



Title	Morphological Features of the Testis among Autoimmune Mouse Model and Healthy Strains
Author(s)	Shouman, Zeinab; Marei, Hany E.; Abd-Elmaksoud, Ahmed; Kassab, Mohamed; Namba, Takashi; Masum, Md. Abdul; Elewa, Yaser Hosny Ali; Ichii, Osamu; Kon, Yasuhiro
Citation	Microscopy and microanalysis, 27(5), 1209-1217 <a href="https://doi.org/10.1017/S1431927621012411">https://doi.org/10.1017/S1431927621012411</a>
Issue Date	2021-08-05
Doc URL	<a href="http://hdl.handle.net/2115/86864">http://hdl.handle.net/2115/86864</a>
Rights	This article has been published in a revised form in [Microscopy and Microanalysis] [ <a href="http://doi.org/10.1017/S1431927621012411">http://doi.org/10.1017/S1431927621012411</a> ]. This version is published under a Creative Commons CC-BY-NC-ND. No commercial re-distribution or re-use allowed. Derivative works cannot be distributed. ©[Cambridge University Press].
Rights(URL)	<a href="https://creativecommons.org/licenses/by-nc-nd/4.0/">https://creativecommons.org/licenses/by-nc-nd/4.0/</a>
Type	article (author version)
File Information	paper.pdf



[Instructions for use](#)

1   **Title: Morphological features of the testis among autoimmune mouse model and  
2   healthy strains**

3   Zeinab Shouman<sup>1,2,†</sup>, Hany El-Sayed Marei<sup>1</sup>, Ahmed Abd-Elmaksoud<sup>1</sup>, Mohammed Kassab<sup>3</sup>, Takashi  
4   Namba<sup>2</sup>, Md. Abdul Masum<sup>2</sup>, Yasser Hosny Ali Elewa<sup>2,4, †</sup>, Osamu Ichii<sup>2,5</sup>, Yasuhiro Kon<sup>2,\*</sup>

5   <sup>1</sup> Department of Cytology and Histology, Faculty of Veterinary Medicine, Mansoura University,  
6   Mansoura 35516, Egypt.

7   <sup>2</sup> Laboratory of Anatomy, Department of Biomedical Sciences, Faculty of Veterinary Medicine,  
8   Hokkaido University, Sapporo 060-0818, Japan.

9   <sup>3</sup> Department of Cytology and Histology, Faculty of Veterinary Medicine, Kafrelsheikh University,  
10   kafrelsheikh 33511, Egypt.

11   <sup>4</sup> Department of Histology and Cytology, Faculty of Veterinary Medicine, Zagazig University,  
12   Zagazig 44519, Egypt.

13   <sup>5</sup> Laboratory of Agrobiomedical Science, Faculty of Agriculture, Hokkaido University, Sapporo, Japan.

14   † Equally contributed author.

15   **\*Corresponding author:** Yasuhiro Kon, DVM, PhD, Laboratory of Anatomy, Department of  
16   Biomedical Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku,  
17   Sapporo 060-0818, Japan

18   Email: y-kon@vetmed.hokudai.ac.jp

19   Tel: +81-11-706-5187

20   Fax: +81-11-706-5189

21

22

23

24

25

26      **Abstract**

27              Autoimmune diseases play a critical role in the progression of infertility in both sexes and  
28              their severity has been reported to increase with age. However, few reports discussed their effect on  
29              the morphological features of the testis. Therefore, we compared the morphological alterations in the  
30              testes of autoimmune mice model (MRL/MpJ-Fas<sup>Lpr</sup>) and its control strain (MRL/MpJ) with that of  
31              their background strain (C57BL/6N) at 3 and 6 months. Furthermore, we analyzed the changes in the  
32              meiotic spermatocytes, Sertoli cells, immune cells and junctional protein by immunohistochemical  
33              staining. The MRL/MpJ-Fas<sup>Lpr</sup> mice showed a significant increase in the serum autoantibodies level,  
34              spleen/ body weight ratio and seminiferous luminal area when compared to other studied strains. On  
35              the other hand, a significant decrease in the testis/ body weight ratio, number of both Sertoli and  
36              meiotic spermatocyte was observed in MRL/MpJ-Fas<sup>Lpr</sup> and MRL/MpJ mice than that in C57BL/6N  
37              mice especially at old age. However, neither immune cells infiltration nor significant difference in ZO-  
38              1 junctional protein expression could be observed among the studied strains. Our findings suggest that,  
39              the increase in autoimmune severity especially with age could lead to infertility through loss of  
40              spermatogenic and Sertoli cells, rather than disturbance of blood-testis barrier.

41

42      **Keywords**

43      Autoimmune disease, blood-testis barrier, MRL/MpJ-Fas<sup>Lpr</sup> mice, seminiferous tubules, Sertoli cells,  
44      Testis.

45     **Introduction**

46         Recently, auto-immune diseases have gained researchers attention due to their association  
47         with occurrence of infertility in both sexes [1]. Systemic lupus erythematosus (SLE) is a systemic  
48         autoimmune disease in which multiple organs are affected and characterized by chronic activation of  
49         immune cells [2]. Despite the fact that female showed sever lesions than that of male in most of  
50         autoimmune diseases, few reports studied the effect of autoimmune disease on testicular morphology.

51         Autoimmune disease can cause autoimmune orchitis which can be classified to primary  
52         autoimmune orchitis that is completely related to antisperm antibody, and secondary autoimmune  
53         orchitis that is mainly caused by systemic autoimmune disease and 50 % related to antisperm antibody  
54         production besides causing apoptosis and loss of cells specially in SLE [3]. Previous studies showed  
55         sperms abnormality related to Sertoli cell dysfunction and decrease testis volume specially in SLE  
56         human patient [4,5]. However, studies related to morphological changes in the testicular structures  
57         associated with autoimmune diseases were scarce.

58         Currently, some autoimmune mouse models were utilized to elucidate the mechanisms  
59         beyond the progression of autoimmune associated lesions. Among these models, the BXSB/MpJ-Yaa  
60         (BXSB-Yaa) which have a mutant gene located on the Y chromosome accelerating the systemic  
61         autoimmune disease in male [6]. Recently, our team showed some histopathological abnormalities in  
62         the testis of the BXSB-Yaa mice (such as an increased number of both residual bodies and apoptotic  
63         germ cells in stage XII, and decreased number of Sertoli cell), and suggested their role in the  
64         pathogenesis of reproductive dysfunction in such autoimmune disease mice model [7]. However, no  
65         information on the morphological alterations associated with systemic autoimmune abnormality in the

66 testes of the autoimmune mice model used MRL/MpJ-Fas<sup>Lpr</sup> (MRL/Lpr) which is a suitable model for  
67 SLE characterized by the development of a systemic autoimmune disease similar to SLE from 3  
68 months of age in both males and females involving several organs causing immune cells infiltration  
69 and enlargement of different organs specially lymph nodes and spleen [8,9]. The MRL/MpJ mice are  
70 the control strain of MRL/Lpr mice and show mild autoimmune disease in older ages [10]. As age is  
71 a factor that affect autoimmune disease, the incidence of autoimmune disease is expected to increase  
72 with age [11]. Furthermore, the C57BL/6N mice are considered healthy strain and the background  
73 strain of the aforementioned mice [12].

74 Spermatogenesis is a very complicated process that includes 12 stages in mice [13]. It can  
75 be affected by many factors, the alteration in such factors will cause imbalanced spermatogenesis [14].  
76 The spermatogenic epithelium consists of Sertoli cells and spermatogenic germ cells (spermatogonia,  
77 spermatocytes and spermatids), apoptosis of such cells is normal and continuously occurring to keep  
78 a balanced cell number throughout lifetime [15]. Interestingly, the testes of MRL/MpJ mice showed 3  
79 characteristic features including a metaphase-specific apoptosis during the first meiotic division [16],  
80 heat-shock resistant spermatocytes [17] and a postnatal oocyte-like cells [18].

81 The knowledge of how systemic autoimmune disease histologically alter testis is limited [7],  
82 therefore the present study was carried out to elucidate to what morphological extent the autoimmune  
83 disease could alter the testicular structure through comparison between the autoimmune disease mice  
84 model (MRL/Lpr), its control parent mice (MRL/MpJ) and its background mice (C57BL/6N) at 3 and  
85 6 months of age providing insight into the pathogenesis of autoimmune disease associated infertility  
86 in male.

