



Title	Pathological Alternations of Mediastinal Fat-Associated Lymphoid Cluster and Lung in a Streptozotocin-Induced Diabetic Mouse Model
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Citation	Microscopy and microanalysis, 27(1), 187-200 https://doi.org/10.1017/S1431927620024824
Issue Date	2021-02
Doc URL	http://hdl.handle.net/2115/84265
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Type	article (author version)
File Information	library 20201102 YA Final Microscopy and microanalysis.asd.pdf



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1 **Original research**

2 **Pathological alternations of mediastinal fat-associated lymphoid cluster and lung in a**
3 **streptozotocin-induced diabetic mouse model**

4

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23 **Running title:** Correlation between diabetes and the development of lung injury and MFALCs.

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Abstract

28 Diabetes is a devastating global health problem and is considered a predisposing factor for lung
29 injury progression. Furthermore, previous reports of the authors revealed the role of mediastinal
30 fat-associated lymphoid clusters (MFALCs) in advancing respiratory diseases. However, no reports
31 concerning the role of MFALCs on the development of lung injury in diabetes have been published.
32 Therefore, the study aimed to examine the correlations between diabetes and the development of
33 MFALCs and the progression of lung injury in a streptozotocin-induced diabetic mouse model.
34 Furthermore, immunohistochemical analysis for immune cells (CD3⁺ T-lymphocytes, B220⁺
35 B-lymphocytes, Iba1⁺ macrophages, and Gr1⁺ granulocytes), vessels markers (CD31⁺ endothelial
36 cells and LYVE-1⁺ lymphatic vessels “LVs”), and inflammatory markers (TNF- α and IL-5) was
37 performed. In comparison to the control group, the diabetic group showed lung injury development
38 with a significant increase in MFALC size, immune cells, LVs, and inflammatory marker, and a
39 considerable decrease of CD31⁺ endothelial cells in both lung and MFALCs was observed.
40 Furthermore, the blood glucose level showed significant positive correlations with MFALCs size,
41 lung injury, immune cells, inflammatory markers, and LYVE-1⁺ LVs in lungs and MFALCs. Thus,
42 we suggested that the development of MFALCs and LVs could contribute to lung injury progression
43 in diabetic conditions.

44 **Keywords:** Diabetes, lung injury, streptozotocin, mediastinal fat-associated lymphoid cluster,
45 lymphatic vessels, immune cells

46 **Introduction**

47 Fat-associated lymphoid clusters (FALCs) are non-encapsulated lymphoid clusters found
48 to be in direct contact with the surrounding adipocytes. FALCs have been reported mainly in
49 association with the mesenteric and mediastinal fat tissues in healthy humans and mice. They are
50 named mesenteric FALCs (Moro, et al., 2010) and mediastinal FALCs “MFALCs” (Elewa, et al.,
51 2014), respectively. Furthermore, the development of such clusters has been demonstrated to be
52 associated with inflammation (Benezech, et al., 2015; Elewa, et al., 2018). Yet, the literature does
53 not report any study concerning the effect of metabolic diseases on the development of MFALCs.
54 Diabetes mellitus (DM) is a metabolic disease characterized by persistent hyperglycemia, with
55 evidence of the development of acute lung injury/acute respiratory distress syndrome (Honiden &
56 Gong, 2009; Zheng, et al., 2017).

57 It has previously been revealed that the histopathological features of lung injury in several
58 mice models include accumulations of mononuclear cells, thickening of the interalveolar septa,
59 collapsed alveoli, and increased collagen fiber deposition in the interalveolar septa, which may even
60 lead to severe lung fibrosis replacing the normal lung architectures (Elewa, et al., 2016; Elewa, et
61 al., 2018). Furthermore, a significantly positive correlation was observed between MFALCs
62 development and lung injury pathogenesis in several mouse models. These included genetic
63 autoimmune diseases (Elewa, et al., 2016; Elewa, et al., 2017); septic condition such as bleomycin
64 induced-pneumonitis (Elewa, et al., 2018); and aseptic infections such as *Mycoplasma pulmonis*
65 (Boonyarattanasoonthorn, et al., 2019). However, the pathological alternations of MFALCs and
66 their possible role in lung injury pathogenesis in DM are not clarified.

67 FALCs mainly comprised macrophages, lymphocytes with no distinguished B and T cell
68 zonation, and few granulocytes (Elewa, et al., 2014; Moro, et al., 2010). Also, several Th2
69 cytokines (such as interleukin 4 “IL-4”, IL-5, and IL13) have been reported to be expressed by
70 FALC c-Kit⁺Sca-1⁺ cells (Moro, et al., 2010). Additionally, an *in-vitro* study revealed that the IL-5
71 regulates B-cell antibody production (Sonoda, et al., 1989). Besides, the role of tumor necrosis

72 factor (TNF) receptors (TNFR1 and TNFR2) in FALC formation has been explicated. In contrast, a
73 defective appearance of FALCs has been revealed in mice lacking such
74 receptors (*Tnfrsf1a*^{-/-}*Tnfrsf1b*^{-/-} mice) (Benezech, et al., 2015). Also, it has been shown that the
75 proinflammatory cytokines, including TNF- α and IL in the lung, act as critical modulators of
76 inflammation in the pathogenesis of lung injury development (Malaviya, et al., 2017).

77 Interestingly, FALCs have been reported to be highly vascularized structures and are found
78 in close association with blood vessels (Benezech, et al., 2015; Moro, et al., 2010). Additionally, the
79 role of lymphatic vessels and high endothelial venules (HEVs) in the development of such clusters
80 in different strains of mice has been previously reported (Elewa, et al., 2014). Such HEVs have
81 been reported as specialized types of post-capillary venules required for lymphocyte trafficking
82 (Buscher, et al., 2016; Rangel-Moreno, et al., 2009). Additionally, there is increasing evidence
83 suggesting that the lung microangiopathy development in diabetic patients is considered a
84 predisposing factor for the development of lung injury (Pitocco, et al., 2012; Sandler, 1990).

85 In this report, the authors examined the morphology of MFALCs after diabetic induction
86 by streptozotocin (STZ) in C57BL/6N (B6) mice. It was hypothesized that the alterations in
87 MFLAC development could play a significant role in the pathogenesis of lung injury progression
88 following diabetic induction. The authors also suggest that LVs could be considered potential
89 avenues for recruiting immune cells into the lungs and into and out of MFALCs. This thus leads to
90 the progression of lung injury and MFALCs development. The authors also postulated that the
91 damaged endothelial cells could contribute to lung injury progression with less effect on MFALCs
92 development.

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98 **Material and Methods**

99 *Experimental animals and ethics statement*

100 Healthy, male B6 mice (aged eight weeks) were purchased from Japan SLC, Inc. (Shizuoka,
101 Japan) and maintained under specific pathogen-free conditions to be used as per the experimental
102 design. The investigators adhered to the Guide for the Laboratory Animal Care and Use approved
103 by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary
104 Medicine, Hokkaido University (approval No. 15–0079).

