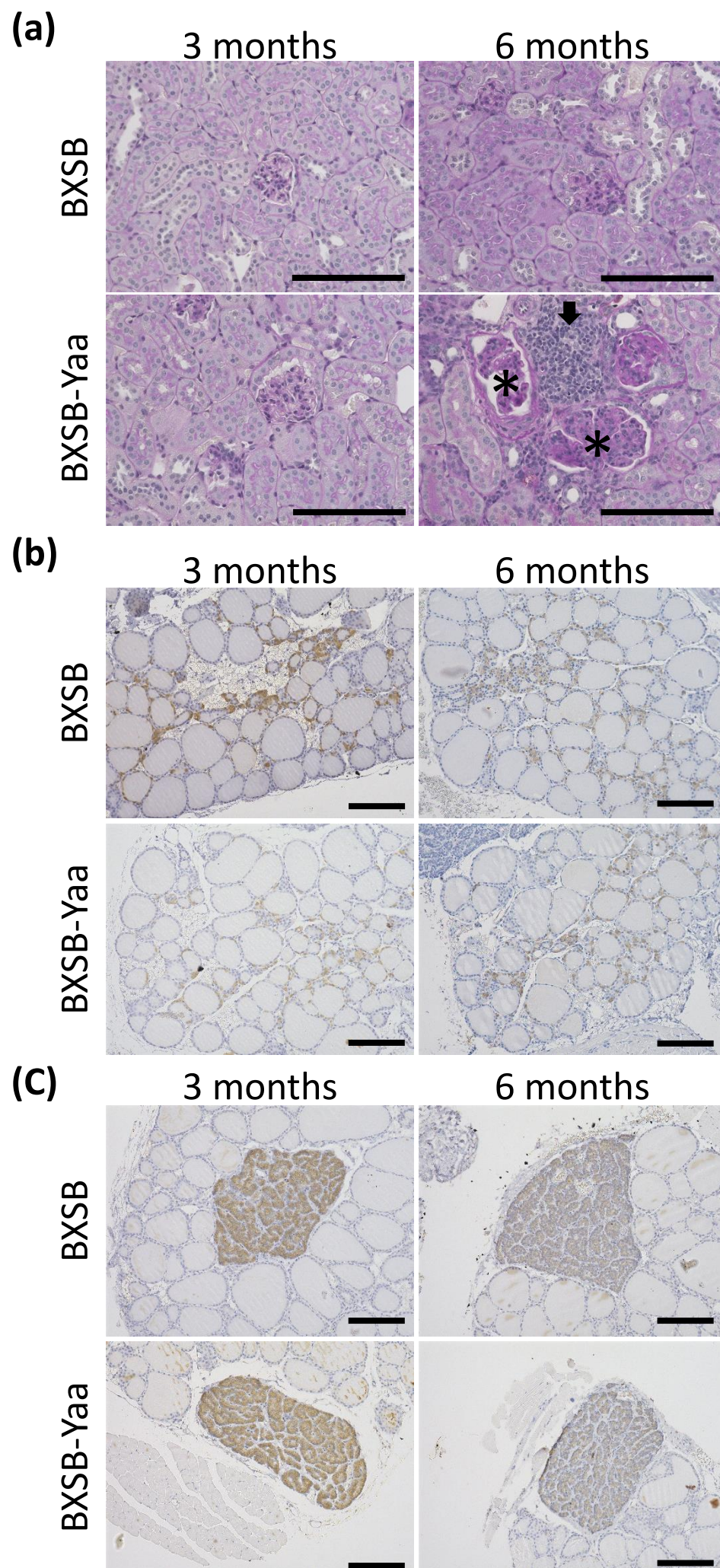


Figure 3

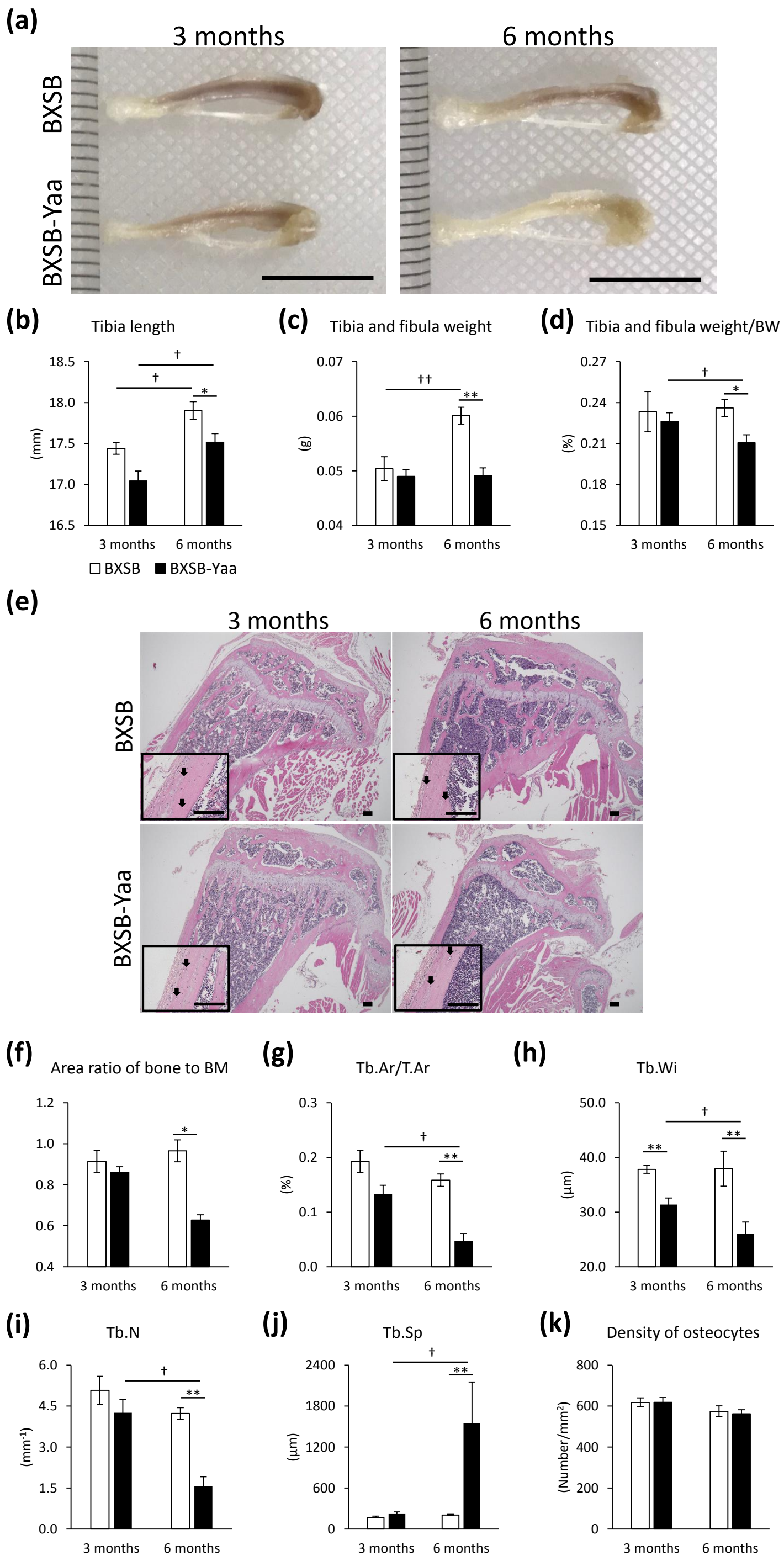


Figure 4