87     **Materials and methods**

88       *Animal*

89           Mice were purchased from Japan SLC, Inc., (Shizuoka, Japan) and kept in the faculty of  
90       veterinary medicine's animal facility until they reached age of 3 months and 6 months, Four mice from  
91       each strain (C57BL/6N, MRL/MpJ, MRL/Lpr ) at age of 3 months and 6 months were used, body  
92       weight was recorded, and then sacrificed under deep anesthesia of a mixture from butorphanol (0.5  
93       mg/kg), midazolam (0.4 mg/kg) and medetomidine (3 mg/kg), according to the animal care and use  
94       of Hokkaido University, Faculty of Veterinary Medicine (approved by the Association for Assessment  
95       and Accreditation of Laboratory Animal Care International, approval No. 16-0124), and organs weight  
96       was recorded.

97

98       *Autoimmune indices measurement*

99           Serum of both MRL/Lpr and MRL/MpJ mice were collected and the double-strand DNA  
100      (dsDNA) levels were analyzed by Enzyme-Linked immunosorbent Assay (ELISA) using autoimmune  
101      disease ELISA kit (Fujifilm, cat no., AKRDD-061, according to manufacture procedures) (Um/mL).  
102      The spleen to body weight ratio was also compared among all studied strains.

103

104       *Sample collection and tissue preparation*

105           Right testes were removed and fixed by paraformaldehyde 4% (for immunohistochemistry  
106      and immunofluorescent staining) while left testes were removed and fixed with Bouin's solution (for  
107      tissue architecture examination and staging of seminiferous tubules). Testes were kept in fixative for  
108      more than 16 hours. Fixed testes were processed in ascending concentration of alcohol followed by  
109      xylene and immersed in paraffin wax then blocks were produced. The blocks were cut into 3 $\mu$ m thick  
110      paraffin sections and stained with hematoxylin-eosin (HE) to identify tissue architecture and periodic

111 acid Schiff combined with hematoxylin (PAS-H) to determine the seminiferous epithelial stage.

112

113 *Immunohistochemistry (IH) and Immunofluorescent (IF) staining*

114 Three µm thick sections were prepared on positively charged slides. Immunofluorescence  
115 (IF) was performed using standard method. IF staining to detect Sertoli cells, and BTB junctional  
116 protein using the following antibodies: GATA-binding protein 4 (GATA4) (Cat. No. sc-25310, Santa  
117 Cruz, California, USA), Zonula occludens-1 (ZO1) (Cat. No. 339100, Invitrogen, California, USA)  
118 respectively. Following deparaffinization the sections were subjected to antigen retrieval heating with  
119 10 mM citrate buffer (pH 6.0) at 105°C for 20 min using autoclave. Then they were washed with  
120 distilled water and incubated with 5% normal donkey serum then the sections were washed with  
121 phosphate buffer saline (PBS) with specific fluorescent for 3 times/5 min each and incubated overnight  
122 with specific primary antibodies at 4°C. The sections were washed in PBS and incubated with specific  
123 fluorescent labelled secondary antibody for 30 mins (Table 1) followed by incubation with Hoechst  
124 33342 solution (Do Jindo, Kumamoto, Japan) at dilution 1:2,000 for 2 mins. They were washed,  
125 mounted and examined under a fluorescent microscope (BZ-X700, All-in-one Fluorescence Keyence,  
126 Osaka, Japan).

127 Immunohistochemical (IH) staining to detect meiotic cells, macrophage, T cells, and B cells  
128 using the following primary antibodies: Synaptonemal complex 3 (SCP3) (ab15093, Abcam,  
129 Cambridge, UK), Iba1 (019-19741, Wako, Osaka, Japan), CD3 (413591F, Nichirei, Tokyo, Japan),  
130 B220 (CL8990A, Cedarlane, Ontario, Canada). Following deparaffinization and rehydration, the  
131 sections were subjected to antigen retrieval using the adequate condition for each antibody (Table1).  
132 Then the sections were soaked in 0.3% H<sub>2</sub>O<sub>2</sub> methanol for 20 mins to block the endogenous peroxidase  
133 activity. They were washed in distilled water and incubated with the 10 % goat serum as blocking

134 serum at room temperature for one hour within humid chamber, followed by overnight incubation with  
135 the specific primary antibody. The sections were washed in PBS (3 times/ 5 mins) and incubated with  
136 the secondary antibodies for 1 hour at room temperature, washed again in PBS and incubated with  
137 streptavidin solution used for 30 minutes, and shortly incubated with 3,3' diaminobenzidine  
138 tetrahydrate H<sub>2</sub>O<sub>2</sub> (DAB) solution followed by rinsing in hematoxylin for 30 seconds and mounting.  
139 Images were taken using microscope (BZ-X700, All-in-one Fluorescence Keyence Microscope, Osaka,  
140 Japan).

141 *Histometric analysis*

142 For histometric analysis, testes from four mice were analyzed (2 sections from each mice)  
143 in all strains at both early (3 months) and late (6 months) age. The HE, PAS-H, and SCP3  
144 immunostained sections were scanned by the nanozoomer scan machine (Hamamatsu photonics Co.,  
145 ltd; Hamamatsu, Japan) and analyzed by nanozoomer digital pathology (NDP) view 2 software. The  
146 HE scanned sections were used to determine the average seminiferous tubule (ST) area, ST lumen  
147 area, ST epithelial area (ST lumen area subtracted from ST epithelial area) (Fig 2j). To determine the  
148 frequency of stages within ST, the ST within PAS stained testicular sections were examined and the  
149 stages were reported according to shape of acrosome and the existence of round spermatid as  
150 previously reported [19]. Furthermore, the numbers of SCP3 positive meiotic cells / ST area were  
151 calculated in nanozoomer scanned SCP3 immuno-stained sections by fixing the number of ST area  
152 from the 2 sections in all studied strains, counting cells in the ST area, and recording their average.  
153 Additionally, digital image from IF stained sections were analyzed by image J software (version 1.32J,  
154 <http://rsb.info.nih.gov/ij>) to detect the GATA4 positive Sertoli cells /ST area, and the positive  
155 ZO-1 cell count. The later were measured by counting the positive cells per ST area [20].

156

157

158     *Statistical analysis*

159         Values are expressed as mean  $\pm$  standard error. Data were analyzed by Kruskal-Wallis test  
160         to compare between the studied group followed by Scheffe's method for multiple comparison when  
161         significant difference among the group were observed ( $P < 0.05$ ). Furthermore, correlation between  
162         two parameters were analyzed using Pearson's correlation test ( $P < 0.05$ ; significant value and  $P < 0.01$ ;  
163         highly significant value).

164

165    **Results**

166    *Alteration in body weight, spleen weight and testis weight in examined strains*

167           The 3 strains showed a variance in the average body weight that's why the relative weight  
168           of organs (organ weight to body weight) (Table 2) was considered. Spleen/body weight ratio showed  
169           a significant increase in MRL/Lpr mice at both 3 and 6 months furthermore, a tendency of increase in  
170           such ratio was observed in MRL/MpJ mice at 6 months. This was confirmed by measuring anti-dsDNA  
171           antibody level in MRL/Lpr and MRL/MpJ sera where a significant increase was observed in MRL/Lpr  
172           mice at 3 months when compared to its control strain (Fig 1a). Also, both strains showed an increased  
173           titer at 6 months than that of 3 months. This result was in accordance with the spleen weight to body  
174           weight ratio (Fig 1b).

175           Testis/body weight ratio showed a significant decrease in both MRL/Lpr and MRL/MpJ  
176           mice at 6 months of age and remained nearly the same in C57BL/6N mice compared to age of 3 months  
177           (Fig 1c). Pearson's correlation test showed a negative correlation between the testis/body weight ratio  
178           and the autoimmune disease indices (serum titer of dsDNA & spleen / body weight ratio) (Table 3).

