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1 Comparative analysis of mediastinal fat-associated lymphoid cluster development and
2 lung cellular infiltration in murine autoimmune disease models and the corresponding
3 normal control strains

4 Short title: Mediastinal lymphoid clusters in murine autoimmune disease models

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16 **Key words:** autoimmune disease model, lymphoid cluster, mediastinal adipose tissue

17
18 Abbreviations: HE, hematoxylin and eosin; LCs, lymphoid clusters; LTi, lymphoid tissue inducer;
19 MFALCs, mediastinal fat-associated lymphoid clusters; MFT, mediastinal fat tissue; NK, natural
20 killer; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus

23 **Summary**

24 We previously discovered mediastinal fat-associated lymphoid clusters (MFALCs) as novel
25 lymphoid clusters (LCs) associated with mediastinal fat tissue (MFT) in healthy mice. However, no
26 data about their morphology in immune-associated disease conditions, and their relationship with
27 lung infiltration, is available to date. In the present study, we compared the morphological features of
28 MFALCs in 4-month-old male murine autoimmune disease models (MRL/MpJ-*lpr* mice and
29 BXSB/MpJ-*Yaa* mice) with those of the corresponding control strains (MRL/MpJ and BXSB/MpJ,
30 respectively). In addition, we analyzed their correlation with lung infiltration. Furthermore,
31 immunohistochemistry for CD3, B220, Iba1, Gr1, and BrdU was performed to detect T- and B-cells,
32 macrophages, granulocytes, and proliferating cells, respectively. The spleen/body weight ratios and
33 anti-dsDNA autoantibody titers were found to be significantly higher in the autoimmune models than
34 in the control strains. Furthermore, the autoimmune model presented prominent MFALCs, with a
35 significantly greater ratio of LC area to total MFT area, and more apparent diffused cellular
36 infiltration into the lung lobes than the other studied strains. Higher numbers of T- and B-cells,
37 macrophages, and proliferating cells, but fewer granulocytes, were observed in the autoimmune
38 models than in the control strains. Interestingly, a significant positive Pearson's correlation between
39 the size of the MFALCs and the density of CD3-, B220-, and Iba1-positive cells in the lung was
40 observed. Therefore, our data suggest a potentially important role for MFALCs in the progression of
41 lung disease. However, further investigation is required to clarify the pathological role of MFALCs
42 in lung disease, especially in inflammatory disorders.

43

44 INTRODUCTION

45 The immune response against microbes and toxins involves cooperation between both innate
46 and adaptive immunity [1]. The innate immune system, which mounts an immediate response against
47 infection, thereby constituting the first line of defense [2], is also required for the activation of the
48 adaptive immune response via antigen presentation. There are two types of adaptive immune
49 responses: humoral immunity, which is mediated by antibody-producing B-cells, and cell-mediated
50 immunity, mediated by T-cells. The lymphocytes of the innate immune system do not express
51 antigen receptors, as observed in T- and B-cells; however, these lymphocytes produce cytokines and
52 rapidly exert cytotoxic activity against virus-infected cells [3,4]. The innate lymphocytes include
53 classical natural killer (NK) and lymphoid tissue inducer (LTi) cells, in addition to the recently
54 reported T helper 2 (Th2)-type innate lymphocytes, such as natural helper (NH) cells [5]. NH cells
55 were first discovered in novel lymphoid structures associated with mesenteric adipose tissue in both
56 humans and mice [6]. These lymphoid structures are referred to as mesenteric fat-associated
57 lymphoid clusters (FALCs). Recently, we found similar lymphoid clusters associated with
58 mediastinal adipose tissue in mice, and referred to these as mediastinal FALCs (MFALCs) [7].

59 Our previous study revealed strain-related differences in MFALCs among three strains of
60 healthy mice: Th1-biased C57BL/6N, Th2-biased DBA/2Cr, and autoimmune-prone MRL/MpJ mice.
61 We revealed that C57BL/6N mice presented a significantly larger number and size of MFALCs
62 compared with DBA/2Cr and MRL/MpJ strains [7]. Interestingly, Th1-biased C57BL/6 mice are
63 susceptible to hypersensitive pneumonitis, a granulomatous inflammatory lung disease caused by
64 repeated inhalation of organic antigens in humans and mice [8,9]; however, Th2-biased DBA/2 mice
65 are resistant [10,11]. This suggests that the development of MFALCs may affect intrapleural immune
66 conditions. However, there have been no data suggesting an association between MFALCs and
67 intrapleural organs such as the lung.

68 In humans, systemic lupus erythematosus (SLE) may affect numerous organs, including

69 intrapleural organs [12]. The most common thoracic manifestation of SLE is pleuritis [13]. Pleuritis
70 occurs in 17–60% of patients at some point during the course of SLE [14]. Although severe
71 parenchymal lung disease is uncommon, pulmonary complications of SLE include acute lupus
72 pneumonitis, diaphragmatic dysfunction and shrinking lung syndrome, cavitating pulmonary nodules,
73 pulmonary hypertension, pulmonary vasculitis, pulmonary embolism (often due to circulating
74 anticardiolipin antibodies), alveolar hemorrhage (reflecting diffuse endothelial injury), and chronic
75 interstitial pneumonitis [15-18].

76 Murine SLE models also exhibit thoracic manifestations [19]. The MRL/MpJ-*lpr* mouse model
77 carries the lymphoproliferation (*lpr*) mutation in the apoptosis-related *Fas* gene, resulting in
78 autoreactive lymphocyte proliferation. This mouse model, which closely mimics SLE in humans,
79 exhibits lymphadenopathy, splenomegaly, hypergammaglobulinemia with anti-double strand DNA
80 (dsDNA) antibodies leading to tissue deposition and injury in various organs, including the lung and
81 kidney [20-22]. These pathophysiological processes are mediated by immune complex deposition,
82 complement activation, and infiltration of inflammatory leukocytes [23,24]. The BXSB/MpJ-*Yaa*
83 model also develops systemic autoimmune disease at around 5–7 months of age, and males show
84 more severe autoimmune symptoms than females due to the Y-linked autoimmune acceleration (*Yaa*)
85 mutation on the Y chromosome [25-27]. Autoimmune disease phenotypes of this strain were
86 characterized by the presence of autoantibodies, hypergammaglobulinemia, splenomegaly,
87 glomerulonephritis, and dacryoadenitis [28]. However, the pathological features of intrapleural
88 organs in SLE remain unclear.