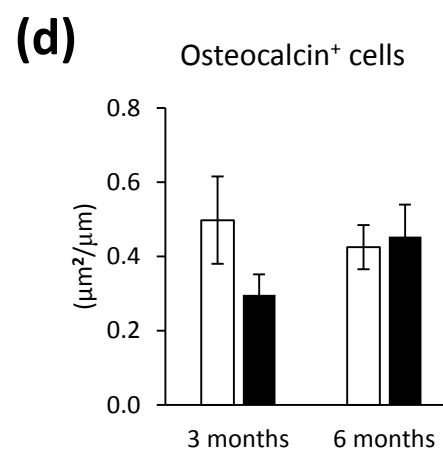
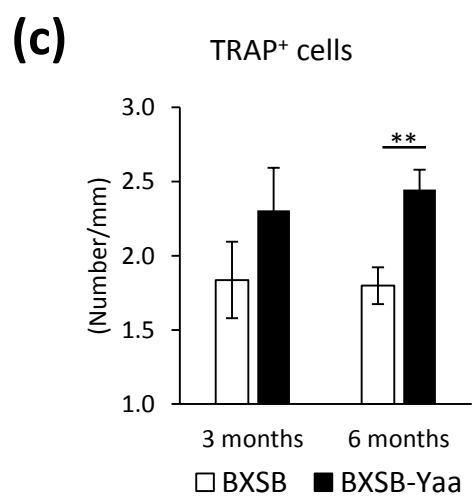
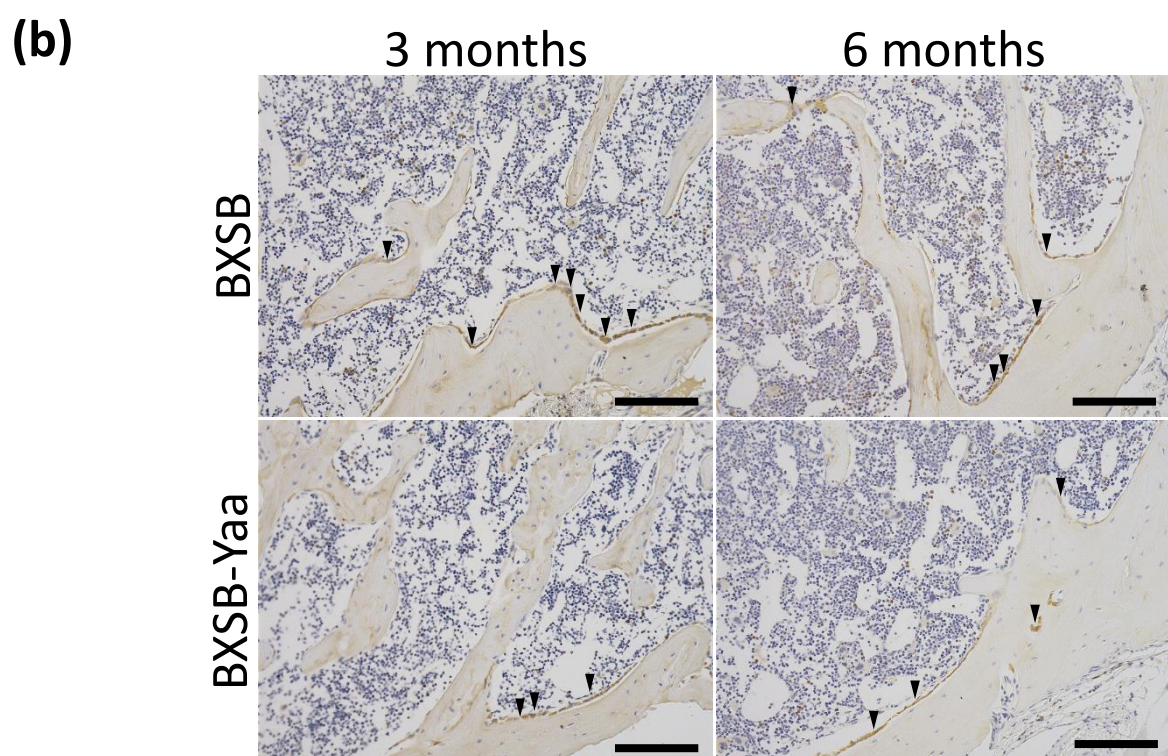