179

180    *Histological comparison of seminiferous tubules of the examined strains in both ages*

181           Histologically, C57BL/6N mice (at both 3 and 6 months) and MRL/MpJ (at 3 months) mice  
182           showed normal regular seminiferous tubular architecture (Fig 2a,b,c) including normal ST lumina area,  
183           and epithelial area and ST area (Fig 2g-i). The average ST lumina area was 2034.2  $\mu\text{m}$  in C57BL/6N  
184           mice at both 3 months, 6 months and 3797.6  $\mu\text{m}$  in MRL/MpJ mice at 3 months. The average ST area  
185           was 29664.6  $\mu\text{m}$  in C57BL/6N mice at both 3 months, 6 months and 37818.6  $\mu\text{m}$  in MRL/MpJ mice  
186           at 3 months. The average ST epithelial area was 27630.4  $\mu\text{m}$  in C57BL/6N mice at both 3 months, 6  
187           months and 34021.1  $\mu\text{m}$  in MRL/MpJ mice at 3 months. On the other hand, the testes of MRL/MpJ  
188           mice at 6 months and of MRL/Lpr mice at both 3 and 6 months of age showed altered morphological

189 features in their ST (Fig 2d-f). Such featured include the appearance of small vacuoles and/or  
190 spermatogenic cell loss in ST lumen. The STs of MRL/Lpr mice at 6 months of age, showed more  
191 dilation and wider luminal area with more spermatogenic cell loss and vacuolation (Fig 2f) than other  
192 studied groups. The average ST luminal area was 5069.3, 8840.9 and 8023.1  $\mu\text{m}$  in MRL/Lpr mice at  
193 3 months, 6 months and MRL/MpJ mice at 6 months, respectively. The average ST area was 37795.4,  
194 44670.2 and 44662.5  $\mu\text{m}$  in MRL/Lpr mice at 3 months, 6 months and MRL/MpJ mice at 6 months,  
195 respectively. The average ST epithelial area was 32726.2, 35829.3 and 36639.5  $\mu\text{m}$  in MRL/Lpr mice  
196 at 3 months, 6 months and MRL/MpJ mice at 6 months, respectively. Furthermore, the STs of  
197 MRL/Lpr mice at 6 months of age showed significant higher values than that of C57BL/6N mice at  
198 both 3 months, 6 months and both MRL/MpJ and MRL/Lpr mice at 3 months.

199

200 *Stage frequency in all studied strains*

201 Staging of PAS stained STs was done to identify the frequency of every stage (Fig 3). Among  
202 all studied strains at both ages (Fig 3). The STs in all studied strains at 3 months as well as in  
203 C57BL/6N mice at 6 months showed high frequency for stages VII. However, a higher frequency for  
204 stage XII was observed in MRL/Lpr mice at 6 months and both stage III and XII in MRL/MpJ mice  
205 at 6 months.

206

207 *Immunostaining of Junctional protein, Sertoli, and meiotic cells*

208 To determine what kind of cell loss is associated with autoimmunity, counting of cells was  
209 carried out after performing both IF and IHC staining of different cell markers (SCP3 for meiotic cells,  
210 GATA4 for Sertoli cells) (Fig 4,5). The morphometrical measurement showed a significant decrease  
211 in SCP3 positive cells in all studied strains (Fig 4g). In addition, the Sertoli cells count in both  
212 MRL/Lpr and MRL/MpJ mice testis at 6 months showed a significant decrease than that of C57BL/6N

213 mice Furthermore, the C57BL/6N mice at 6 months showed a significant increase in the Sertoli cell  
214 count than that of 3 months (Fig 5h).

215

216 *Immune cells infiltration and ZO-1 junction protein*

217 To examine effect of autoimmunity on immune cell infiltration in testis and BTB,  
218 immunohistochemical staining was performed to detect both immune cells (CD3 for T cells, B220 for  
219 B cells, Iba1 for macrophages) and ZO-1 junctional protein as a major junctional protein for BTB. The  
220 ZO1 junctional protein appeared as whitish precipitates at the basal compartment of STs in sites of  
221 tight junction of BTB (Fig 5 a-f), it was counted in area of ST as well as in relation to Sertoli cells.  
222 Interestingly, a non-significant decrease in ZO-1<sup>+</sup>cells/ST area was observed in testes of both  
223 MRL/MpJ and MRL/Lpr mice at 6 months than other studied groups (Fig 5g). Furthermore, in all  
224 studied strains our results revealed the presence of few CD3<sup>+</sup> T lymphocytes, B220<sup>+</sup> B lymphocytes  
225 and Iba1 macrophages in the interstitial tissue but there was no infiltration in the STs (Fig 6).

226 Furthermore, Pearson's correlation analysis revealed positive correlation between  
227 testis/body weight ratio and both Sertoli cell count and SCP3 positive meiotic cell count (Table 4).  
228 However, a negative correlation was observed between testis/body weight ratio and other  
229 morphometric measurement including ST area, ST lumen area, ST epithelial area, and ZO-1 positive  
230 cells /GATA4 positive cells.

231     **Discussion and conclusion**

232         In human, SLE is one of the autoimmune diseases that have great impact on the occurrence  
233         of infertility in both sexes. An abnormal semen was reported in human patient with SLE and owed that  
234         to Sertoli cells dysfunction due to decreased inhibin secretion from Sertoli cells [5]. Previous studies  
235         demonstrated a decrease in the testicular volume in SLE patients related to semen abnormalities and  
236         suggesting ST sever lesions [21]. Other reports suggested that the effect of autoimmunity on fertility  
237         could be due to disruption of immune privilege related to T cells reaction toward certain antigens [22].  
238         However, the mechanism beyond the effect of autoimmunity on testicular integrity is not yet elucidated.  
239         Therefore, we examined the morphology of testes from MRL/Lpr mice that showed autoimmune  
240         disease at both early and late age as an attempt to investigate such mechanism. We also investigated  
241         the testes from MRL/MpJ mice that is known to develop an autoimmune disease at a later onset than  
242         MRL/Lpr mice [9] and compared with the testes of their healthy background strain (C57BL/6N mice)  
243         at both early (3 months) and late (6 months) age. The present investigation revealed higher  
244         autoimmune indices in MRL/Lpr mice at both ages with a significant increase in such indices in  
245         MRL/MpJ mice at 6 months, however, no difference in C57BL/6N mice at both ages. Therefore our  
246         results indicate that MRL/Lpr mice developed an autoimmune disease in both early and late age, but  
247         a mild autoimmune disease could be observed in MRL/MpJ mice in late age, however, no development  
248         of autoimmunity in C57BL/6N mice at both ages.

249         In parallel with the findings of Otsuka et, al. concerning the decrease in MRL/MpJ testis  
250         weight due to apoptosis [23], our investigation revealed high frequency of stage XI and XII within the  
251         ST of both MRL/Lpr and MRL/MpJ specially at 6 months that indicate apoptosis. Our report of a high  
252         frequency for stage VII in the ST of C57BL/6N mice in both ages could indicate normal fertility.  
253         Additionally, the present study elucidated that a significant decrease in testes weight associated with  
254         aging in both MRL/Lpr and MRL/MpJ mice as compared to C57BL/6N mice. Our result clarified that

255 such decrease in testes weight could be due to hollowness of the dilated ST lumen with germ cell loss  
256 and epithelial vacuolation. Similar results were also reported in passive experimental autoimmune  
257 orchitis [22].

258 Interestingly, SCP3 is considered a marker for meiotic cells (primary spermatocyte) [24],  
259 our study revealed a significant decrease in SCP3 positive cells in all studied strains in association  
260 with aging. Previous study showed that the decrease in meiotic cells was contributed to their apoptosis  
261 [25].

262 Our study revealed a significant decrease in GATA4 positive Sertoli cells in both MRL/Lpr  
263 and MRL/MpJ especially at 6 months but an increased value was detected in C57BL/6N mice. Such  
264 observation could be due to the effect of autoimmune disease in MRL/Lpr and MRL/MpJ but a normal  
265 age-related change in C57BL/6N mice. This was in parallel with previous report [26]. Furthermore, it  
266 has been revealed that the deletion of GATA4 leads to testicular atrophy and associated with  
267 development of infertility [27]. Interestingly, previous reports have been clarified the role of Sertoli  
268 cells in controlling the number of spermatogenic cells as well as the number of produced sperms [28,  
269 29, 30]. Based on the aforementioned findings, we could suggest that the decrease in meiotic germ  
270 cells number in the current investigation is mainly due to the decrease in Sertoli cells number.  
271 Moreover, the loss of such germ cell could be attributed to apoptosis which is a main feature related  
272 to autoimmune orchitis [31].

273 The Fas system (Fas and FasL) has been reported to be involved in the induction of germ  
274 cell apoptosis [32, 33]. Furthermore, it has been recorded that there was an increase in metaphase  
275 specific apoptotic cells in MRL/MpJ mice testis in comparison to C57BL/6N mice testis in stage XII  
276 [16]. Similarly, our results revealed increased frequency of stage XII in both MRL/Lpr and MRL/MpJ  
277 mice testis at 6 months suggesting an increased apoptosis in such strains.

278 Additionally, in order to examine the involvement of immune cells infiltration and the

279 disturbance in BTB with the morphological alteration of testes of studied strains, we examined the  
280 infiltration of various immune cells (B, T lymphocytes and macrophages) as well as the disturbance  
281 in BTB junctional protein ZO-1 expression which is considered as a major component of BTB tight  
282 junctional protein. Our result revealed absence of immune cells infiltration in STs in all studied strains  
283 which is in accordance with previous report [34]. However, a non-significant decrease in ZO-1 protein  
284 expression in STs of MRL/MpJ and MRL/Lpr mice especially with old age. Previous report revealed  
285 a disturbance of BTB as a result of destruction of ZO-1 protein [35].