105

106 *Experimental design*

107 B6 mice were administered intraperitoneal (i.p.) injection of STZ (Calbiochem, San Diego,
108 CA, USA) dissolved in 0.05 M sodium citrate buffer (CB) (pH 4.5) at a dose of 40 mg/kg body
109 weight (BW), which represented the diabetic group. The remaining mice were injected CB and
110 comprised the control group (n = 5 mice/group). The protocol of STZ- diabetic induction was
111 performed according to the previous report (Ichii, et al., 2010; Tamura, et al., 2005). Briefly, mice
112 of either the diabetic or control groups received two rounds of i.p. injections, i.e., either STZ or CB
113 (for five consecutive days/round), respectively. The first round was completed at eight weeks, and
114 the second round was injected at 12 weeks of age. The sampling was done at 15 weeks of age
115 (Figure 1a).

116

117 *Blood glucose level analysis and sampling for histopathological observation*

118 At 15 weeks of age, mice from both control and the diabetic group were weighed and sacrificed
119 under deep anesthesia (using a mixture of midazolam at a dose of 4.0 mg/kg, medetomidine at a
120 dose of 0.3 mg/kg, and butorphanol at a dose of 5.0 mg/kg). Blood glucose level was measured
121 using Medisafe Fit blood glucose meters and its TIPs (MEDISAFE Fit Smile, TERUMO, Tokyo,
122 Japan) according to the manufacturer's instructions. Mice were considered diabetic when the blood
123 glucose level was ≥ 200 mg/dL. On the other hand, the control group's blood glucose level was

124 found to be ≤ 100 mg/dL (Figure 1b). The pancreas, mediastinal fat tissue, and lung were
125 immediately fixed in 4% paraformaldehyde overnight at 4° C for histopathological analysis. Also,
126 the spleen of mice in each group was weighted, and the average spleen/BW in each group was
127 calculated.

128

129 *Tissue preparation for histopathological observation*

130 Following overnight fixation, the specimens were washed with distilled water and subjected to
131 dehydration in ascending graded ethanol. The samples were then cleared in xylene (3 changes/30
132 min each) and embedded in melted paraffin (3 changes/60 min each). Paraffin blocks were prepared,
133 cut using microtome at three μm thickness, and subjected to either routine histological staining or
134 immunohistochemical staining following deparaffinization and hydration.

135

136 The sections were stained with either hematoxylin and eosin (H&E) or Masson's trichrome (MT) to
137 detect the degree of lung fibrosis for routine histological examination. To detect different types of
138 collagen in lung, both groups' lung sections were immunostained with rabbit polyclonal
139 anti-collagen I antibody (ab21286, abcam, Tokyo, Japan) diluted at 1:300, and rabbit polyclonal
140 anti-COL3A1 (22734-1-AP, Proteintech, Manchester, United Kingdom) diluted at 1:1000. For
141 pancreatic sections, immunohistochemical staining was performed to detect insulin positive beta
142 cells using rat monoclonal anti-insulin antibody (MAB1417, diluted at 1:300, R&D, Funakoshi Co.,
143 Ltd., Tokyo, Japan). Furthermore, to detect different immune cells (B- lymphocytes, T-lymphocytes,
144 macrophages, and granulocytes) within the MFALCs and lung tissue, immunohistochemical
145 staining was performed using rat monoclonal anti-B220 at 1:1600 dilution (Cat. No. CL8990NA,
146 Cedarlane, ON, Canada), rabbit monoclonal anti-CD3 at 1:200 dilution (Cat. No. 413601, Nichirei,
147 Tokyo, Japan.), rabbit anti-Iba1 at 1:1000 dilution (Cat. No. 019-19741, Fujifilm Wako, Osaka,

148 Japan), and rat monoclonal anti-Gr-1 antibodies at 1:800 dilution (Cat. No. MAB1037, R and D
149 system, MN, USA), respectively. Furthermore, for the analysis of the degree of development of
150 vasculature within MFALCs and lung tissue, immunohistochemical staining was performed using
151 rat monoclonal anti-peripheral node addressin PNAd at 1:500 dilution (Cat. No.12, Biolegend, San
152 Diego, CA, USA), rabbit anti-LYVE-1 at 1:500 dilution (Cat. No. AG-25T- 0103, AdipoGen, San
153 Diego, CA, USA), and rabbit monoclonal anti-CD31 antibodies at 1:100 dilution (Cat. No.
154 Ab28364, Abcam), to detect the expression of HEVs, LVs, and ECs lining the blood capillaries and
155 vessels. Additionally, immunohistochemistry was performed to detect the expression pattern of the
156 proinflammatory cytokines in both MFALCs and lung tissue using rabbit anti-TNF- α (Cat. No.
157 ab6671, Abcam) and IL-5 (LS-C177535–100, Life Span Bioscience) antibodies (at dilution of 1:200,
158 and 1:500, respectively). The immunohistochemical staining was performed as per the author's
159 previous report (Elewa, et al., 2016; Elewa, et al., 2014; Elewa, et al., 2018). Except for the
160 immunohistochemical staining of Gr-1, CD3, and CD31, the antigen retrieval was performed by
161 heating the section at 105 °C for 15 min in 10 mM CB (pH 6.0) solution. For Gr-1, the sections
162 were incubated at 37 °C for 5 min in 0.1% pepsin/0.2 M HCl. For CD3 and CD31, antigen retrieval
163 was performed by heating the section at 110 °C for 20 min in Tris solution.

164

165 *Morphometrical measurements:*

166 For histomorphometry, photomicrographs were captured from the H&E-, MT, and
167 immune- stained sections (three different tissue sections from mice of each group) at 200x
168 magnification using a BZ-X710 microscope. They were subjected to the following measurements
169 using the BZ-X analyzer (Keyence, Osaka, Japan) software:

170 (a) The percentage of ratios for islets area/pancreatic area; lymphoid clusters (LCs)
171 area/total mediastinal fat tissue (MFT) area; HEVs area/LCs area; LVs area/LCs area;
172 LVs area/lung field area; CD31⁺ capillary endothelial cells area/LCs area; and CD31⁺
173 capillary endothelial cells area/lung field area. Briefly, using the BZ-X analyzer, the

174 areas of the pancreatic islet, LCs, HEVs, LVs, CD31⁺ blood capillary were measured in
175 the captured photographs and divided into the corresponding field area of either
176 pancreas, MFT, LCs, or lung. The average ratios were then reported and compared
177 among the groups, as previously reported (Elewa, et al., 2014).

178 (b) The percentage of aniline blue⁺ collagen areas was measured from photographs of
179 MT-stained sections using the BZ-X analyzer and divided to that of the corresponding
180 field area of lung sections as previously revealed (Elewa, et al., 2018).

181 (c) The percentages of ratios for the positive area of immune cells (T-lymphocytes,
182 B-lymphocytes, macrophages, and granulocytes), TNF- α , and IL-5⁺ cells/LCs area was
183 measured in three different MFT sections/mice. To calculate the positive area ratios for
184 the immunopositive cells, the BZ-X analyzer was used to measure the positive area for
185 different immunopositive cells and divided it into the field area. Then the averages of
186 the percentages were calculated and compared between the two groups.