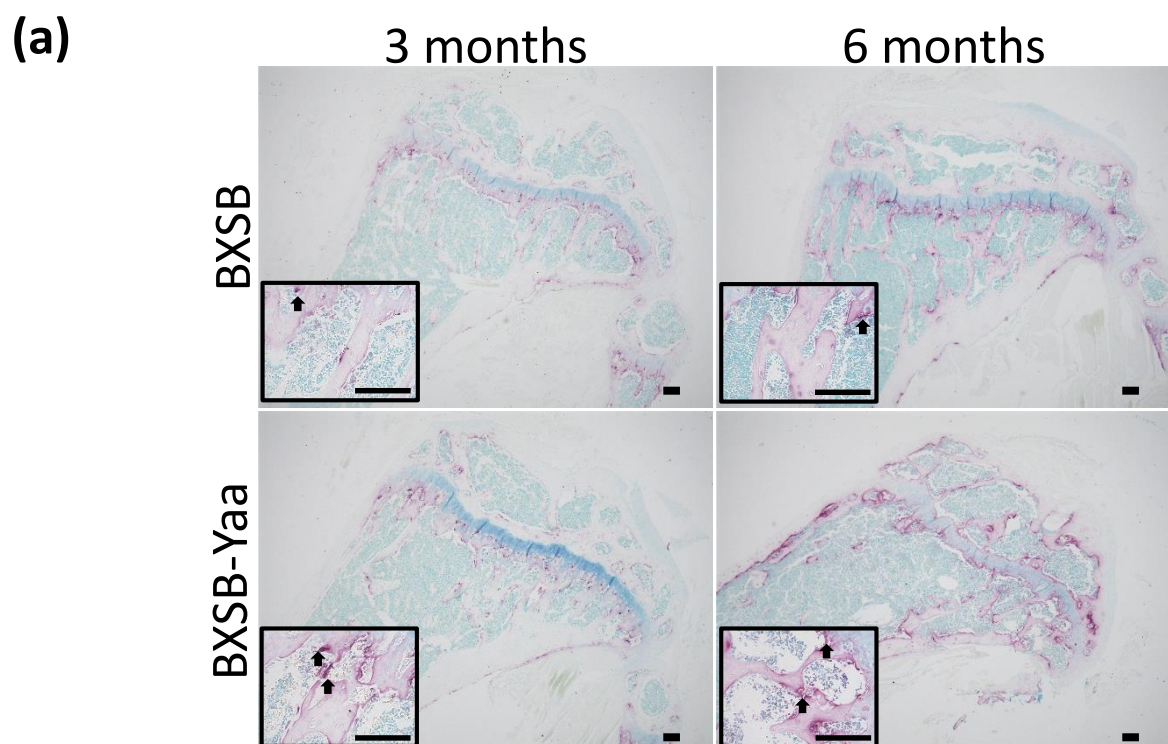
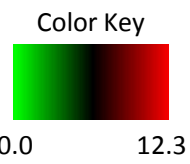