89 In the present study, we demonstrate that MFALCs are significantly larger in size in
90 autoimmune disease model mice than in the corresponding control strains. Additionally, we show
91 that the size of these MFALCs is positively correlated with immune cell infiltration into the lung
92 tissue. This is the first report of the development of MFALCs in autoimmune disease model mice,
93 and of the pathological correlation between MFALCs and inflammatory processes in the lung. These

94 data emphasize the crucial roles of MFALCs in immunological functions in the intrathoracic
95 environment.

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119 **MATERIALS AND METHODS**

120 *Experimental animals*

121 Autoimmune disease model mice (MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa*) and healthy control
122 mice (MRL/MpJ, and BXSB/MpJ) were utilized in the present study. Mice were purchased from
123 Japan SLC, Inc., (Shizuoka, Japan) (n = 5/strain) and examined at 4 months of age. In handling the
124 experimental animals, the investigators adhered to the Guide for the Care and Use of Laboratory
125 Animals, Hokkaido University, Graduate School of Veterinary Medicine (approved by the
126 Association for Assessment and Accreditation of Laboratory Animal Care International).

127

128 *Tissue preparation and microscopy*

129 Following euthanasia by deep anesthesia, blood was collected from the carotid arteries and the
130 mediastinal fat tissue (MFT), the lungs, and spleens were removed and fixed with 4%
131 paraformaldehyde. In order to preserve lung architecture, the lungs were inflated with 4%
132 paraformaldehyde prior to immersion in fixative solution. After overnight fixation, specimens were
133 dehydrated in graded alcohol and embedded in paraffin. Subsequently, 3- μ m-thick paraffin sections
134 of MFTs were deparaffinized, rehydrated, stained with hematoxylin and eosin (HE), and observed
135 under a light microscope.

136

137 *Immunohistochemistry*

138 Immunohistochemical analysis for B220, CD3, Gr1 (Ly-6G), and Iba1 was performed using
139 3- μ m-thick paraffin sections to detect B-cells, pan-T-cells, granulocytes, and macrophages,
140 respectively. Immunohistochemical procedures were performed according to our previous report
141 [29]. In order to detect cell proliferation, BrdU⁺ cells were observed within the MFALCs and lungs
142 of the strains studied. The detection of BrdU⁺ cells was performed according to our previous report
143 [7]. The details of the antigen retrieval method used, as well as of the sources and dilutions of

144 antibodies used are listed in Table 1. Sections of the spleen were used as positive controls and
145 stained simultaneously with the MFTs and lung lobes (cranial right, middle right, caudal right,
146 accessory right lobes, and left lobe) using the abovementioned antibodies. The sections representing
147 negative controls were incubated in 0.01 M phosphate buffered saline without primary antibody
148 (data not shown).

149

150 *ELISA for serum autoantibody*

151 Serum levels of anti-dsDNA autoantibodies were determined by ELISA using dsDNA-coated
152 plates purchased from Alpha Diagnostic Intl. Inc (M-5110; San Antonio, TX, USA). The assay was
153 performed according to the manufacturer's instructions

154

155 *Histoplanimetry*

156 For histoplanimetry, 5 mice were analyzed per strain. The spleen weights and spleen/body
157 weight ratios were compared between the different mouse strains. For measurement of the lymphoid
158 cluster (LC) area / the total MFT area ratio, light micrographs of HE-stained MFT sections from
159 each strain were prepared. The areas of LCs and the total areas of the mediastinal white adipose
160 tissue within the MFT were measured using ImageJ software (ver. 1.32j, <http://rsb.info.nih.gov/ij>)
161 and the lymphoid cluster (LC) area / the total MFT area ratio was calculated as reported previously
162 [7].

163 Based on the immunohistochemistry staining images, the percentages of B220-, CD3-, Gr1-,
164 and Iba1-positive cells in MFALCs were calculated in 3 different sections from each mouse. These
165 were presented as the positive cell index for each cell population by counting the number of
166 immunopositive cells and dividing them by the total number of mononuclear cells within the
167 MFALCs. In addition, the densities of immunopositive cells / unit area (0.094 mm^2) in different lung

168 lobes were compared between the different strains.

169

170 *Statistical analysis*

171 All numerical results are presented as the means \pm standard error (SE). The results for the
172 different groups were compared using analysis of variance (ANOVA). We used the nonparametric
173 Mann-Whitney *U* test for comparison between the autoimmune disease mouse models and the
174 normal control strains ($P < 0.05$). The correlation between the density of CD3-, B220-, Gr1-, and
175 Iba1-positive cells in different lung lobes, and the percentage of lymphoid cluster areas in the total
176 MFT areas, in the mouse strains was analyzed by Pearson's correlation test (*significant value, $P <$
177 0.05 , **highly significant value, $P < 0.01$).

178

179 **Results**

180 *Autoimmune disease onset*

181 Spleen weights and spleen/body weight ratios were found to be significantly higher in the
182 autoimmune mouse models (MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa*) than in the controls (MRL/MpJ,
183 and BXSB/MpJ) (Fig. 1a and b respectively). The spleen weights of the MRL/MpJ-*lpr* and
184 BXSB/MpJ-*Yaa* mice were almost 3.2-fold and 3.3-fold higher than those of the MRL/MpJ and
185 BXSB/MpJ mice, respectively. Moreover, the spleen/body weight ratios of the MRL/MpJ-*lpr* and
186 BXSB/MpJ-*Yaa* mice were almost 2.8-fold and 3.1-fold higher than those of the MRL/MpJ and
187 BXSB/MpJ mice, respectively. With respect to serum anti-dsDNA antibody levels, a significantly
188 higher titer was observed in MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa* mice than in MRL/MpJ and
189 BXSB/MpJ mice (Fig. 1c).

190

191 *Morphological features of MFALCs*

192 Analysis of the HE-stained sections indicated that MFALCs were observed in the white adipose
193 tissue of the MFTs, in all mice examined, with varying sizes among the studied strains (Fig. 2). The
194 size of the MFALCs was more prominent in the autoimmune models, MRL/MpJ-*lpr* and
195 BXSB/MpJ-*Yaa* (Figs. 2b and d) than in the MRL/MpJ, and BXSB/MpJ mice (Figs. 2a and c). With
196 respect to the histological index of MFALCs development (as expressed by the ratio of the LC area
197 to the total MFT area), a significantly higher LC/MFT area ratio was observed in the autoimmune
198 disease models than in the control strains (Fig. 2e).