187 (d) The percentage of positive index ratios for insulin⁺ β - cells/islet area, immune
188 cells/lung field, TNF- α , IL-5⁺ cells/lung field, and CD31⁺ endothelial cells/large blood
189 vessels within the lung sections were compared among both the groups. The number of
190 immunopositive cells in five random fields within each of the three different
191 sections (at 200 \times magnification) was counted to calculate the positive index ratio. Then,
192 the number was divided by the area of the same corresponding field. Next, the average
193 index ratios/group was calculated and compared among both the groups.

194 Furthermore, the degree of lung injury among both the control and diabetic groups was
195 analyzed by comparing the average score of the grades for lung fibrosis based on a scale criterion
196 (Ashcroft, et al., 1988). Briefly, grading of different microscope fields was detected on a scale from
197 zero to 8 (grade 0: Normal lung structure; grade 1: Slight thickening of the alveolar wall with few
198 cellular infiltrations; grade 3: Slight thickening of the alveolar wall with moderate cellular
199 infiltrations; grade 5: Numerous cellular infiltrations with mild fibrosis, and clear damage to the

200 lung architecture; grade 7: Severe damage of lung tissue with clear fibrous masses; grade 8: Total
201 replacement of lung tissue with fibrous tissue). The average of odd number scores for different
202 microscopic fields/lung sections was recorded. The intervening even-numbered score was
203 considered in case of any suspicion while deciding between two odd-numbered grades.

204

205 *2.6. Statistical analysis:*

206 To compare the differences between diabetic and control groups, the Mann–Whitney *U*-test was
207 performed, and statistical significance was determined at *p* values ≤ 0.05 . All numerical data have
208 been presented as mean \pm standard error (SE). Moreover, to assess the correlation between two
209 variables, Spearman’s correlation was used (*significant value, $p \leq 0.05$; and ** highly significant
210 value, $p \leq 0.01$).

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227 **Results**

228 *Diabetic index*

229 Both blood glucose level and histoplanimetical measurements of the ratio of islet area/pancreatic
230 tissue, as well as insulin+ β cell index ratio (number of insulin+ β cell/islet area), were analyzed as
231 diabetic indices to confirm the diabetic induction following two rounds of STZ IP injection (5
232 consecutive days/round). The blood glucose level was measured in the serum collected at 15
233 weeks of age in diabetic and control groups (Figure 1a). The diabetic group showed a significantly
234 higher blood glucose level than the control group (Figure 1b). Moreover, immunohistochemical
235 staining of insulin revealed fewer insulin-positive β cells in the diabetic group's pancreatic islets
236 than that of the control group (Figure 1c). Histoplanimetical measurements of the pancreatic islet
237 in the diabetic group revealed a significant decrease in its size (ratio of islet area/pancreatic area)
238 (Figure 1d) and the insulin+ β cell index ratio as compared to the control group.

239

240 *Effect of diabetic induction on the morphological features of MFALCs and spleen/BW ratio*

241 In order to investigate the effect of diabetes induction on the body immune status, the LC/MFT
242 area ratio (as localized intrathoracic effect) and the spleen/BW ratios (as generalized effect) were
243 analyzed. Examination of H&E-stained mediastinal fat tissue sections revealed an increase in the
244 sizes of MFALCs in the diabetic group compared to those in the control group (Figure 2a). The
245 LC/MFT area ratio among both groups was also compared to investigate the degree of MFALCs
246 development. Interestingly, the diabetic group revealed a significantly higher LC/MFT area ratio
247 than the control groups (Figure 2b). On the other hand, no significant differences could be
248 observed in spleen/BW ratios between the two groups (Figure 2c).

249

250 *Effect of diabetic induction on the histopathological features of the lung*

251 The lung tissue among diabetic and control groups was compared to examine the effect of

252 diabetes induction on lung lesions' progression. Analysis of H&E-stained lung sections in the
253 control group revealed normal lung architecture with a thin alveolar wall, inter-alveolar septa, and
254 a limited number of immune cell infiltrations. However, the diabetic group's lungs showed large
255 accumulations of mononuclear cells, alveolar collapse, and some areas showed diffuse alveolar
256 damage (Figure 3a). Furthermore, the MT staining of diabetic lung sections revealed numerous
257 aniline blue⁺ collagen fibers accumulation specially around blood vessels, the bronchi, and
258 interalveolar spaces with the presence of numerous congested blood vessels. However, only a few
259 collagen fibers could be detected in the interalveolar spaces of the control group lung (Figure 3b).
260 Histopathological quantification of the degree of lung injury was done using a standardized
261 scoring system (Ashcroft, et al., 1988). Interestingly, the diabetic group showed a significantly
262 higher lung injury score than the control group (Figure 3c). Furthermore, the percentage of aniline
263 blue⁺ area of the diabetic group was significantly higher than that of the control group (Figure 3d).
264 To detect different types of collagen deposition, the lung sections from both groups were
265 immuno-stained with collagen I (Figure 4a) and col3A1 (Figure 4b) antibodies. Similar to the
266 MT-stained lung sections, more positively stained collagen fibers deposition was mainly observed
267 around the bronchi and large blood vessels of diabetic group than that of control group.

268

269 *Effect of diabetic induction on the immune cell populations within MFALCs and infiltration into* 270 *the lung*

271 To determine the effect of diabetes on the immune cell populations within both MFALCs and lung
272 tissue, immunohistochemical staining for B220-, CD3-, Iba1-, and Gr1- was performed. This
273 staining was done to detect B-lymphocytes, T-lymphocytes, macrophages, and granulocytes,
274 respectively. Immunohistochemical analysis B220-, CD3-, Iba1-, and Gr1-⁺ cells within the
275 MFALCs of the diabetic group revealed a significant increase (2.05, 1.46, 1.81, 5.37 fold,
276 respectively) in the ratio of immune cell⁺ area/LCs area as compared to that of the control group
277 (Figure 5a). Also, the diabetic group's lung showed more immune cell infiltration than that of the

278 control group. The percentages of immune cell⁺ index ratio were compared among both groups to
279 assess immune cell infiltrations into the lung. Interestingly, the diabetic group revealed a
280 significantly higher positive immune cell density than the control group. The diabetic group also
281 showed 4.03, 4.30, 3.30, 2.95-fold increase for the B220-, CD3-, Iba1-, and Gr1-⁺ cell density,
282 respectively, than that of the control group (Figure 5b).

283

284 *Histopathological correlations between the diabetic index, and parameters of the lung and*
285 *MFALCs*

286 The correlations between diabetic index (blood glucose level, and Insulin⁺ index ratio),
287 and parameters of the lung (lung injury score, aniline blue⁺ area, immune cells) and MFALCs
288 (MFALCs size, immune cell populations of “B-lymphocytes, T-lymphocytes, macrophages, and
289 granulocytes,” and the ratio of HEVs area) was analyzed. As shown in Table 1, significantly
290 positive correlations were observed between blood glucose and parameters of the lung and
291 MFALCs (except for the HEVs within MFALCs, where a non-significantly positive correlation
292 was observed). However, for the insulin⁺ index ratio, significant negative correlations to MFALCs
293 size, degree of lung injury, immune cells within MFALCs, and lung was indicated. Additionally,
294 significant positive correlations were observed between the HEVs and MFALCs size, and immune
295 cell populations within the MFALCs, as well as with lung parameters. Furthermore, a positive
296 correlation was detected between lung aniline blue⁺ area and immune cell populations with
297 MFALCs. Such correlations showed significant differences with all immune cell populations
298 except for the CD3⁺ T-lymphocytes.