Figure 5



		3 months		6 months	
		BXSB	BXSB-Yaa	BXSB	BXSB-Yaa
Gene on Yaa locus	<i>Ofd1</i>	1.0	0.8	0.7	0.8
	<i>Trppc2</i>	0.0	0.0	0.0	0.0
	<i>Rab9</i>	1.0	1.7 **	0.8	1.9 **
	<i>Tceanc</i>	1.0	1.3	0.6	1.0
	<i>Egfl6</i>	1.3	0.5	0.7	0.3
	<i>Tmsb4x</i>	1.0	2.0 *	1.0	2.9 **
	<i>Tlr8</i>	1.0	1.8 *	0.8	3.2 **
	<i>Tlr7</i>	1.0	3.0 **	0.9	4.0 **
	<i>Prps2</i>	1.1	2.0 **	1.3	2.6 **
	<i>Frmpd4</i>	1.4	2.2	1.8	5.4
	<i>Msl3</i>	2.0	3.1	2.2	12.3 ††
	<i>Arhgap6</i>	1.0	1.3	0.8	3.0 ††
	<i>Amelx</i>	1.4	1.7	4.1	3.6 †
	<i>Hccs</i>	1.0	1.8	1.1	2.0
	<i>Mid1</i>	1.0	1.2	0.8	1.7 *
Inflammatory cytokine	<i>Il1a</i>	1.0	0.9	0.8	1.7 ††
	<i>Il1b</i>	1.1	1.1	2.6 †	3.9 ††
	<i>Il6</i>	1.0	0.7	1.5	1.1
	<i>Il8</i>	1.0	0.8	0.6	0.4
	<i>Tnf</i>	1.1	0.6	0.8	1.4 †

Table 1. Correlations between bone morphological parameters and autoimmune disease indices or gene expression.