199

200 *MFALC immune cell populations*

201 In order to assess the population of immune cells in the MFALCs, immunohistochemical
202 analysis for CD3, B220, Iba1, and Gr1 was performed to detect pan-T-cells, B-cells, macrophages,
203 and granulocytes, respectively. CD3-, B220-, and Iba1-positive cells (Figs. 3a-c) were found to

204 predominate in all strains examined, with the presence of a few Gr1-positive cells (Fig. 3d).

205 In order to assess the immune cell populations in MFALCs, the percentages of immunopositive
206 cells in the total cells detected in MFALCs were analyzed in the different mouse strains and
207 represented as cell indices (Fig. 3e). A significant difference was observed between the cell indices
208 of CD3-, B220-, and Iba1-populations in the autoimmune disease models and the corresponding
209 control strains (Figs. 3e). No significant difference was observed between the strains in terms of the
210 percentage of Gr1-positive cells (data not shown).

211

212 *Proliferating cells in the MFALCs*

213 BrdU-positive cells, whose numbers varied by strain, were observed in the MFALCs (Fig. 4). A
214 greater number of BrdU-positive cells was observed within the MFALCs of the autoimmune disease
215 model mice (Fig. 4b and d) than in the MFALCs of the control strains (Fig. 4a and c). In particular,
216 the number of BrdU-positive cells in the MFALCs of MRL/MpJ-*lpr* mice was greater than in those
217 of the other strains studied (Fig. 4b).

218

219 *Histopathological features of the lungs*

220 Analysis of the HE-stained sections indicated that MRL/MpJ (Figs. 5a and b) mice showed
221 normal histological lung architecture, characterized by normal blood vessels, thin interalveolar
222 septum, and a low degree of cell infiltration. However, the lungs in MRL/MpJ-*lpr* and
223 BXSB/MpJ-*Yaa* mice, as well as BXSB/MpJ mice, showed several histopathological alterations
224 characterized by the presence of numerous congested blood vessels, high numbers of extravasated
225 red blood cells, large accumulations of mononuclear cells, thicker interalveolar septa, and
226 numerous abnormal and collapsed alveoli (Figs. 5c-h).

227

228 *Immune cell infiltration in the lung lobes*

229 Immunohistochemical analysis revealed the infiltration of CD3-, B220-, Iba1-, and Gr1-positive
230 cells in the mouse lungs (Figs. 6a-d). Mononuclear cells positive for CD3-, B220-, and Gr1 were
231 mainly observed in the interalveolar septum and interstitium (Figs. 6a, b, and d). Iba1-positive cells
232 were mainly observed in the connective tissues of the peribronchial regions as well as in the lung
233 dust cells (Fig. 6d).

234 Within the same strain, no significant difference was observed in the density of immunopositive
235 cells in the left and right (cranial, middle, caudal, and accessory) lobes (data not shown). However, a
236 significant difference was observed in the density of CD3-, B220-, and Iba1-positive cell infiltration
237 between the lungs of autoimmune disease model mice (MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa* mice)
238 and those of the control strains (Fig. 6e). There was a significant difference in the density of
239 Gr1-positive cell infiltration between MRL/MpJ-*lpr* mice and the corresponding normal control
240 strain; however, no significant difference was observed between the lung of BXSB/MpJ-*Yaa* mice
241 and its counterpart control strain (Fig. 6e).

242

243 *Correlation between the MFLAC development, autoimmune disease indices, and cellular infiltration*
244 *of the lungs*

245 Significant positive correlation was observed between the autoimmune disease model mice
246 (MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa*) and the control strains in terms of the size of the MFALCs and
247 the spleen weights, spleen/body weight ratios, and serum anti-dsDNA antibody levels (Table 2). The
248 size of the MFALCs was also significantly positively correlated with the density of CD3-, B220- and
249 Iba1-positive cell infiltration in the lung lobes (Table 3). Positive correlation was also observed
250 between the size of MFALCs and the density of Gr-1 positive cell infiltration in the lung lobes
251 (Table 3). The later correlations were found to be significant between the BXSB/MpJ-*Yaa* mice and
252 corresponding controls ($r = 0.733^*$). However, no significant correlation was observed between
253 MRL/MpJ-*lpr* and the counterpart control strain ($r = 0.077$).

254 **Discussion**

255 In previous studies, we demonstrated the presence of LCs associated with MFT, similar to those
256 discovered in the mesenteric adipose tissue of both humans and mice [6], in apparently normal mice
257 strains. These were termed MFALCs [7]. In addition, we showed that there are significant
258 differences in MFALC size in normal mice, and determined that the predominant cells in MFALCs
259 were lymphocytes [7]. However, there have been no data on the morphology of such MFALCs in
260 autoimmune disease model mice. We reported an association between the LCs and mediastinal white
261 adipose tissue. In addition, we found that the autoimmune disease models (MRL/MpJ-*lpr* and
262 BXSB/MpJ-*Yaa*) exhibit better developed MFALCs than the control strains. White adipose tissue,
263 which secretes adipokines such as leptin, adiponectin, visfatin, and resistin, is considered the largest
264 endocrine organ. These adipokines function as hormones in glucose homeostasis and appetite
265 regulation, and as cytokines in the immune system, where they mediate effects on autoimmunity
266 [30,31,32,33]. In addition, they have been reported to play roles in autoimmune rheumatic diseases,
267 such as SLE and rheumatoid arthritis [33].

268 In the present study, the autoimmune disease models, MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa*,
269 were reported to develop SLE-like disorders characterized by lymphoid activation and hyperplasia,
270 nephritis, splenomegaly, and elevated serum autoantibodies against dsDNA [20,34]. MRL/MpJ-*lpr*
271 and BXSB/MpJ-*Yaa* mice presenting MFALCs of larger size also exhibited higher spleen weights
272 and spleen/body weight ratios as well as higher serum anti-dsDNA autoantibody levels than the
273 normal control strains. Numerous mononuclear cells within the MFALCs of the autoimmune disease
274 model mice showed bromodeoxyuridine-incorporating activity, suggesting local cell proliferation [7].
275 Therefore, we suggest that the presence of prominent lymphoproliferation in the MFTs of the
276 autoimmune model mice examined may represent an SLE-like phenotype in addition to other
277 previously reported phenotypes.