299

300

301 *Immunohistochemical staining of the PNAd⁺ HEVs, LYVE-1⁺ LVs, and CD31⁺ endothelial cells*
302 *within MFALCs and lung*

303 Immunohistochemical staining for PNAd was performed to determine the effect of

304 diabetic induction on HEVs in the MFALCs and the lungs. PNAd⁺ HEVs were more abundant in
305 the MFALCs of the diabetic group than that of the control group (Figure 6a). However, such
306 PNAd⁺ HEVs were not detected in the lung of either group (data not shown). Quantitative
307 measurements of the PNAd⁺ HEVs area ratio within the MFALCs of the diabetic group showed
308 significantly higher percentages than that of the control group (Figure 6b).

309 Immunohistochemistry of the LYVE-1⁺ LVs demonstrated more developed LVs in both
310 MFALCs and the diabetic group's lung than that of the control group (Figure 7a). Similarly, the
311 morphometrical measurements of the ratio of LYVE-1⁺ LVs area within both MFALCs
312 (Figure 7b) and lung (Figure 7c) indicated significantly higher percentages in the diabetic group
313 than that in the control group.

314 To compare the abundance of blood capillaries in the MFALCs and the lungs of both
315 diabetic and control groups, immunohistochemical analysis for CD31⁺ endothelial cells was
316 performed. Interestingly, the MFALCs and lung of the diabetic group showed less CD31⁺ blood
317 capillary than that of the control group. Also, few CD31⁺ endothelial cells were observed in the
318 large blood vessels of the diabetic group lung than the control group (Figure 8a). Interestingly, the
319 quantitative index of the percentages of CD31⁺ capillary area ratio in the MFALCs (Figure 8b) and
320 the lungs (Figure 8c) of the diabetic group was significantly decreased compared to that in the
321 control group. Furthermore, a significant decrease in the index ratio of CD31⁺ in endothelial
322 cells/blood vessel walls within the diabetic lung was observed compared to that in the control group
323 (Figure 8d).

324 *Immunohistochemical staining of the proinflammatory cytokine (TNF- α , and IL-5) positive cells*
325 *within MFALCs and lung*

326 Immunohistochemical staining for the TNF- α ⁺ cells in MFALCs revealed that most of the
327 immune cells within the MFALCs reacted positively to TNF- α and the MFALCs of the diabetic group
328 showed more abundantly positive cells (Figure 9a). Histomorphometrically, a significantly higher

329 TNF- α ⁺ area ratio was observed in the MFALCs of the diabetic group as compared to that of the
330 control group (Figure 9b). Similarly, the control group's lung showed few TNF- α ⁺ cells, but more
331 abundant TNF- α ⁺ cells (mainly macrophages, neutrophils, and lymphocytes) were observed in the
332 diabetic group's lung. Furthermore, a positive TNF- α reaction was observed in the endothelial cells
333 lining the blood vessels and epithelial lining of the bronchiole in the diabetic group (Figure 9a).
334 TNF- α ⁺ index ratio in the diabetic group's lungs was significantly higher than those in the control
335 group (Figure 9c).

336 Immunohistochemical staining for the IL-5⁺ cells in the MFALCs revealed that most of the
337 immune cells within the MFALCs reacted positively to IL-5 and the MFALCs of the diabetic group
338 showed higher IL-5⁺ cells than the control group (Figure 10a). Quantitative measurement of the IL-5⁺
339 area ratio in the MFALCs of the diabetic group revealed a higher percentage than the control group,
340 though the difference was non-significant (Figure 10b). Also, more abundant IL-5⁺ cells were
341 observed in the diabetic lung than that of the control group. The immunopositivity was mainly
342 observed in macrophage, neutrophils, and lymphocytes (Figure 10a). Interestingly, a significantly
343 higher IL-5⁺ index ratio was observed in the diabetic group's lungs than those in the control group
344 (Figure 10c).

345 *Histopathological correlations between blood glucose level and both vessel areas and*
346 *proinflammatory markers in the MFALCs and lung of diabetic and control groups*

347 The pathological correlations between blood glucose level and vessel areas (CD31⁺ blood
348 capillaries and LYVE-1⁺ LVs) in MFALCs and lung were examined (Figure 11a). Significantly
349 negative correlations were observed between blood glucose level and blood capillary areas within
350 both MFALCs and lungs. However, significant positive correlations were reported between blood
351 glucose levels and blood capillaries in both MFALCs and lungs.

352 Furthermore, the blood glucose level showed a significantly positive correlation with
353 proinflammatory cytokines (TNF- α and IL-5) within both MFALCs and lung (Figure 11b).

354 **Discussion**

355 DM is a chronic metabolic disease characterized by an abnormality in carbohydrate
356 metabolism and an increased blood glucose level. This may be due to impaired body ability to
357 produce or respond to the insulin hormone and is classified into either type 1 or type 2 DM,
358 respectively (Bajpai, 2018; Kolluru, et al., 2012). STZ is an alkylating agent, previously used as a
359 chemotherapeutic substance for treating pancreatic islet tumors because of its preferential
360 accumulation in pancreatic β -cells via the GLUT2 transporter system, which led to the cytotoxicity of
361 β -cells (Eleazu, et al., 2013; Ventura-Sobrevilla, et al., 2011). STZ is one of the most widely used
362 diabetogenic drugs that induce diabetes in experimental animal models because of their selectively
363 damaging effect on β -cells (Akbarzadeh, et al., 2007; Goud, et al., 2015).

364 In addition to the toxic effect of STZ on β -cells, STZ toxicity has also been reported from
365 other vital organs (including kidney, eye, nerves, and brain), leading to the development of diabetic
366 nephropathy, retinopathy, neuropathy, and cerebrovascular diseases (Fong, et al., 2004; Kario, et al.,
367 2005; Tervaert, et al., 2010; Tesfaye, et al., 2005). On the other hand, and unlike other vital organs,
368 the lungs have been reported to be less sensitive to the toxicity of STZ (Samarghandian, et al., 2014).
369 Despite that fact, pulmonary dysfunction and dyspnea have been associated with Type I DM (Scano,
370 et al., 1999). Interestingly, it has been established that the progression of lung damage and asthma
371 associated with DM is owed to an increased level of inflammatory mediators and oxidative stress in
372 the bronchoalveolar lavage fluid (Samarghandian, et al., 2014).

373 The current investigation indicated the development of lung injury and fibrosis in the
374 diabetic group compared to the control group. Similarly, recent reports revealed that alteration in the
375 genes involved in stress and collagen leads to the lungs' functional impairment in
376 streptozotocin-induced diabetic rats (Talakatta, et al., 2018; van Lunteren, et al., 2014). Also,
377 pulmonary interstitial fibrosis has been reported to develop in STZ-induced diabetic rat following
378 hyperglycemia (Talakatta, et al., 2018; Zou, et al., 2017). Furthermore, the author's previous reports
379 have suggested the possible role of MFALCs in the pathogenesis of lung injury and fibrosis in both

380 autoimmune disease mice model (Elewa, et al., 2016) and bleomycin-induced pneumonitis mice
381 model (Elewa, et al., 2018). However, the role of MFALCs in the progression of lung injury in the
382 STZ-induced diabetic mice model and the effect of diabetes on the pathological alterations of
383 MFALCs is not yet clarified. Interestingly, more developed MFALCs were observed in the diabetic
384 group than that of the control group in the present study population. Besides, a significantly positive
385 correlation was observed between the progression of lung injury and the size of MFALCs and the
386 blood glucose level as a diabetic index, suggesting that MFALCs could play a role in the pathogenesis
387 of lung lesions in DM. However, further investigations are required to clarify if reprogramming
388 MFALCs through targeting some immune cells/ LVs or HEVs could help in controlling the lung
389 injury development.