	Strains	Parameters					
		TF/B	Bone area	Tb.Ar/T.Ar	Tb.Wi	Osteoclast	Osteoblast
S/B	All mice	-0.407*	0.117	-0.660**	-0.665**	0.470**	-0.01
	BXSB-Yaa	-0.144	0.401	-0.476*	-0.399	0.161	0.074
Anti-dsDNA antibody	All mice	-0.331	-0.523*	-0.621**	-0.452*	0.702**	0.079
	BXSB-Yaa	-0.233	0.048	0.033	0.300	0.583	0.314
<i>Ofd1</i>	All mice	0.321	-0.296	-0.049	0.092	-0.218	-0.01
	BXSB-Yaa	0.771	-0.214	-0.095	-0.738*	-0.619	-0.214
<i>Rab9</i>	All mice	-0.100	-0.591**	-0.259	-0.680**	0.094	0.022
	BXSB-Yaa	0.600	-0.310	-0.095	-0.762*	-0.738*	-0.071
<i>Tceanc</i>	All mice	-0.132	-0.286	-0.236	-0.534*	0.230	-0.179
	BXSB-Yaa	0.029	0.071	-0.286	-0.238	0.238	-0.036
<i>Egfl6</i>	All mice	-0.312	0.263	0.032	0.238	0.082	0.091
	BXSB-Yaa	-0.257	0.476	0.048	0.500	0.381	-0.357
<i>Tmsb4x</i>	All mice	-0.056	-0.575*	-0.397*	-0.684**	0.288	-0.221
	BXSB-Yaa	0.371	-0.738*	-0.524	-0.881**	-0.619	0.250
<i>Tlr8</i>	All mice	-0.449*	-0.050	-0.574**	-0.618**	0.539**	0.054
	BXSB-Yaa	-0.181	0.091	-0.389	-0.221	0.276	0.005
<i>Tlr7</i>	All mice	-0.399*	0.175	-0.397*	-0.684**	0.288	-0.221
	BXSB-Yaa	0.011	0.456	-0.221	-0.482	0.009	-0.670*
<i>Prps2</i>	All mice	-0.282	-0.558*	-0.585*	-0.686**	0.399	0.216
	BXSB-Yaa	0.429	-0.738*	-0.5	-0.524	-0.095	0.286
<i>Frmpd4</i>	All mice	-0.240	-0.432	-0.318	0.071	0.393	0.231
	BXSB-Yaa	-0.486	-0.607	-0.214	0.000	0.143	0.486
<i>Msl3</i>	All mice	-0.188	-0.639**	-0.544*	-0.364	0.488*	0.223
	BXSB-Yaa	-0.600	-0.929**	-0.667	-0.429	0.024	0.357
<i>Arhgap6</i>	All mice	-0.418	-0.577*	-0.505*	-0.377	0.680**	0.093
	BXSB-Yaa	-0.886*	-0.595	-0.357	0.143	0.619	0.750
<i>Amelx</i>	All mice	-0.086	-0.047	-0.038	0.350	0.224	0.600*
	BXSB-Yaa	-0.657	-0.69	-0.476	0.048	0.452	0.571
<i>Hccs</i>	All mice	0.018	-0.444	-0.36	-0.495*	0.159	0.132

	BXSB-Yaa	0.200	-0.357	-0.143	-0.548	-0.381	-0.071
<i>Mid1</i>	All mice	-0.276	-0.585*	-0.255	-0.327	0.486*	0.152
	BXSB-Yaa	-0.657	-0.333	-0.167	0.071	0.500	0.714
<i>Il1a</i>	All mice	-0.252	0.212	-0.326	-0.444*	0.010	0.154
	BXSB-Yaa	-0.133	0.362	-0.550*	-0.536*	-0.124	0.143
<i>Il1b</i>	All mice	-0.306	0.225	-0.579**	-0.440*	0.111	0.072
	BXSB-Yaa	-0.125	0.159	-0.554*	-0.468	-0.041	0.214
<i>Il6</i>	All mice	0.385	0.265	0.051	0.036	-0.294	0.100
	BXSB-Yaa	-0.200	-0.429	-0.619	-0.714*	-0.214	-0.100
<i>Il8</i>	All mice	0.046	-0.017	0.235	0.078	-0.142	-0.236
	BXSB-Yaa	0.700	0.25	0.143	-0.643	-0.357	-0.600
<i>Tnf</i>	All mice	-0.050	-0.066	0.026	0.037	0.047	0.056
	BXSB-Yaa	-0.432	-0.095	-0.218	-0.024	0.037	0.136

Spearman's rank correlation coefficients. *: $P < 0.05$, **: $P < 0.01$, $n \geq 4$. BXSB-Yaa: BXSB/MpJ-Yaa, S/B: Ratio of spleen weight to body weight, TF/B: Ratio of tibia and fibula weight to body weight, Bone area: Area ratio of bone to bone marrow, Tb.Ar/T.Ar: The ratio of bone area to tissue area, Tb.Wi: Trabecular width, Tb.N: Trabecular number, Tb.Sp: Trabecular separation, Osteoclasts: Number of TRAP+ osteoclasts, Osteoblasts: Numerical values of osteocalcin+ osteoblasts.