286 In conclusion, the testis of autoimmune disease mice model MRL/Lpr and its parent strain  
287 MRL/MpJ mice showed a decreased weight and an altered histological feature as compared to their  
288 background strain C57BL/6N especially at 6 months of age at which the autoimmune disease  
289 progressed including dilated ST with hollowness (wide lumen) and vacuolated epithelium suggested  
290 that the effect of autoimmune disease could be through loss of germ cells through apoptosis rather  
291 than their effect on immune cells infiltration or BTB disturbance.

## 292 **Acknowledgments and funding**

293 This research was supported by Egypt-Japan Education Partnership (EJEP) joint supervision  
294 scholarship.

## 295 **Declaration of Interest**

296 The present manuscript was approved by all authors to be submitted to Experimental animal journal  
297 and was not submitted to other journal, so we declare that there are no conflicts of interest.

## 298 **Abbreviations**

299 **SLE** systemic lupus erythematosus

300 **Fas L** Fas Ligand

301 **HE** hematoxylin-eosin HE

302 **PAS-H** periodic acid Schiff combined with hematoxylin

303 **dsDNA** double stranded DNA

304     **ST** seminiferous tubules

305     **IH** immunohistochemistry

306     **IF** immunofluorescent

307     **GATA4** GATA -Binding Protein 4

308     **SCP3** Synaptonemal complex protein 3

309     **ZO-1** Zonula occludens-1

310     **BTB** blood-testis barrier

311     **References**

- 312       1. Carp HJA. Selmi C, Shoenfeld Y. The autoimmune bases of infertility and pregnancy loss.  
313              Journal of Autoimmunity 2012; 38(2–3), J266-J274.
- 314       2. Ray S, Sonthalia N, Kundu S, et al. Autoimmune Disorders: An Overview of Molecular and  
315              Cellular Basis in Today's Perspective. J Clin Cell Immuno. 2012; 2215-2230.
- 316       3. Silva CA, Coccuzza M, Carvahlo JF, et al. Diagnosis and classification of autoimmune orchitis.  
317              Autoimmunity Rev. 2014;13: 431–434
- 318       4. Soares PMF, Borba EF, Bonfa E, et al. Gonad Evaluation in Male Systemic Lupus Erythematosus.  
319              Arthritis and rheumat. 2007; 56(7): 2352–2361.
- 320       5. Suehiro RM, Borba EF, Bonfa E, et al. Testicular Sertoli cell function in male systemic lupus  
321              erythematosus. Rheumatol. 2008; 47:1692–1697.
- 322       6. Izui S, Merino R, Fossati L, Iwamoto M. The role of the Yaa gene in lupus syndrome. Int Rev  
323              Immunol. 1994;11(3):211-230.
- 324       7. Otani Y, Ichii O, Masum MA, et al. BXSB/MpJ-Yaa mouse model of systemic autoimmune  
325              disease shows increased apoptotic germ cells in stage XII of the seminiferous epithelial cycle.  
326              Cell and Tissue Research. 2020;10.1007/s0041-020-03190-0.
- 327       8. Perry D, Sang A, Yin Y, et al. Review Article Murine Models of Systemic Lupus Erythematosus.

- 328 J Biomed Biotechnol. 2010; 2011: 271694.
- 329 9. Hewicker M, Kromschroder E, Trautwein G. Detection of circulating immune complexes in
- 330 MRL mice with different forms of glomerulonephritis. Z Versuchstierkd. 1990;33(4):149-56.
- 331 10. Hang L, Aguado MT, Dixon FJ, et al. Induction of severe autoimmune disease in normal mice
- 332 by simultaneous action of multiple immunostimulators. J Exper Med. 1985; 161: 423-428.
- 333 11. Goronzy JJ, Weyand CM. Immune aging and autoimmunity. Cell. Mol. Life Sci. 2012; 69:1615–
- 334 1623.
- 335 12. Kalley VE, Roths JB. Interaction of mutant lpr gene with background strain influences renal
- 336 disease. Clin immnopathol. 1985;37(2):220-229.
- 337 13. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in
- 338 the rat. Ann N Y Acad Sci. 1952; 55: 548-573.
- 339 14. de Kretser DM, Loveland KL, Meinhhardt A, et al. Spermatogenesis. Hum Reproduct. 1998;
- 340 13(□□ □ □)
- 341 15. Moreno RD, Reyes JG, Farias JG, et al. Spermatogenesis at the extreme: oxidative stress as a
- 342 converging mechanism of testicular damage due to pathological and environmental exposure.
- 343 Testis: Anat Physiol Pathol. 2012; chapter 1.
- 344 16. Kon Y, Endoh D. Morphological study of metaphase-specific apoptosis in MRL mouse
- 345 testis. Anat Histol Embryol. 2000;29(5):313-319.
- 346 17. Namiki Y, Kon Y, Kazusa K, et al. Quantitative trait loci analysis of heat stress resistance of
- 347 spermatocytes in the MRL/MpJ mouse. Mamm Genome. 2005;16(2):96-102.
- 348 18. Otsuka S, Konno A, Hashimoto Y, et al. Oocytes in newborn MRL mouse testes. Biol Reprod.
- 349 2008;79(1):9-16.
- 350 19. Ahmed EA, de Rooij DG. Staging of Mouse Seminiferous Tubule Cross-Sections. Meiosis,
- 351 Volume 2: Cytological Meth. 2009; 558.

- 352 20. Tripathi UK, Chhillar S, Kumaresan A, et al. Morphometric evaluation of seminiferous tubule  
353 and proportionate numerical analysis of Sertoli and spermatogenic cells indicate differences  
354 between crossbred and purebred bulls. *Vet. World.* 2015; EISSN: 2231-0916.
- 355 21. da Silva CAA, Bonfá E, EF Borba, et al. Reproductive health in male systemic lupus  
356 erythematosus. *Bras J Rheumatol.* 2009; 49(3):207-227.
- 357 22. Ihng KSK, Teuscher C. Mechanisms of autoimmune disease in the testis and ovary. *Human*  
358 *Reproduc Update.* 1995; 1(1) 35-50.
- 359 23. Otsuka S, Namiki Y, Ichii, O et al. Analysis of factors decreasing testis weight in MRL mice,  
360 *Mamm Genome.* 2010; 21(3-4): 153-161.
- 361 24. Heyting C. Synaptonemal Complexes: Structure and function. *Curr. Opin. Cell Biol.* 1996; 8:  
362 389-396.
- 363 25. Yuan L, Liu J-G, Zhao J, et al. The Murine SCP3 Gene Is Required for Synaptonemal Complex  
364 Assembly, Chromosome Synapsis, and Male Fertility. *Molecular Cell.* 2000; 5: 73–83.
- 365 26. Takano H, Kazuhiro ABE. Age-related histologic changes in the adult mouse testis. *Arch histol*  
366 *jap.* 1987; 50(5): 533-544.
- 367 27. Kyrölähti A, Euler R, Bielinska M, et al. GATA4 regulates Sertoli cell function and fertility in  
368 adult male mice. *Molecul Cellul Endocrinol.* 2011; 333: 85-95.
- 369 28. Johnson L, Thompson JrDL, Varner DD. Role of Sertoli cell number and function on regulation  
370 of spermatogenesis. *Anim Reprod Sci.* 2008; 105:23–51.
- 371 29. Johnson L, Zane RS, Petty CS, et al. Quantification of the human Sertoli cell population: Its  
372 distribution, relation to germ cell numbers, and age-related decline. *Biol Reprod.* 1984. 31:758–  
373 795.
- 374 30. Sharpe RM, McKinnell C, Kivlin C, et al. Proliferation and functional maturation of Sertoli  
375 cells, and their relevance to disorders of testis function in adulthood. *Reprod.* 2003; 125:769–

- 376 784.
- 377 31. Jacobo P, Guazzone VA, Theas MS, et al. Testicular autoimmunity. Autoimmun Rev. 2011; 10:
- 378 201–204.
- 379 32. Riccioli A, Salvati L, D'Alessio A, et al. The Fas system in the seminiferous epithelium and its
- 380 possible extra-testicular role. Andrologia, 2003; 35: 64–70.
- 381 33. lee J, Richburg JH, Younkin SC, et al. The Fas system is a key regulator of germ cell apoptosis
- 382 in the testis. Endoc Soc. 1997; 138: 5.
- 383 34. Fanning AS, Jameson BJ, Jesaitis LA, et al. The tight junction protein ZO-1 establishes a link
- 384 between the transmembrane protein occludin and the actin cytoskeleton. J Biol Chem. 1998; 273:
- 385 29745–29753.
- 386 35. Wittchen ES, Haskins J, Stevenson BR. Protein interactions at the tight junction. Actin has
- 387 multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. J Biol
- 388 Chem. 1999; 274: 35179–35185.