278 It has been reported that pulmonary involvement may be an important contributor to morbidity

279 and mortality in human SLE [15,35,36]. The MRL/MpJ-*lpr* mouse model has been used to study the
280 progress of pulmonary inflammation in SLE [37]. Perivascular lymphocytic infiltration in the lung
281 has been reported to precede renal involvement in such murine models of lupus [38]. Lung disorders
282 secondary to SLE include interstitial pneumonitis with mononuclear cellular infiltration, interstitial
283 fibrosis, edema, and pulmonary vasculitis [35]. Accordingly, our findings revealed a greater
284 accumulation of mononuclear cells with histopathological changes such as numerous congested
285 blood vessels, thicker interalveolar septa, and several abnormal and collapsed alveoli in the lungs of
286 MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa* mice as well as in BXSB/MpJ mice than in the other strains
287 studied. The interstitial mononuclear infiltration in the lung lobes observed in these strains may
288 reflect the inflammatory processes associated with interstitial pneumonitis in human SLE [15,36].

289 CD3-positive T cells, B220-positive B cells, Iba1-positive macrophages, and Gr-1-positive
290 granulocytes were observed in both MFALCs and the lung. Interestingly, the autoimmune disease
291 models showed significantly higher numbers of CD3-, B220-, and Iba1- positive cells, in both the
292 MFALCs and the lung, than the control strains. Furthermore, both the MFALCs and lungs in
293 MRL/MpJ-*lpr* mice showed a higher number of CD3-positive cells than those of the other strains.
294 Massive T-cell proliferation was also reported in the spleen and mesenteric lymph node of
295 MRL/MpJ-*lpr* mice compared with C57BL/6 mice [38]. Furthermore, it has been reported that the
296 majority of proliferating lymphoid cells in the lymph nodes of MRL/MpJ-*lpr* mice are T-cells rather
297 than B-cells [39]. Such lymphoproliferation was reported to be due to a massive accumulation of
298 double-negative (CD3⁺/CD4⁻/CD8⁻) T-cells in the secondary lymphoid organs [40] resulting from *lpr*
299 mutation of *Fas* [41,42]. On the other hand, the BXSB/MpJ-*Yaa* mice presented a higher number of
300 B220-positive cells, in both MFALCs and lungs, than the other strains studied. Furthermore, we
301 previously reported that BXSB/MpJ-*Yaa* mice develop dacryoadenitis with T- and B-cell infiltration,
302 and demonstrated that B-cells were the predominant infiltrating cells in the lacrimal glands of these
303 mice [28]. Moreover, in BXSB/MpJ-*Yaa* mice, B-cells with the *Yaa* mutation are intrinsically biased

304 towards nucleolar antigens due to increased expression of Toll-like receptors in duplicated regions of
305 the Y chromosome [43]. Therefore, the increased cell infiltration of the lungs would reflect the
306 autoimmune disease conditions in MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa* mice due to the mutation of
307 immune-associated genes (*lpr* mutation of *Fas* in MRL/MpJ-*lpr* mice and mutation in Y
308 chromosome in BXSB/MpJ-*Yaa*). These mutations may characterize the specific immune cell
309 populations of the MFALCs and lungs in each strain.

310 Our data reveal a significantly positive correlation between the size of MFALCs and
311 autoimmune disease indices, including the spleen weights, spleen/ body weight ratios, and serum
312 autoantibody levels, as well as cellular infiltration in the lung, in the autoimmune disease models and
313 control strains. These data clearly indicate a pathological association between MFALCs and
314 autoimmune disease-associated lung pathology. However, differences in the spleen weights and
315 serum autoantibody levels between strains did not fully correspond to differences in MFALC size or
316 density of cellular infiltration in the lung. Briefly, MRL/MpJ mice showed a higher titer of
317 anti-dsDNA antibodies than the other healthy controls, but presented the smallest MFALCs, lowest
318 levels of cellular infiltration in the lung, and lowest spleen weights among the examined strains.
319 Furthermore, although BXSB/MpJ mice presented a relatively higher positive B-cell density in the
320 lung than MRL/MpJ mice, the spleen weights and serum anti-dsDNA antibody levels were
321 comparable to those of the latter strain. These results indicate that the spleen weights and serum
322 anti-dsDNA antibody levels were not directly associated with the development of MFALCs or
323 cellular infiltration in the lung. In addition, the data suggest that not only autoimmune disease
324 phenotypes, but also other factors, are involved in these processes.

325 As reported in our previous study, the MFALCs of Th1-prone C57BL/6 mice were found to
326 be larger in size than those of Th2-prone DBA/2 mice [7]. Furthermore, we recently reported that
327 not only male BXSB/MpJ-*Yaa* mice, but also female BXSB/MpJ mice, clearly exhibit symptoms
328 of age-related autoimmune disease onset, such as increased spleen weights, elevated serum

329 autoantibodies, and glomerulonephritis, without the contribution of the *Yaa* mutation [44, 45]. For
330 the BXSB/MpJ strain, age-related autoimmune disease onset was attributed to the expression of
331 immune-associated genes in the telomeric regions of chromosome 1 [45]. Therefore, gene
332 mutations as well as genetic backgrounds associated with the immune response may also be
333 important in the development of MFALCs and lung cellular infiltration in murine autoimmune
334 disease models.

335 In conclusion, our findings indicate a possible role for MFALCs in the development of
336 autoimmune-associated lung diseases. In particular, the elucidation of a positive correlation
337 between the size of MFALCs and density of lung infiltration with mononuclear cells provides
338 insights into the pathogenesis of several intrapleural diseases.

339

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343 wrote the manuscript. OI designed the experiments and wrote the manuscript. YK conceptualized the
344 research, designed the experiments, and wrote the manuscript.

345 **Conflict of Interest**

346 The authors declare no conflict of interest.

347

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454 chromosome 1 and *Yaa*. *Genes Immun* 2014; **15**:182–189.
- 455

456 **Figure Legends**

457 **Figure 1:** Indices of autoimmune disease onset in mice

458 Graph showing the spleen weight (n = 5 mice for each strain) (a), spleen / body weight ratio (n = 5
459 mice for each strain) (b), and serum anti-double strand DNA (dsDNA) level (n = 4 mice for each
460 strain) (c). Statistically significant difference, as determined using the Mann–Whitney *U*-test ($P <$
461 0.05), between autoimmune disease models (MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa*) and the control
462 strains (MRL/MpJ, BXSB/MpJ), respectively, is indicated by asterisk (*). Values are shown as the
463 means \pm SE.

464

465 **Figure 2:** Histological features of mediastinal fat-associated lymphoid clusters (MFALCs) in mice

466 Light microscopic images of hematoxylin and eosin (HE)-stained mediastinal fat tissues (MFTs) of
467 MRL/MpJ, MRL/MpJ-*lpr*, BXSB/MpJ, and BXSB/MpJ-*Yaa* mice, respectively (a-d). Note that
468 MFALCs (indicated by arrows) are more prominent in both MRL/MpJ-*lpr* (b) and BXSB/MpJ-*Yaa*
469 mice (d). (e) Graph showing the ratio of lymphoid cluster (LC) area to the total MFT area in the
470 HE-stained sections ($\times 40$). Statistically significant difference, as determined using the
471 Mann–Whitney *U*-test ($P < 0.05$); n = 5 mice for each strain, between autoimmune disease models
472 (MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa*) and the control strains (MRL/MpJ, BXSB/MpJ), respectively,
473 is indicated by asterisk (*). Values are shown as the means \pm SE.