390 The present study revealed a significant increase in the quantitative index of immune cells (mainly for
391 B-lymphocytes, T-lymphocytes, and macrophages) and the TNF- α and IL-5⁺ cells in both MFALCs
392 and lung of the diabetic group as compared to the control group. Similarly, intense cellular infiltration
393 has been reported in the interstitial tissue of the lungs of diabetic group rats compared to the control
394 rats (Talakatta, et al., 2018). Primarily, macrophages and monocytes produce TNF- α (Aggarwal, et
395 al., 2012; Suzuki, et al., 2013). Interestingly, the role of adipose tissue macrophages in FALCs
396 formation has been reported. Such a function is because the adipose tissue macrophages are
397 considered the primary TNF source, which stimulates TNF receptor on the stromal cells (Benezech,
398 et al., 2015). Furthermore, other functions of TNF- α have been illustrated, including promoting
399 leukocyte accumulation and tissue remodeling (Aggarwal, 2003; Mukhopadhyay, et al., 2006).
400 Additionally, IL-5 has been reported to regulate B1 cells' self-renewal and B cell antibody production
401 (Erickson, et al., 2001).

402 Angiopathy or abnormal angiogenesis is one of DM's major complications (Martin, et al.,
403 2003; Xu, et al., 2012). Interestingly, a complicated alveolar-capillary network was observed in the
404 healthy lung. Microvascular damage was observed in such capillary network in the lung in
405 association with DM and led to an increased risk of several pulmonary diseases (De Santi, et al.,

406 2017; Ehrlich, et al., 2010). The present study revealed a capillary defect in the MFALCs and lung of
407 the diabetic group. Simultaneously, a significant decrease in the CD31+ capillary endothelial lining
408 cells of the diabetic group than the control group was also found. Similarly, endothelial cell
409 dysfunction has been reported in patients with DM (Kolluru, et al., 2012).

410 On the other hand, the MFALCs and lung of the diabetic group showed a significant increase
411 in the LVs area ratios than that of the control group in the present study. Also, a significantly positive
412 correlation between blood glucose level and the quantitative index of LVs was observed.
413 Interestingly, it has been clarified that the pulmonary LVs could transport a larger number of immune
414 cells than do other LVs in different organs because of the lack of smooth muscle cell coverage of
415 pulmonary collecting LVs, and thus showed more permeability with facilitated cellular extravasation
416 (Reed, et al., 2019). Therefore, the authors suggest that the LV development observed in the
417 MFALCs and lungs of the diabetic group could play a significant role in immune cell trafficking
418 among them, and in turn, provide a possible mechanism in lung injury progression associated with
419 DM induction.

420 HEVs are specialized post-capillary venules that play an essential role in lymphocyte
421 migration into and out of the lymph nodes, tonsils, Peyer's patches, and mucosa-associated lymphoid
422 tissue (MALT) as secondary lymphoid structures (Girard & Springer, 1995). Additionally, previous
423 studies by the authors have revealed the role of HEVs in MFALC development in both healthy
424 (Elewa, et al., 2014) and diseased (Elewa, et al., 2016; Elewa, et al., 2018) conditions. Similarly, the
425 present study revealed more developed HEVs in the MFALCs of the diabetic group than in the
426 control group. It showed a significantly positive correlation with MFALC development and lung
427 injury progression, suggesting the possible role of such venules in the intrathoracic immune
428 hemostasis associated with diabetic conditions.

429 **Conclusion**

430 We have investigated the effects of diabetes induction on the pathological alterations of MFALCs

431 and their possible role in lung injury progression. The diabetic group showed a significant increase
432 in MFALCs size, immune cells, inflammatory cytokines, HEVs, and LVs area compared with the
433 control group. Furthermore, significant positive correlations were observed between MFALCs size,
434 and quantitative indices of MFALCs parameters, and both the degree of lung injury and parameters
435 of the lung. The data also revealed significantly positive correlations between blood glucose levels
436 and quantitative parameters of LVs and HEVs. A significantly negative correlation was observed
437 between blood glucose level and quantitative parameters of blood capillaries. Therefore, the present
438 study hypothesized the possible role of MFALCs development as an additional mechanism in the
439 pathogenesis of lung injury progression in diabetic mice. However, further investigations are
440 required to clarify various signals involved in the development of MFALCs and the possible role of
441 different cell populations within MFALCs in the mechanism of lung injury progression following
442 diabetic conditions. Understanding such signals and the role of various immune cells within
443 MFALCs could provide some therapeutic approaches for advancing lung lesions associated with
444 metabolic disorders such as diabetes by targeting MFALCs.

445

446 **ACKNOWLEDGMENTS**

447 This research was sponsored by Hokkaido University Tenure Track Program and a grant-in-aid for
448 the Japan Society for the Promotion of Science KAKENHI (No 20K07420).

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581

582 **Figure 1.** Experimental design and diabetic index: **(a)** Scheme of the experimental protocol
583 showing schedule for intraperitoneal injection of either streptozotocin (STZ) or 0.05 M sodium
584 citrate buffer (CB) in the diabetic group (DG) and control group (CG), respectively. **(b)** Blood
585 glucose level. **(c)** Immunohistochemical staining of insulin⁺ β -cells. **(d)** Ratio of islet
586 area/pancreatic area. **(e)** Index ratio of insulin-positive β -cells. Comparison between the control and
587 diabetic groups (n = 5 mice in each group), analyzed by the Mann–Whitney *U*-test ($p \leq 0.05$).
588 Statistically significant difference indicated by an asterisk (*). Values = mean \pm SE.

589

590 **Figure 2.** Degree of MFALC development and spleen/body weight (BW) ratio: **(a)** H&E-stained
591 mediastinal fat tissue (MFT), MFALCs (arrows). **(b)** the ratio of LC area/total MFT. **(c)** Percentage
592 of the spleen/BW ratio. Comparison between the control and diabetic groups (n = 5 mice of each
593 group), analyzed by the Mann–Whitney *U*-test ($p \leq 0.05$). Statistically significant difference
594 indicated by an asterisk (*). Values = mean \pm SE.

595

596 **Figure 3.** Histopathological features of the lungs **(a)** H&E-stained lung tissue. **(b)** Masson's
597 trichrome staining of lung sections showing aniline blue⁺ collagen fibers (arrows), congested blood
598 vessels (BV). **(c)** Lung injury score. **(d)** Comparison of the percentage of aniline blue⁺ collagen
599 areas in lung tissues of diabetic and control groups. Comparison between the control and diabetic
600 groups (n = 5 mice of each group), analyzed by the Mann–Whitney *U*-test ($p \leq 0.05$). Statistically
601 significant difference indicated by an asterisk (*). Values = mean \pm SE.