389 **Figure legends**

390 **Figure 1:** Charts showing spleen weight to body weight ratio with a significant increase in MRL/Lpr

391 mice compared to MRL/MpJ and C57BL/6N mice (a). A significant increase in dsDNA antibody level

392 in MRL/Lpr mice compared to MRL/MpJ mice (b) and testis weight to body weight ratio with

393 significant decrease in both MRL/MpJ and MRL/Lpr mice compared to C57BL/6N mice (c). Data

394 was analyzed by Kruskal-Wallis test followed by Scheffe's method ( $P <0.05$ ),  $n=4$  in each

395 experimental group. The letters a, b, c, d, e and f significant difference between groups (a=C57BL/6N

396 3m, b=MRL/MpJ 3m, c=MRL/Lpr 3m, d=C57BL/6N 6m, e=MRL/MpJ 6m, f=MRL/Lpr 6m). Values

397 are given as mean  $\pm$ SE.

398 **Figure 2:** Photomicrograph of H&E staining of Bouins fixed C57BL/6N ST at 3 months (a) and 6

399 months (b) showing a regular ST architecture, MRL/MpJ ST at 3 months (c) and 6 months (d) showing

400 a regular tubules that altered to wide lumen dilated ST with vacuolation (arrow heads) at 6 months of  
401 age, and MRL/Lpr ST at 3 months (e) some showed regular tubules while other showed loss of cells  
402 (arrows) which gathered in lumen and beginning of epithelial vacuolation (arrow heads) and 6 months  
403 (f) showed wide lumen dilated ST with vacuolated epithelium (arrow heads), the chart (g,h,i) shows  
404 the average ratio of luminal, tubular and epithelial area in all studied strains and j shows the area  
405 measured for lumen and ST. Data was analyzed by Kruskal-Wallis test followed by Scheffe's method  
406 ( $P < 0.05$ ),  $n=4$  in each experimental group. The letters a, b, c, d, e and f significant difference between  
407 groups (a=C57BL/6N 3m, b=MRL/MpJ 3m, c=MRL/Lpr 3m, d=C57BL/6N 6m, e=MRL/MpJ 6m,  
408 f=MRL/Lpr 6m). Values are given as mean  $\pm$ SE. scale bars=100 $\mu$ m.

409 **Figure 3:** Photomicrograph of PAS staining of Bouins fixed C57BL/6N ST at 3 months (a) and 6  
410 months (b), MRL/MpJ ST at 3 months (c) and 6 months (d), and MRL/Lpr ST at 3 months (e) and 6  
411 months (f), while charts (g) and (h) show frequency of stage occurrence in all strains in which all  
412 strains showed highest occurrence of stage 7 at 3 months of age, while at age of 6 months C57BL/6N  
413 showed frequent occurrence of stage 7, MRL/MpJ mice showed frequent occurrence of stage 3 and  
414 stage 12 and MRL/Lpr mice showed frequent occurrence of stage ). Data was analyzed by Kruskal-  
415 Wallis test followed by Scheffe's method ( $P < 0.05$ ),  $n=4$  in each experimental group. The letters a, b,  
416 c, d, e and f significant difference between groups (a=C57BL/6N 3m, b=MRL/MpJ 3m, c=MRL/Lpr  
417 3m, d=C57BL/6N 6m, e=MRL/MpJ 6m, f=MRL/Lpr 6m). Values are given as mean  $\pm$ SE. scale  
418 bars=100 $\mu$ m.

419 **Figure 4:** NDP Photomicrograph of immunohistochemistry staining against SCP3 of C57BL/6N ST  
420 at 3 months (a) and 6 months (b), MRL/MpJ ST at 3 months (c) and 6 months (d), and MRL/Lpr ST  
421 at 3 months (e) and 6 months (f), the positive reaction was observed as brown reaction in the nucleus  
422 of spermatocytes (arrow heads), while chart (g) shows counting of +ve cells/area in all studied strains.  
423 Data was analyzed by Kruskal-Wallis test followed by Scheffe's method ( $P < 0.05$ ),  $n=4$  in each

424 experimental group. The letters a, b, c, d, e and f significant difference between groups (a=C57BL/6N  
425 3m, b=MRL/MpJ 3m, c=MRL/Lpr 3m, d=C57BL/6N 6m, e=MRL/MpJ 6m, f=MRL/Lpr 6m). Values  
426 are given as mean  $\pm$ SE. scale bars=25 $\mu$ m.

427 **Figure 5:** Photomicrograph of immunofluorescence staining of ZO-1 (white), GATA4 (red) and  
428 Hoechst (blue) in C57BL/6N ST at 3 months (a) and 6 months (b), MRL/MpJ ST at 3 months (c) and  
429 6 months (d), and MRL/Lpr ST at 3 months (e) and 6 months (f), while chart (g) shows counting of  
430 ZO-1 positive cells in ST area in all studied strains. Data was analyzed by Kruskal-Wallis test followed  
431 by Scheffe's method ( $P < 0.05$ ), n=4 in each experimental group. The letters a, b, c, d, e and f significant  
432 difference between groups (a=C57BL/6N 3m, b=MRL/MpJ 3m, c=MRL/Lpr 3m, d=C57BL/6N 6m,  
433 e=MRL/MpJ 6m, f=MRL/Lpr 6m). Values are given as mean  $\pm$ SE. scale bars=100 $\mu$ m for low  
434 magnification and 30 $\mu$ m for magnified inset.

435 **Figure 6:** NDP photomicrograph of immunohistochemical staining of MRL/Lpr mice STs for CD3,  
436 B220 and Iba1 at 6 months of age showing no infiltration of such cells in the STs.

437 **Tables**

438 **Table 1:** Antibodies used in immunostaining, expressing cells, dilution, antigen retrieval method,  
439 secondary antibody, blocking serum and method.

Primary antibody	Target cell	Dilution	Antigen retrieval method	Secondary antibody
GATA4 (goat)	Sertoli cells and interstitial cells besides nonspecific reaction in acrosome	1:100	10 mM CB (pH 6.0) 105 °C for 20 minutes	anti-goat-IgG (produced in donkey) Alexa Fluor 546, Santa Cruz, USA
SCP3 (rabbit)	Meiotic cell	1:750	10 mM CB (pH 6.0) 105 °C for 20 minutes	anti-Rabbit IgG (produced in goat) biotin labelled (H1903,Histofine)
ZO-1 (rabbit)	BTB junctional protein (Sertoli cell)	1:500	10 mM CB (pH 6.0) 105 °C for 20 minutes	anti-rabbit-IgG (produced in donkey) Alexa Fluor 647, Santa Cruz, USA

Ibal (rabbit)	Macrophage	1:200	0.3 % Pepsin 37 °C for 5 minutes	anti-Rabbit IgG (produced in goat) biotin labelled (H1903,Histofine)
CD3 (rabbit)	T lymphocytes	1:100	10 mM CB (pH 6.0) 105 °C for 20 minutes	anti-Rabbit IgG (produced in goat) biotin labelled (H1903,Histofine)
B220 (Mouse)	B lymphocytes	1:1600	0.3 % Pepsin 37 °C for 5 minutes	anti-mouse IgG (produced in goat) biotin labelled (H1605, Histofine)

440

441 **Table 2:** Shows the average body weight, average spleen weight/body weight, and average testis

442 weight/body weight in all studied strains.

	Average (gm)	Body weight	Average /body weight		Average Testis weight/body weight	
	3 M	6 M	3 M	6 M	3 M	6 M
C57BL/6N	24.9425	29.6875	0.003141	0.003129	0.00705	0.00701
MRL/MpJ	45.8345	46.244	0.001977	0.002225	0.004409	0.004127
MRL/Lpr	45.01225	52.64625	0.005943	0.015105	0.003828	0.003569

443

444 **Table 3:** Pearson correlation between autoimmune indices (spleen weight/Body weight and dsDNA

445 antibody level) and testis weight to body weight ratio.

	Testis weight/Body weight	
Spleen weight/Body weight	ρ	-0.528
	P	0.078
dsDNA antibody level	ρ	-.644*
	P	0.024

446 \*Significant, P <0.05.