474

475 **Figure 3:** Immune cells from mediastinal fat-associated lymphoid clusters (MFALCs) in mice

476 Light microscopic photographs showing immunohistochemical staining of mouse MFALCs with
477 CD3 (a), B220 (b), Iba1 (c), and Gr-1 (d). Numerous CD3-positive T-cells, B220-positive B-cells,
478 and Iba1-positive macrophages (a-c), but only few Gr-1-positive granulocytes (d), are present in
479 mouse MFALCs, and their appearance differs between the strains. Arrows indicate immunopositive
480 cells. Bars (a-d) = 100 μ m. Graphs showing the indices of immunopositive cells for CD3 (a), B220

481 (b), and Iba1 (c) in MRL/MpJ, MRL/MpJ-*lpr*, BXSB/MpJ, and BXSB/MpJ-*Yaa* mice. Statistically
482 significant difference, as determined using the Mann–Whitney *U*-test ($P < 0.05$); $n = 5$ mice for each
483 strain, between autoimmune disease models (MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa*) and the control
484 strains (MRL/MpJ, BXSB/MpJ), respectively, is indicated by asterisk (*). Values are shown as the
485 means \pm SE.

486

487 **Figure 4:** Proliferating cells in the mediastinal fat-associated lymphoid clusters (MFALCs) of the
488 mice

489 Immunohistochemistry for BrdU in MRL/MpJ (a), MRL/MpJ-*lpr* (b), BXSB/MpJ (c), and
490 BXSB/MpJ-*Yaa* (d) mice. Fewer BrdU-positive cells are visible within the MFALCs of MRL/MpJ
491 and BXSB/MpJ mice (a, c) than in the MFALCs of MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa* mice (b, d) at
492 4 months.

493

494 **Figure 5:** Histopathological features of the lungs in mice

495 Light microscopic photographs of hematoxylin and eosin (HE)-stained mouse lung sections of
496 MRL/MpJ (a and b), MRL/MpJ-*lpr* (c and d), BXSB/MpJ (e and f), and BXSB/MpJ-*Yaa* (g and h),
497 mice. The squares in a, c, e, and g indicate the same areas as those in b, d, f, and h, respectively. A
498 greater accumulation of mononuclear cells (asterisks), thicker interalveolar septa (arrows), congested
499 blood vessels (arrowheads), and collapsed alveoli (ca) are visible in the lung tissue of MRL/MpJ-*lpr*,
500 BXSB/MpJ, and BXSB/MpJ-*Yaa* mice (c-h) than in that of MRL/MpJ mice (a and b). Bars = 300
501 μm (a, c, e, and g). Bars = 50 μm (b, d, f, and h).

502

503 **Figure 6:** Immune cells in the mouse lungs

504 (a-d) Light microscopic photographs showing immunohistochemical staining of the mouse lungs
505 with CD3 (a), B220 (b), Iba1 (c), and Gr-1 (d). Immunopositive cells (indicated by arrows) are

506 present in the lungs of MRL/MpJ, MRL/MpJ-*lpr*, BXSB/MpJ, and BXSB/MpJ-*Yaa* mice, and their
507 appearance differs between the strains. In particular, the MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa* mice
508 show a greater abundance of positive cells within the infiltration area. Bars = 50 μ m. (a-d) Graph
509 showing the average density of CD3- (a), B220- (b), Iba1- (c), and Gr1- (d) positive cells/unit area in
510 all the lobes in the lungs of MRL/MpJ, MRL/MpJ-*lpr*, BXSB/MpJ, and BXSB/MpJ-*Yaa* mice.
511 Statistically significant difference, as determined using the Mann–Whitney *U*-test ($P < 0.05$); $n = 5$
512 mice for each strain, between autoimmune disease models (MRL/MpJ-*lpr* and BXSB/MpJ-*Yaa*) and
513 the control strains (MRL/MpJ, BXSB/MpJ), respectively, is indicated by asterisk (*). Values are
514 shown as the means \pm SE.
515

516 **Table 1:** List of antibodies, working dilutions, and methods for antigen retrieval.

517

Antibody	Source	Dilution	Antigen retrieval	Heating condition
Rabbit anti-CD3	Nichirei (Tokyo, Japan)	1 : 200	Target retrieval solution (high pH) (produced by Dako)	105°C, 20 min
Rat anti-B220	Cedarlane (Ontario, Canada)	1 : 1600	10 mM citrate buffer (pH 6.0)	105°C, 20 min
Rabbit anti-Iba1	Wako (Osaka, Japan)	1 : 1200	10 mM citrate buffer (pH 6.0)	105°C, 20 min
Rat anti-Gr1	R and D system (Minneapolis, USA)	1 : 800	0.1% pepsin/ 0.2 N HCl	37°C, 5 min
Rat anti-BrdU	Abcam (Tokyo, Japan)	1 : 200	10 mM citrate buffer (pH 6.0)	105°C, 20 min

518

519 Table 2. Pearson's correlation between the average number of CD3-, B220-, Iba1-, and Gr1-positive
 520 cells in all lung lobes and the proportion of lymphoid cluster area in the total MFT areas (expressed
 521 as percentages) in autoimmune disease model mice and the normal control strains.

Immune cells		CD3	B220	Iba1	Gr1
MRL/MpJ and	<i>r</i>	0.865 ^{**}	0.944 ^{**}	0.656 [*]	0.733 [*]
MRL/MpJ- <i>lpr</i>	<i>P</i>	0.001	0.000	0.040	0.016
BXSB/MpJ and	<i>r</i>	0.935 ^{**}	0.948 ^{**}	0.762 [*]	0.077
BXSB/MpJ- <i>Yaa</i>	<i>P</i>	0.000	0.000	0.010	0.832

522 Pearson's correlation test, n = 10, (* Significant, $P < 0.05$; ** Highly significant, $P < 0.01$).

523

524

525 Table 3. Pearson's correlation between spleen weights, spleen/body weight ratios, serum anti-dsDNA
 526 antibody level, and the proportion of lymphoid cluster area in total MFT areas (expressed as a
 527 percentage) in autoimmune disease model mice and the normal control strains.