602

603 **Figure 4.** Collagen fibers deposition in the lungs. **(a)** Immunohistochemical staining of lung
604 sections with the collagen I antibody. **(b)** Immunohistochemical staining of lung sections with the
605 Col3A1 antibody. Diabetic group showed more deposition of collagen in the lung tissue around the
606 large blood vessels and in the peri-bronchial tissue.

607

608

609 **Figure 5.** Immune cell populations in mediastinal fat-associated lymphoid clusters (MFALCs) and
610 lungs of diabetic and control groups. **(a)** Percentages of the immune cells (B-lymphocytes,
611 T-lymphocytes, macrophages, and granulocytes) positive area ratio in MFALCs. **(b)** Percentages of
612 the immune cells (B-lymphocytes, T-lymphocytes, macrophages, and granulocytes) positive index
613 ratio in lung tissues. Comparison between the control and diabetic groups (n = 5 mice of each
614 group), analyzed by the Mann–Whitney *U*-test ($p \leq 0.05$). Statistically significant difference
615 indicated by an asterisk (*). Values = mean \pm SE.

616

617 **Figure 6.** The occurrence of high endothelial venules (HEVs) in MFALCs of diabetic and control
618 groups. **(a)** Immunohistochemical images depict PNA⁺ HEVs in MFALCs. Arrows indicate
619 PNA⁺ HEVs in MFALCs. **(b)** Percentage of the ratio of HEVs area within the MFALCs.
620 Comparison between the control and diabetic groups (n = 5 mice of each group), analyzed by the
621 Mann–Whitney *U*-test ($p \leq 0.05$). Statistically significant difference indicated by an asterisk (*).
622 Values = mean \pm SE.

623

624 **Figure 7.** Lymphatic vessels (LVs) in MFALCs and lung of diabetic and control
625 groups. **(a)** Immunohistochemical staining for the LVs marker “LYVE-1. Arrows indicate LYVE-1⁺
626 LVs. **(b)** Percentage of the LYVE-1⁺ LVs area ratio within the MFALCs. **(c)** Percentage of the ratio
627 of LYVE-1⁺ LVs area within the lung. Comparison between the control and diabetic groups (n = 5
628 mice of each group), analyzed by the Mann–Whitney *U*-test ($p \leq 0.05$). Statistically significant
629 difference indicated by an asterisk (*). Values = mean \pm SE.

630

631 **Figure 8.** Blood capillaries in MFALCs and lung of diabetic and control
632 group. **(a)** Immunohistochemical staining for the endothelial cell marker “CD31”. Notice CD31⁺
633 endothelial cells lining the blood capillaries (arrows) and blood vessels (black arrowheads), and

634 CD31 negative endothelial cells (red arrowheads). **(b)** Percentage of the ratio of LYVE-1⁺ LVs area
635 within the MFALCs. **(c)** Percentage of the ratio of CD31⁺ capillary area within the lung. **(d)** Index
636 ratio of CD31⁺ endothelial cells/blood vessel wall within the lung. Comparison between the control
637 and diabetic groups (n = 5 mice of each group), analyzed by the Mann–Whitney *U*-test ($p \leq 0.05$).
638 Statistically significant difference indicated by an asterisk (*). Values = mean \pm SE.

639

640 **Figure 9.** Analysis of the proinflammatory cytokines (TNF- α) in MFALCs and lung of diabetic and
641 control groups. **(a)** Immunohistochemical staining for the TNF- α ⁺ cells in MFALCs and lungs.
642 Notice the positive reaction for the neutrophils (black arrowheads), macrophages (red arrowheads),
643 lymphocytes (green arrowheads), lining epithelium of bronchiolar wall (B). The diabetic lung
644 showed TNF- α ⁺ reaction in the lining epithelium of bronchioles (black arrows) and the endothelial
645 cells lining the blood vessels (red arrows). **(b)** Percentages of the TNF- α positive area ratio in
646 MFALCs. **(c)** Percentages of the TNF- α positive index ratio in lung tissues. Comparison between
647 the control and diabetic groups (n = 5 mice of each group), analyzed by the Mann–Whitney *U*-test
648 ($p \leq 0.05$). Statistically significant difference indicated by an asterisk (*). Values = mean \pm SE.

649

650 **Figure 10.** Analysis of the proinflammatory cytokines (IL-5) in MFALC and lung of diabetic and
651 control group. **(a)** Immunohistochemical staining for the IL-5⁺ cells in MFALCs and lungs. Notice
652 the positive reaction for neutrophils (black arrowheads), macrophages (red arrowheads),
653 lymphocytes (green arrowheads). **(b)** Percentages of the IL-5 positive area ratio in MFALCs. **(c)**
654 Percentages of the IL-5 positive index ratio in lung tissues. Comparison between the control and
655 diabetic groups (n = 5 mice of each group), analyzed by the Mann–Whitney *U*-test ($p \leq 0.05$).
656 Statistically significant difference indicated by an asterisk (*). Values = mean \pm SE.

657

658 **Figure 11.** Correlations between blood glucose level, vessel markers, and proinflammatory markers
659 in MFALCs and the control and diabetic groups' lungs. **(a)** Spearman's correlation test for the

660 correlation between blood glucose level and relative area ratio of vessel markers (CD31⁺ blood
661 capillary and LYVE-1⁺ LVs). **(b)** Spearman's correlation test for the correlation between blood
662 glucose level and the relative ratio of proinflammatory cytokines (TNF- α and IL-5). ρ : Spearman's
663 rank-order correlation coefficient, $n = 5/\text{group}$, and 10 for both groups. * Significant value, $p \leq 0.05$;
664 ** highly significant value, $p \leq 0.01$.

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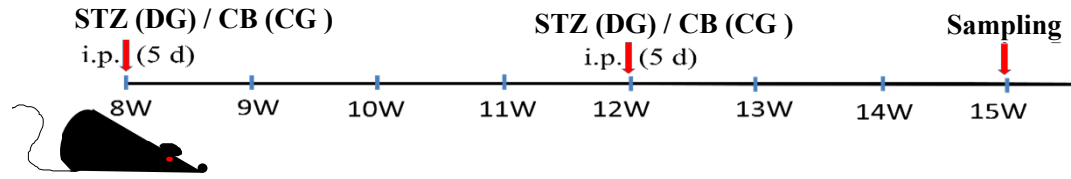
668

Table 1: Spearman correlations between diabetic indices and parameters for both MFALCs and lung in control and diabetic mice groups.