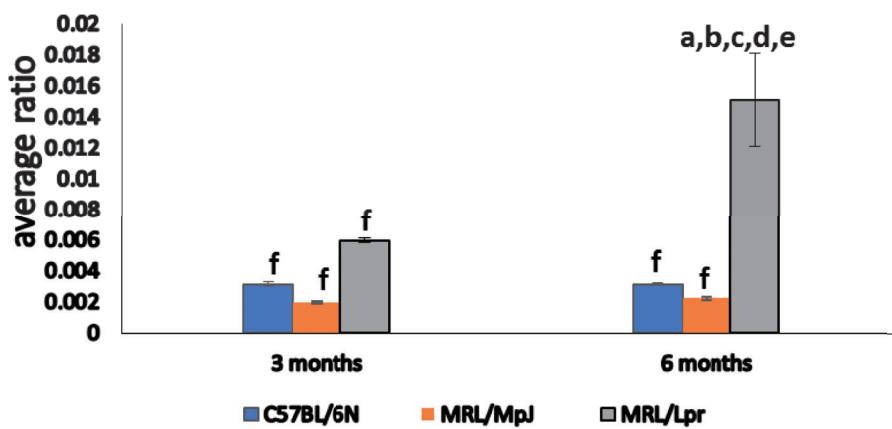
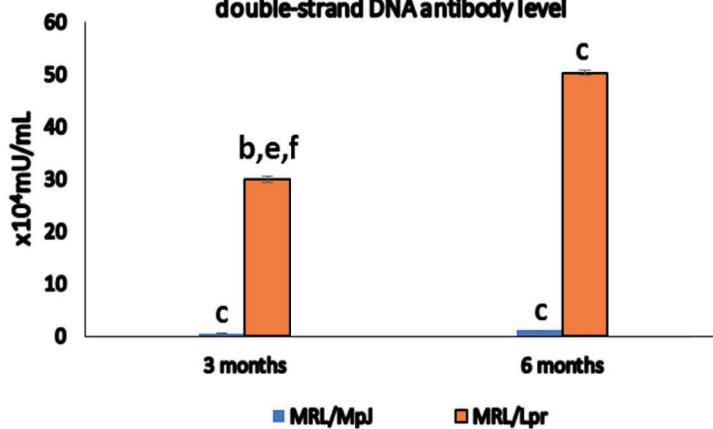
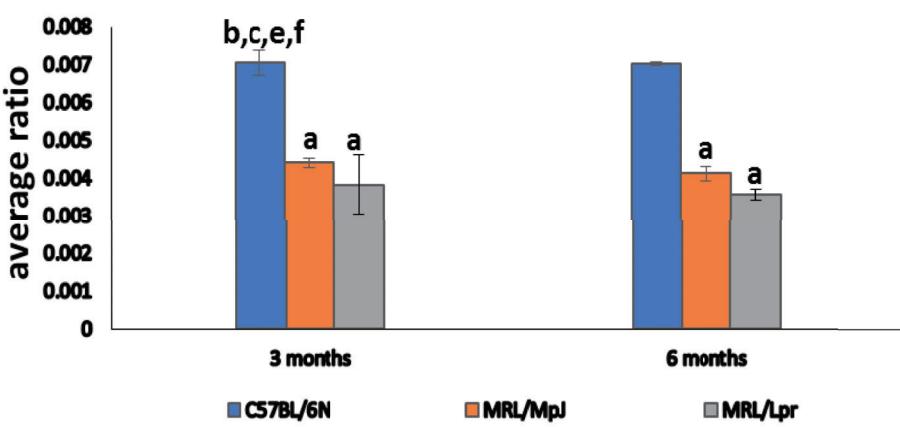
447

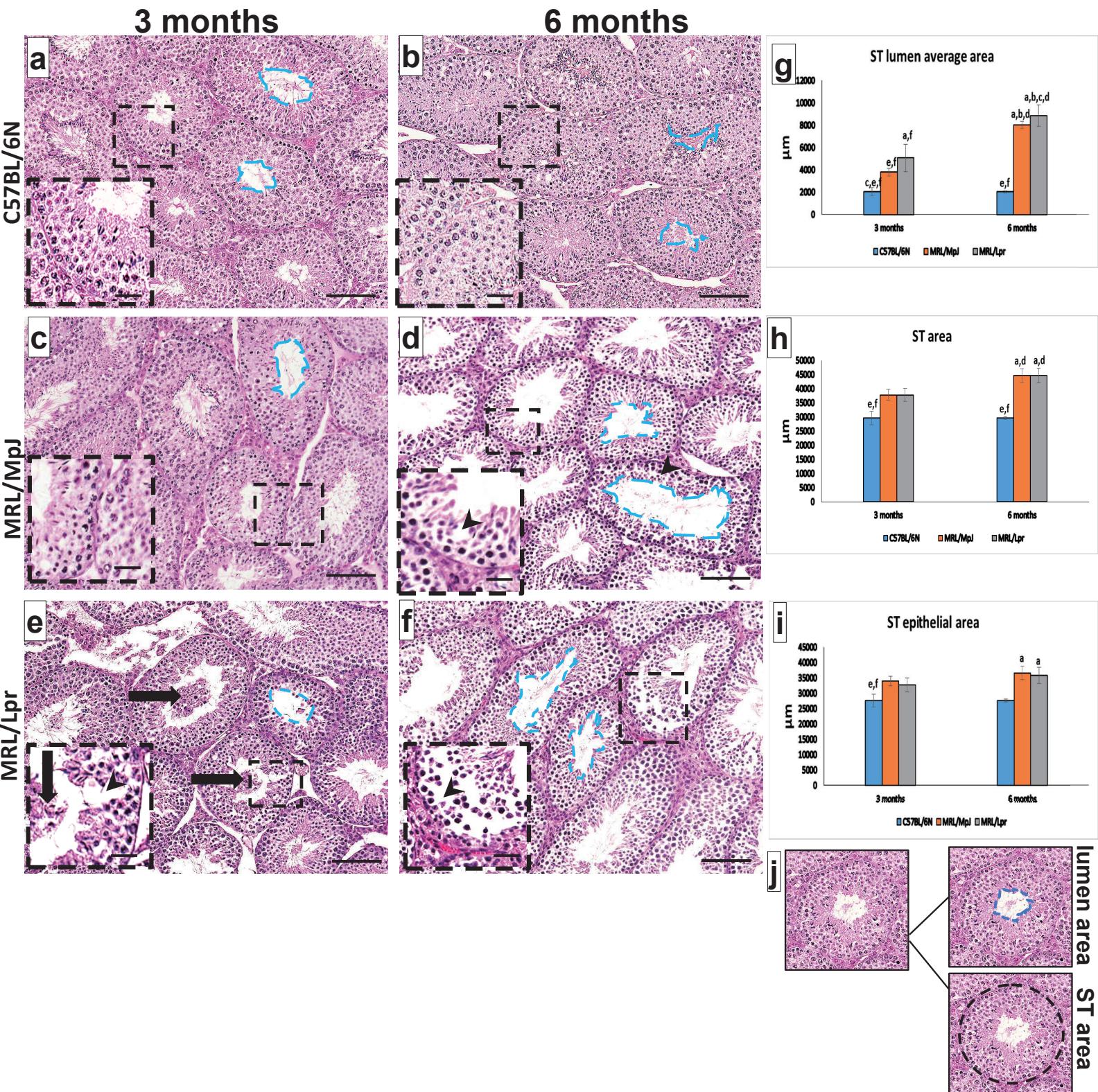
448

449      **Table 4:** Pearson correlation between testis weight to body weight ratio (Testis/Body weight) and  
450      other morphometric measurement.

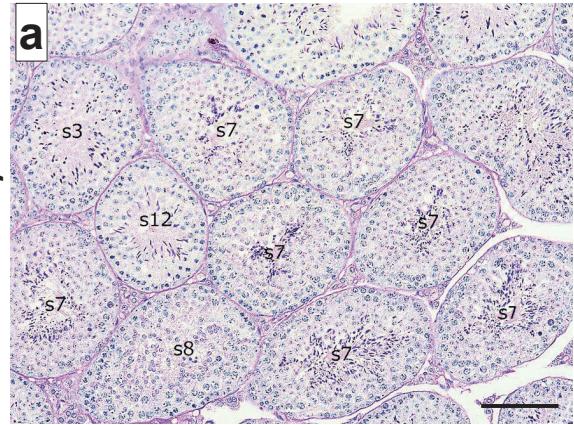
	ST luminal area	ST area	ST epithelial area	SCP3 <sup>+</sup> cells/area	StageXII	Zo-1/GATA4	GATA4/area (Sertoli cells)
Testis / Body weight	$\rho$	-.839**	-.837**	-.722**	0.033	-.608**	-.0463
	p	0.001	0.001	0.001	0.895	0.007	0.053

451      \*Significant, P <0.05 while \*\* highly significant, P <0.01.