Mouse strain		Spleen weight	Spleen/body weight ratios	Serum anti-dsDNA antibody level
MRL/MpJ and	r	0.647 [*]	0.685 [*]	0.816 [*]
MRL/MpJ- <i>lpr</i>	<i>P</i>	0.043	0.029	0.013
BXSB/MpJ and	r	0.685 [*]	0.632 [*]	0.745 [*]
BXSB/MpJ- <i>Yaa</i>	<i>P</i>	0.029	0.050	0.034

528 Pearson's correlation test, n ≥ 8, (*, Significant, P < 0.05; **, Highly significant, P < 0.01).

Figure 1

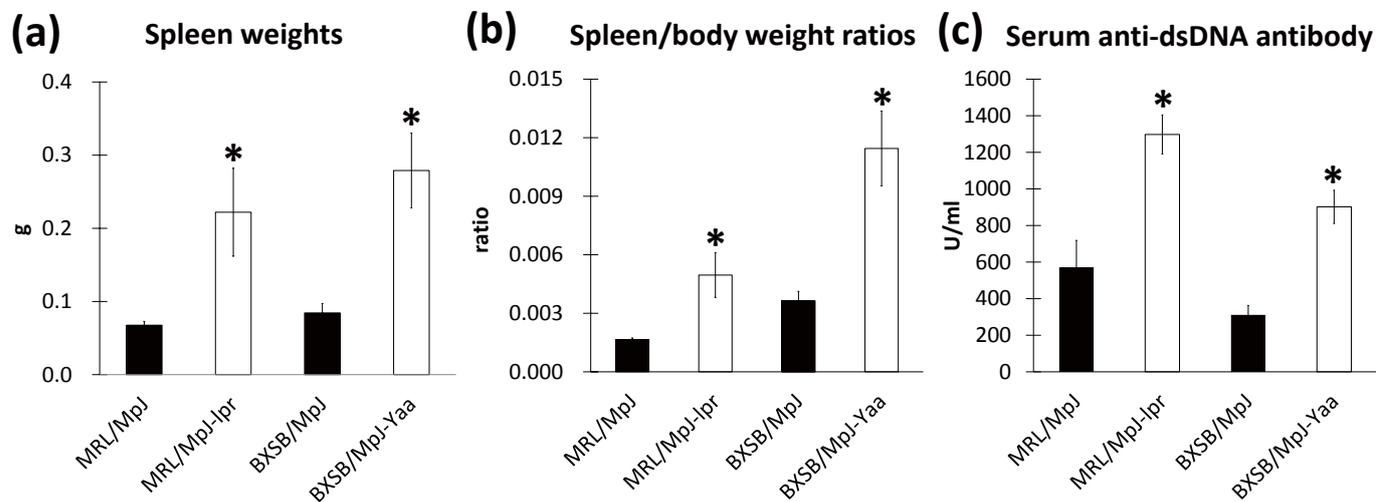
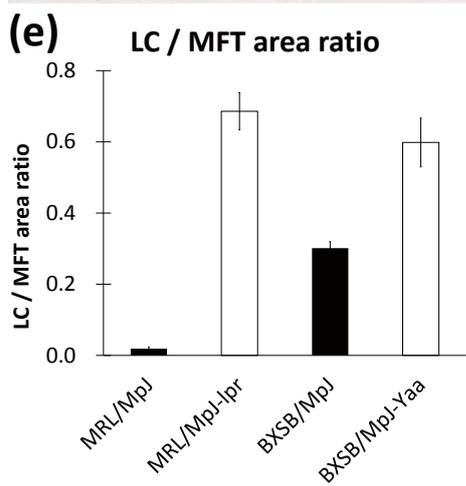
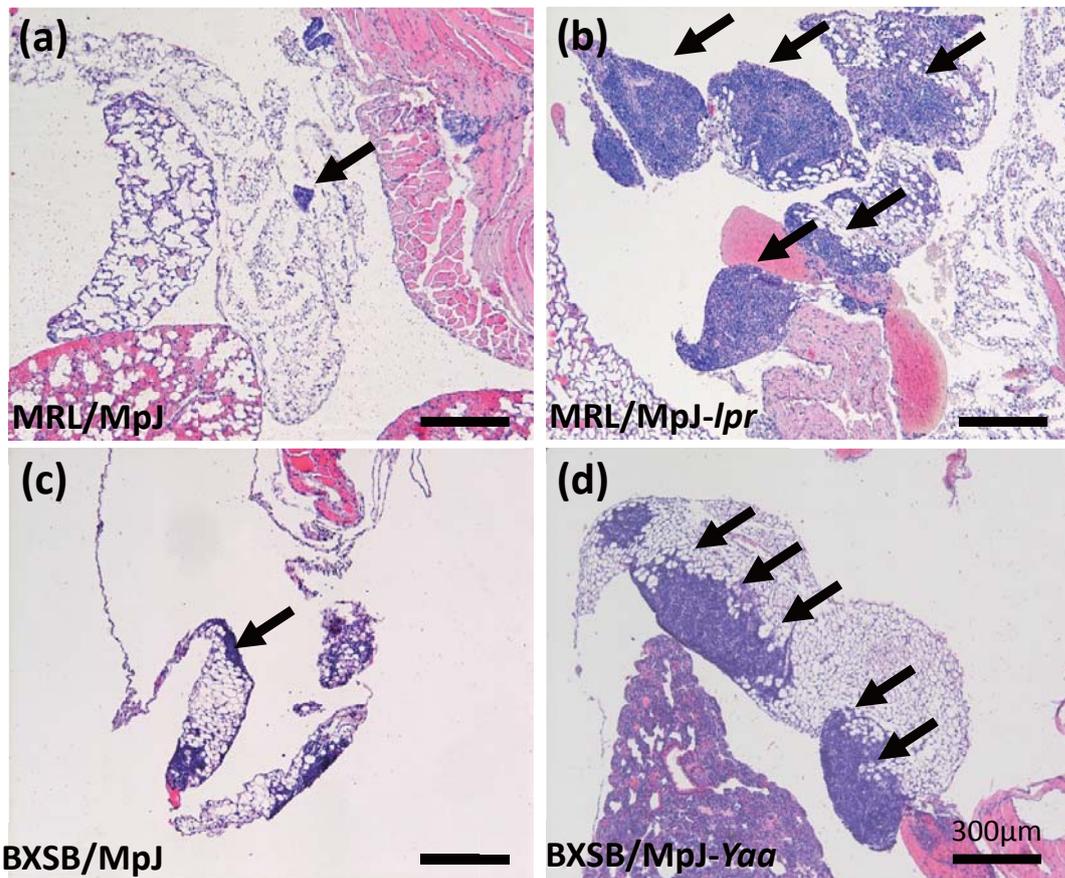
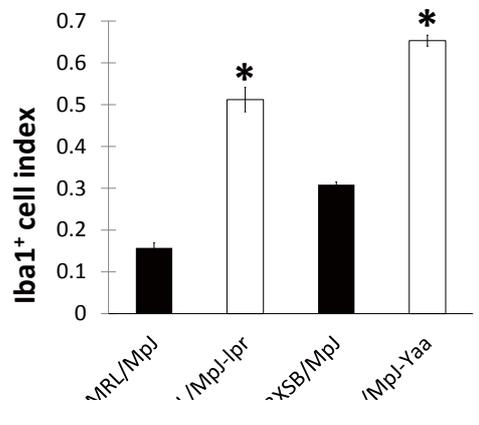
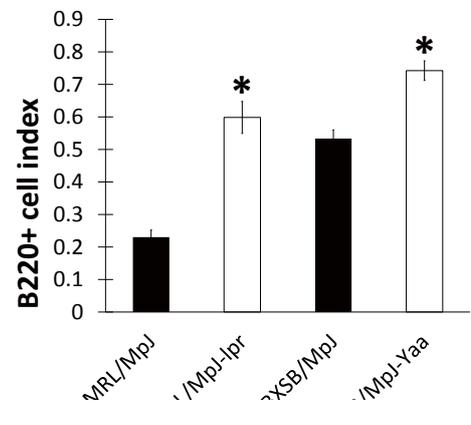
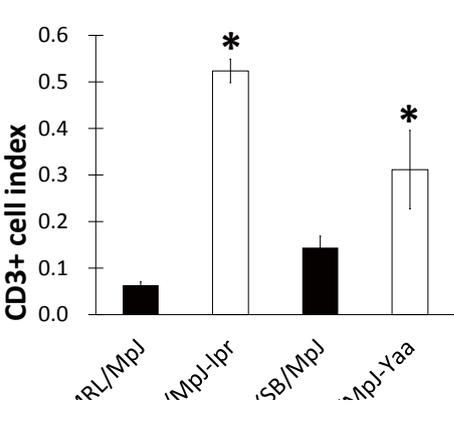
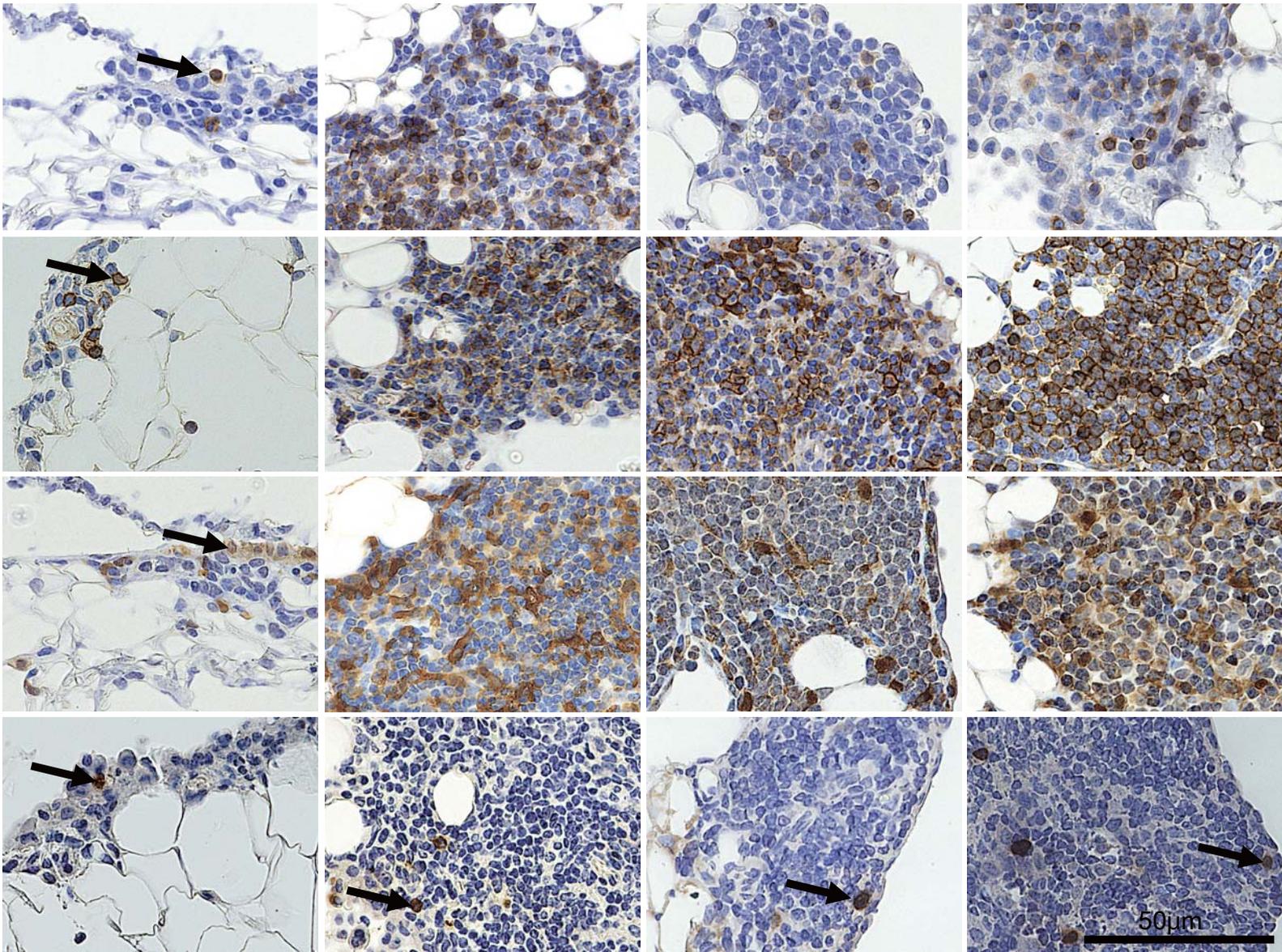