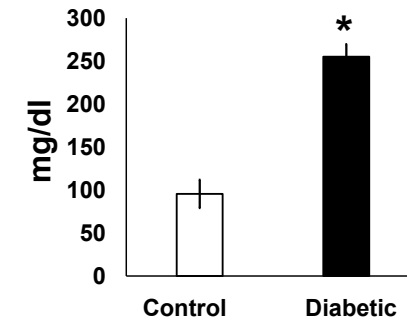
Table 1		Parameters for diabetic indices			Parameters for MFALCs					
			Blood glucose	Insulin+ cell density	MFALC	B220	CD3	Iba1	Gr1	HEVs
Parameters for diabetic index	Blood glucose	ρ	-	-0.906**	0.796**	0.669*	0.802**	0.769**	0.802**	0.584
		P	-	0.001	0.006	0.035	0.005	0.001	0.005	0.07
	Insulin+ cell density	ρ	-0.906**	-	-0.842**	-0.806**	-0.879**	-0.879**	-0.782**	-0.612
		P	0.001	-	0.002	0.005	0.001	0.001	0.008	0.060
Parameters for lung	Lung aniline blue positive area	ρ	0.681*	-0.685*	0.818**	0.758*	0.539	0.685*	0.709*	0.794**
		P	0.030	0.029	0.004	0.011	0.108	0.029	0.022	0.006
	Lung injury score	ρ	0.740*	-0.774**	0.774*	0.817**	0.634**	0.756**	0.591*	0.799**
		P	0.014	0.009	0.014	0.004	0.004	0.001	0.072	0.006
	B220	ρ	0.699*	-0.794**	0.673*	0.964**	0.527	0.733*	0.661*	0.818**
		P	0.024	0.006	0.033	0.001	0.117	0.016	0.038	0.004
	CD3	ρ	0.815**	-0.855**	0.952**	0.733*	0.770**	0.733*	0.758*	0.673*
		P	0.004	0.002	0.001	0.016	0.009	0.016	0.011	0.033
	Iba1	ρ	0.796**	-0.903**	0.794**	0.758**	0.855**	0.976**	0.842**	0.661*
		P	0.006	0.001	0.006	0.011	0.002	0.001	0.002	0.038
	Gr1	ρ	0.802**	-0.782**	0.855**	0.612	0.697**	0.782**	1.000**	0.697*
		P	0.005	0.008	0.002	0.060	0.025	0.008	0.001	0.025

MFALC: % of LCs area/total MFTs area. ρ : Spearman's rank order correlation coefficient. n= 5/group (control, and diabetic groups). *: Significant, $P < 0.05$. **: highly significant, $P < 0.01$.

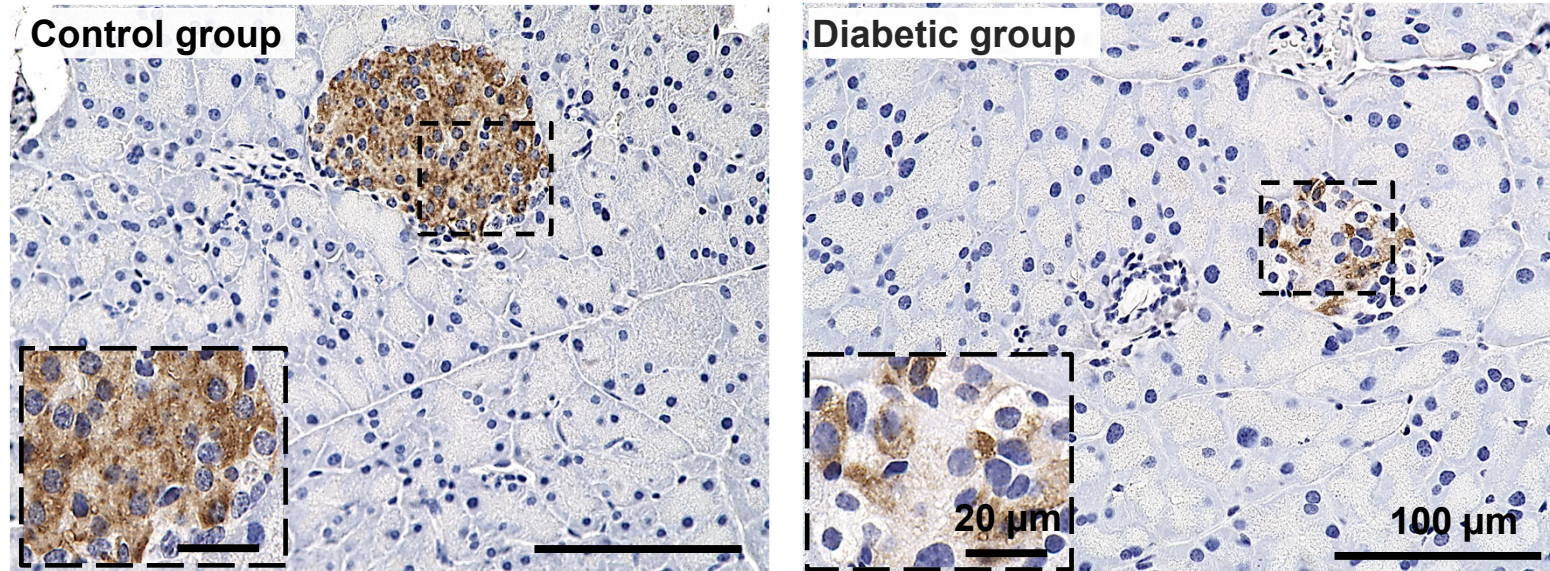
a) Experimental protocol



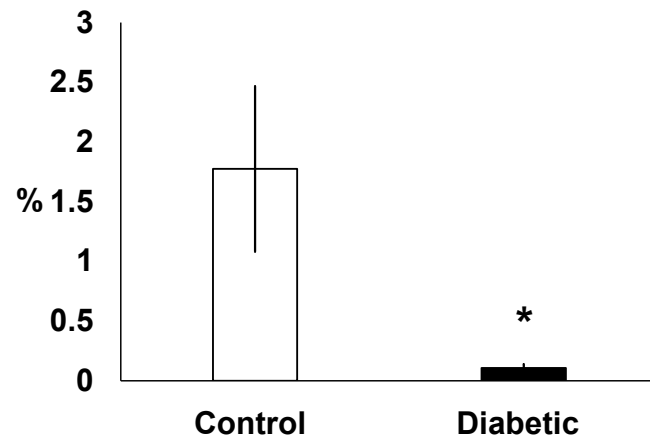
b) Blood glucose level



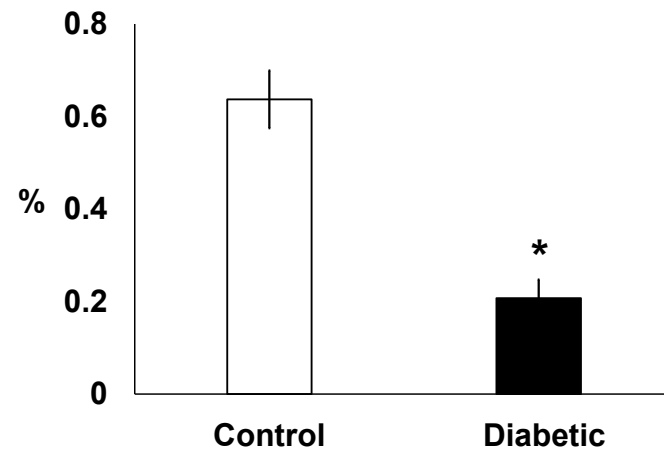
c) Immunohistochemical staining of insulin positive beta cells