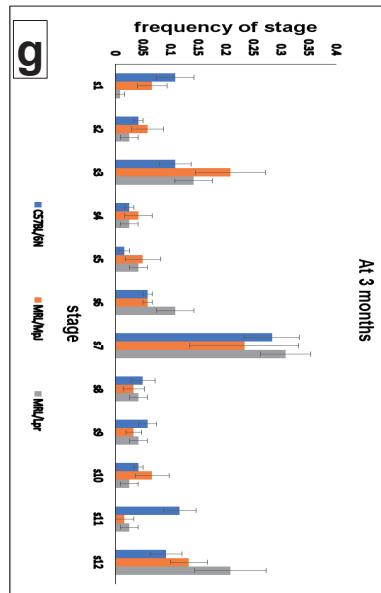
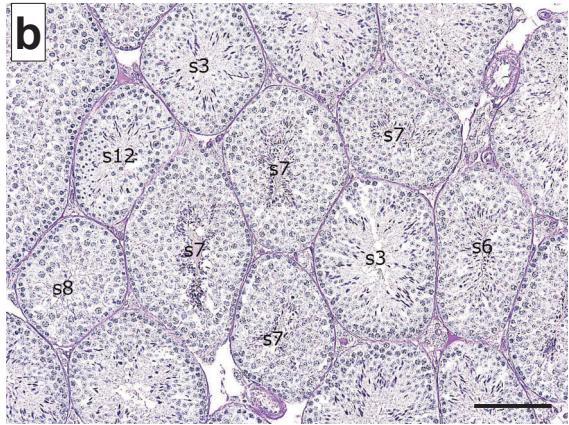
**a****Spleen weight/ Body weight****b****double-strand DNA antibody level****c****Testis weight/ Bodyweight**



### 3 months



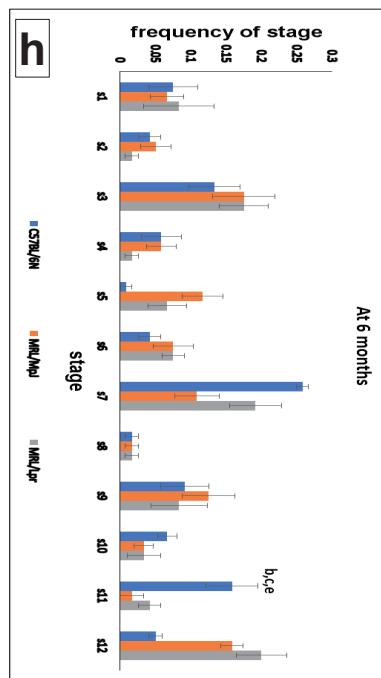
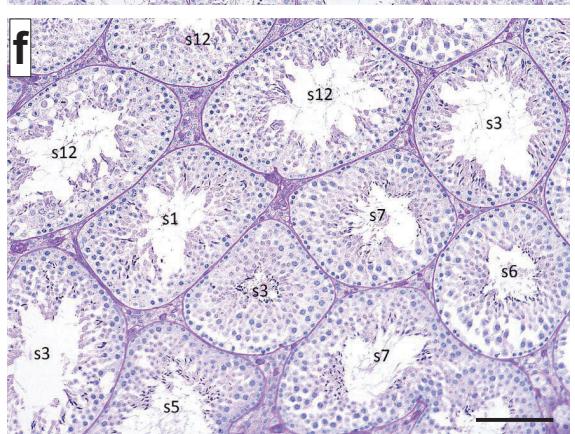
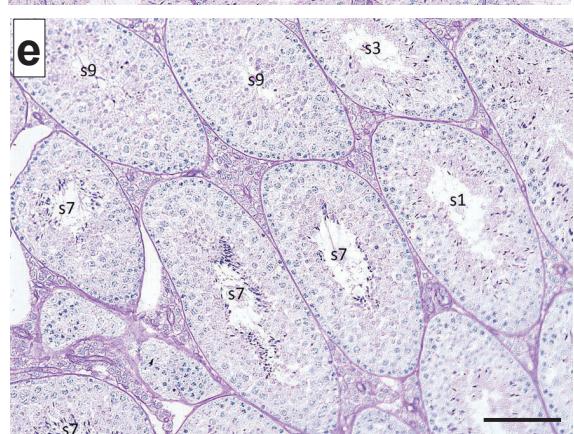
### 6 months