Figure 2





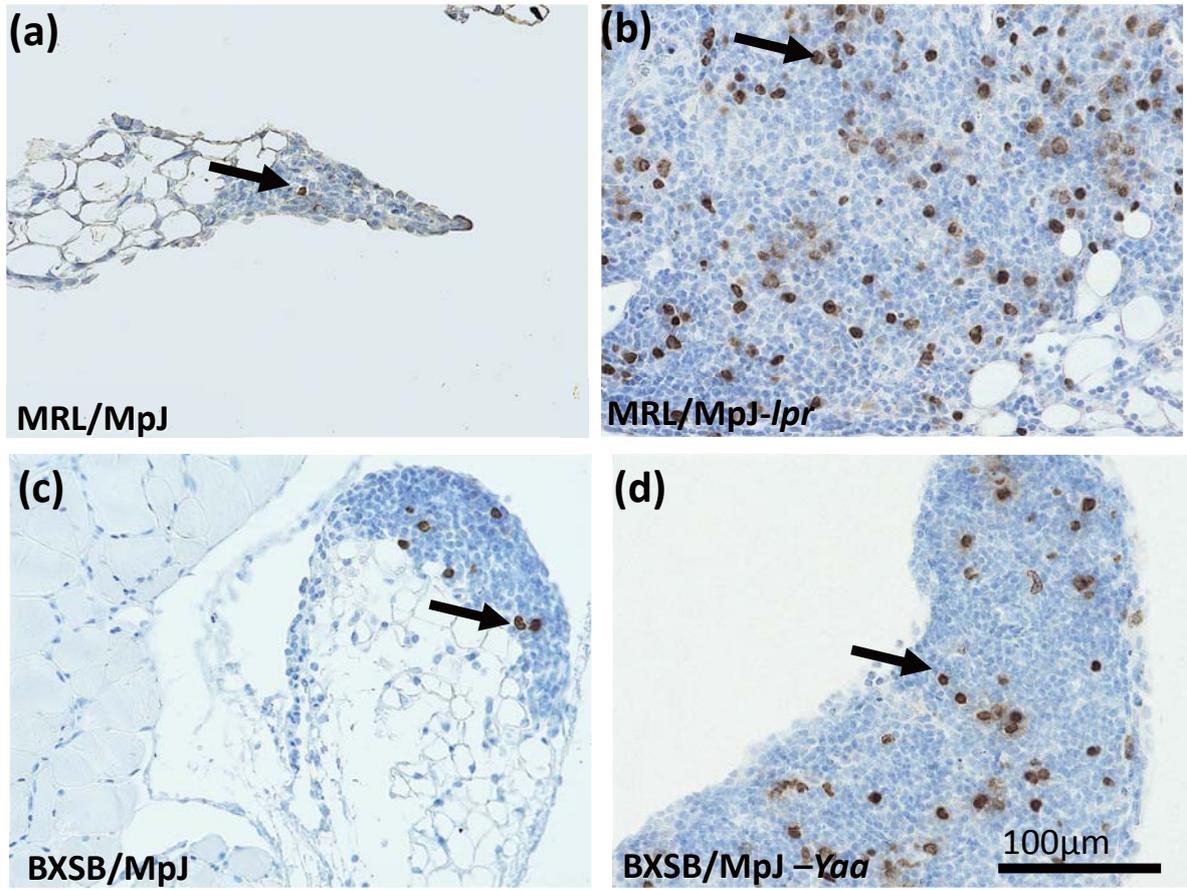


Fig. 4

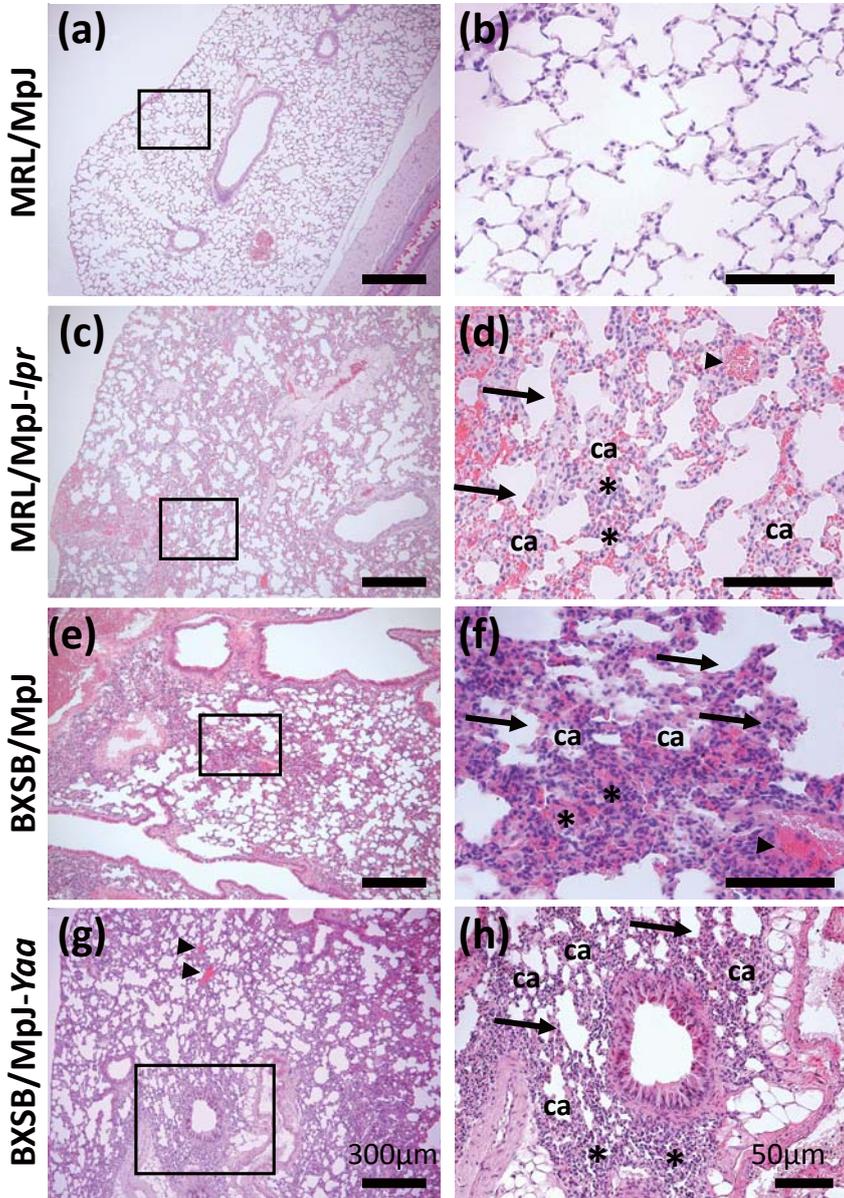


Fig. 5