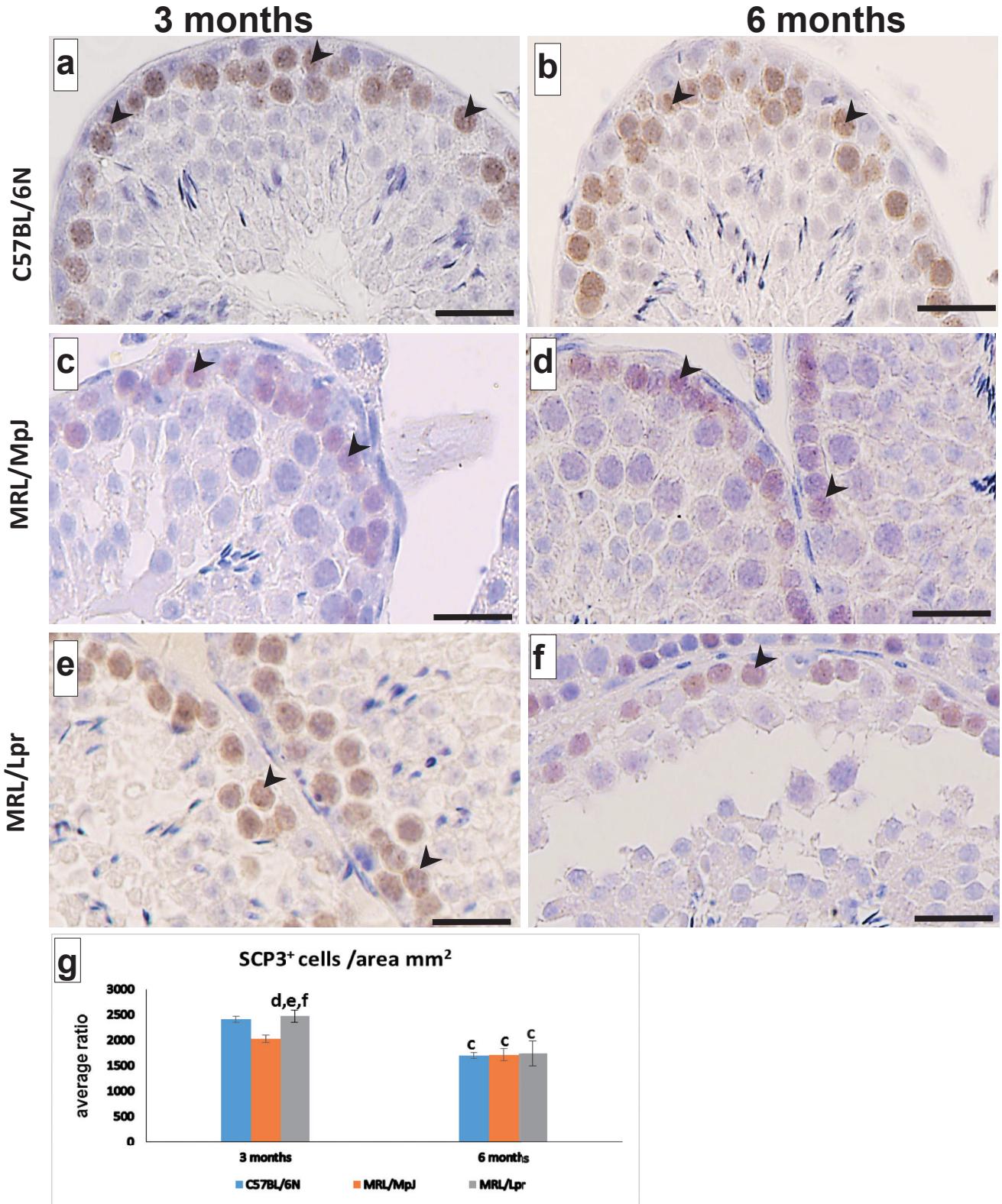


N

MRL/MpJ

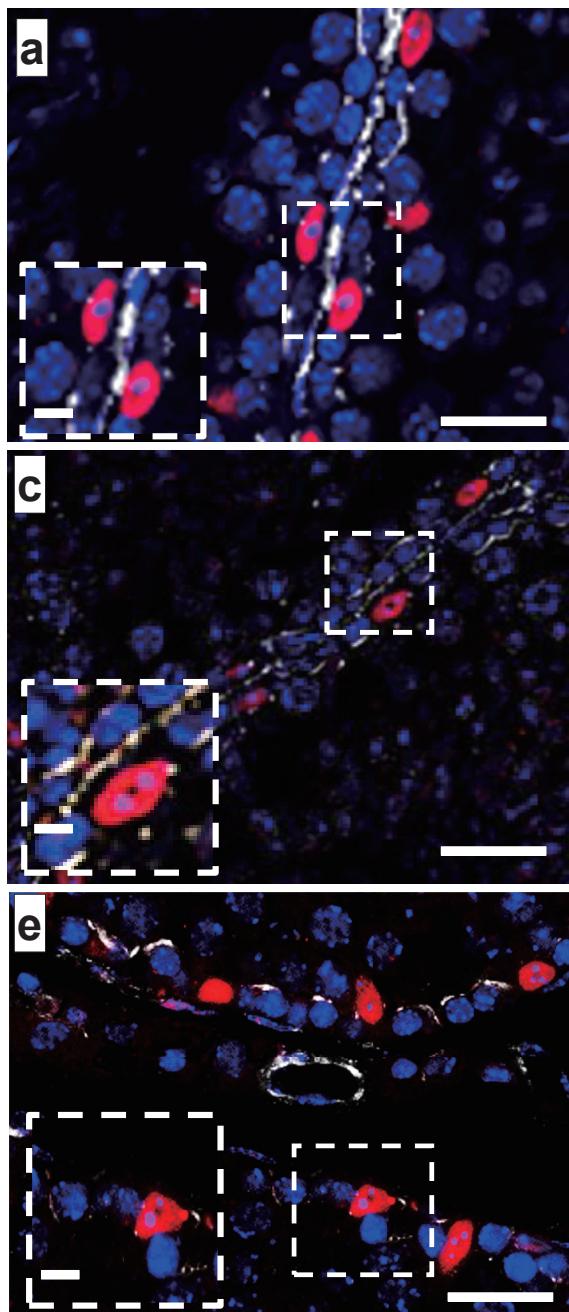
MRL/Lpr



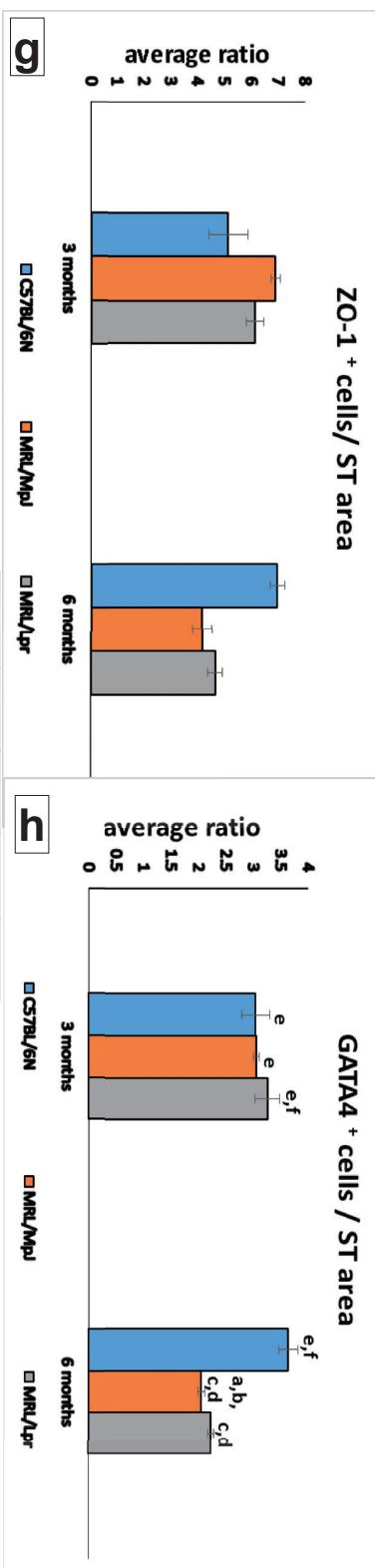
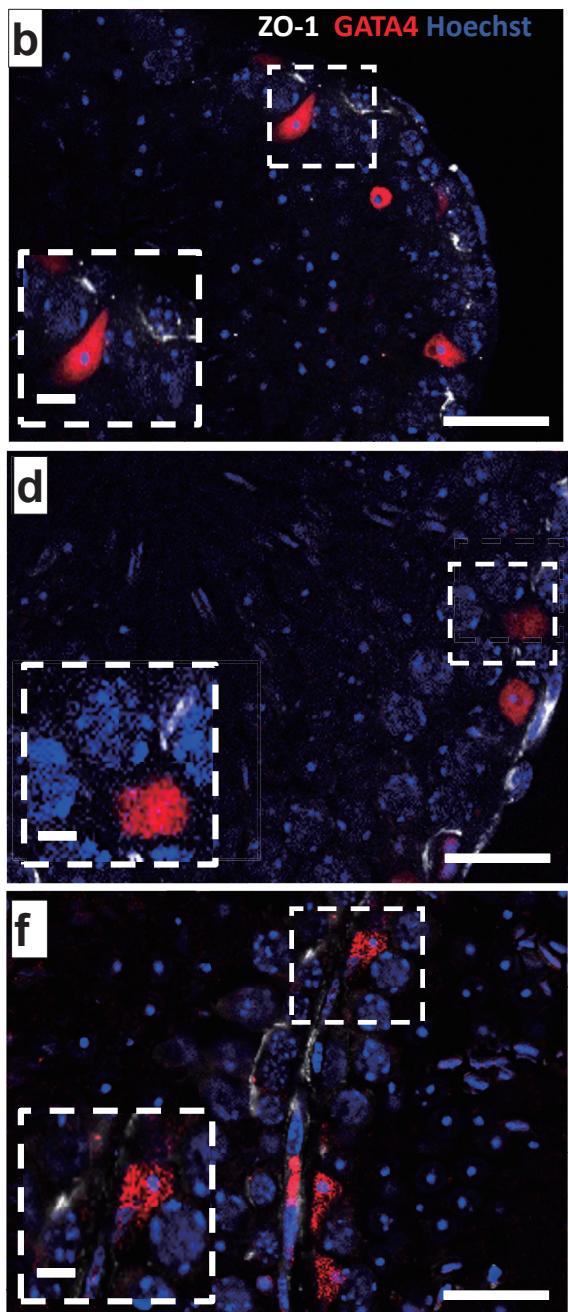


C57BL/6N  
MRL/Mpj  
MRL/Lpr

3 months

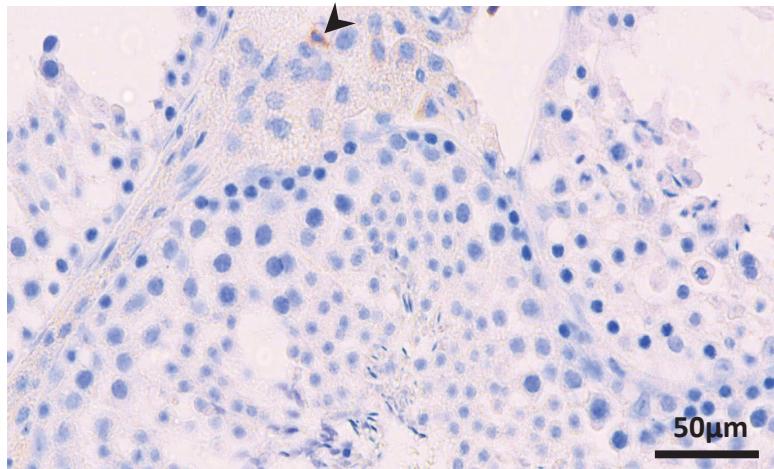


6 months

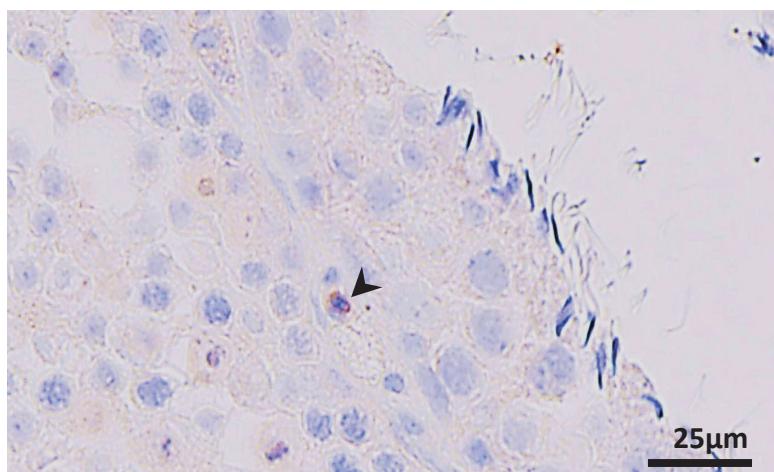


## MRL/Lpr 6 months

CD3



B220



Iba1