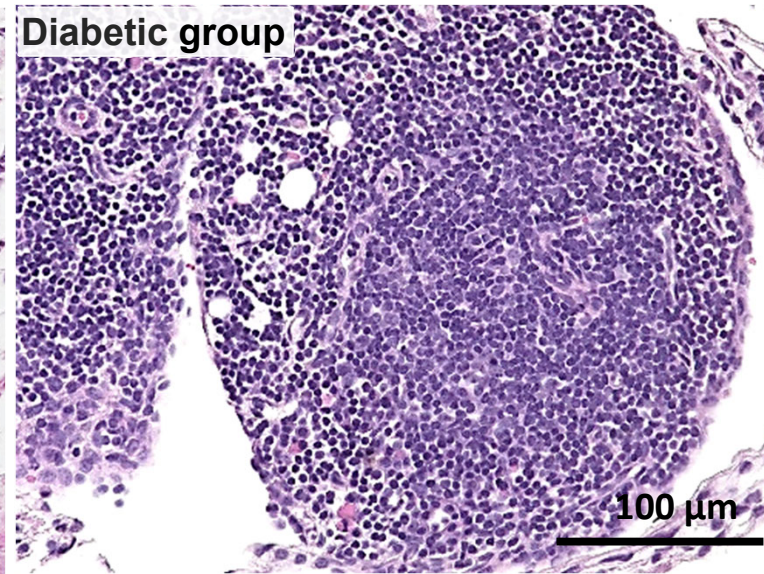
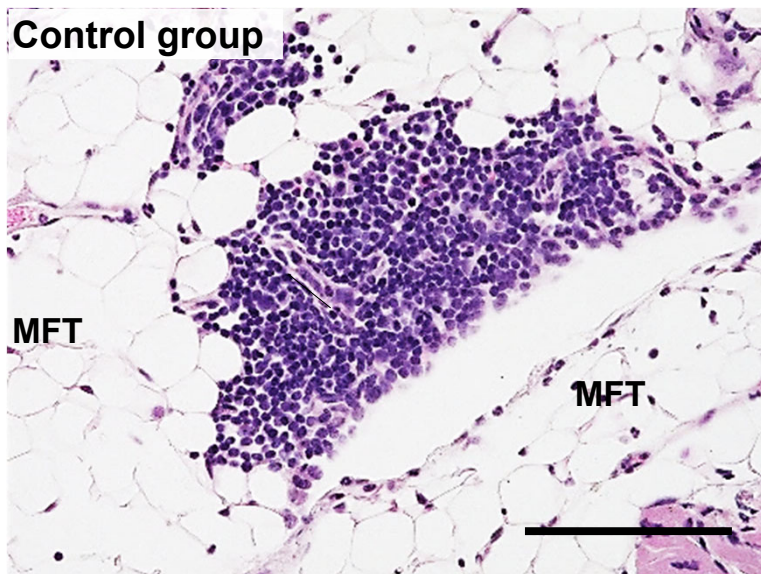
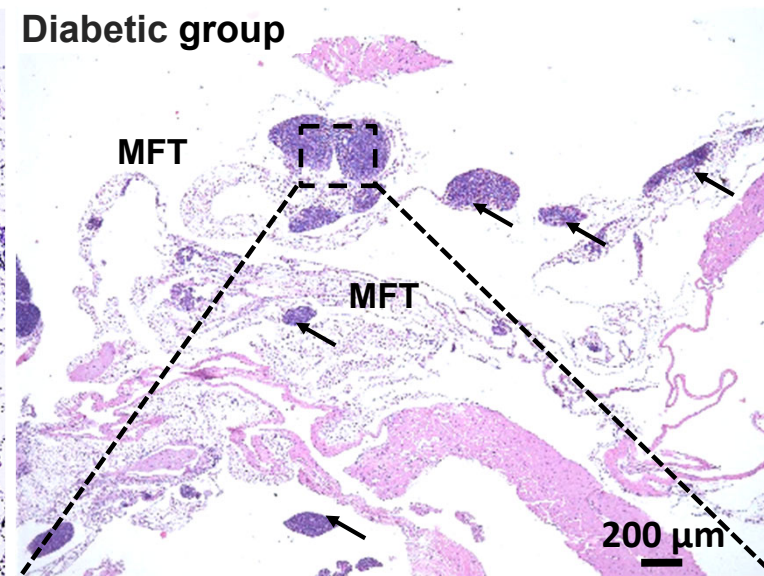
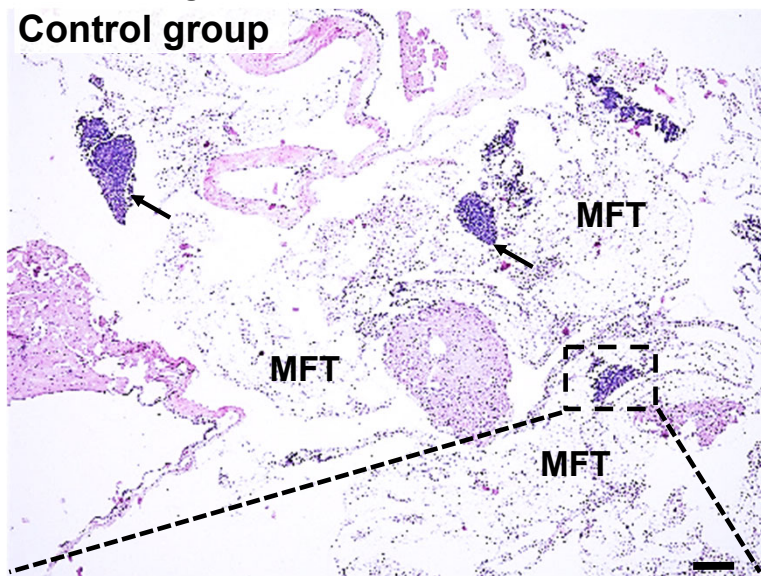
d) Ratio of islet area/ pancreatic area (μm^2)



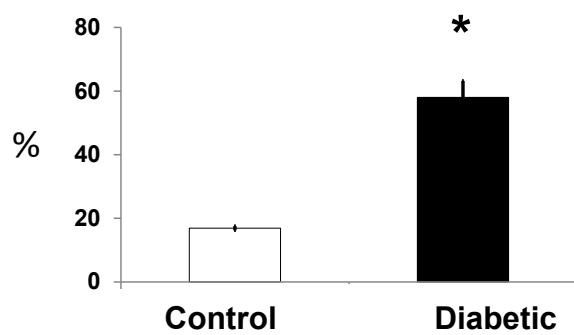
e) β - cells Index ratio (Insulin positive number/ islet area)



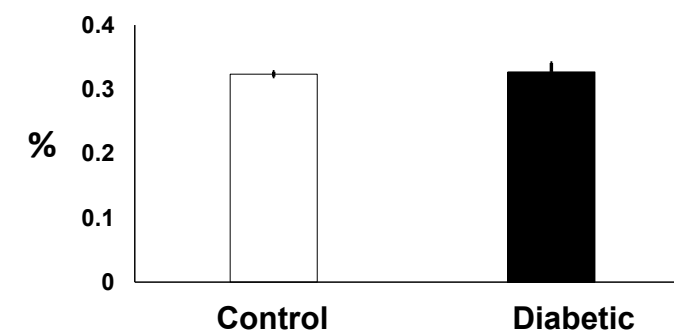
a) H&E staining of mediastinal fat tissue

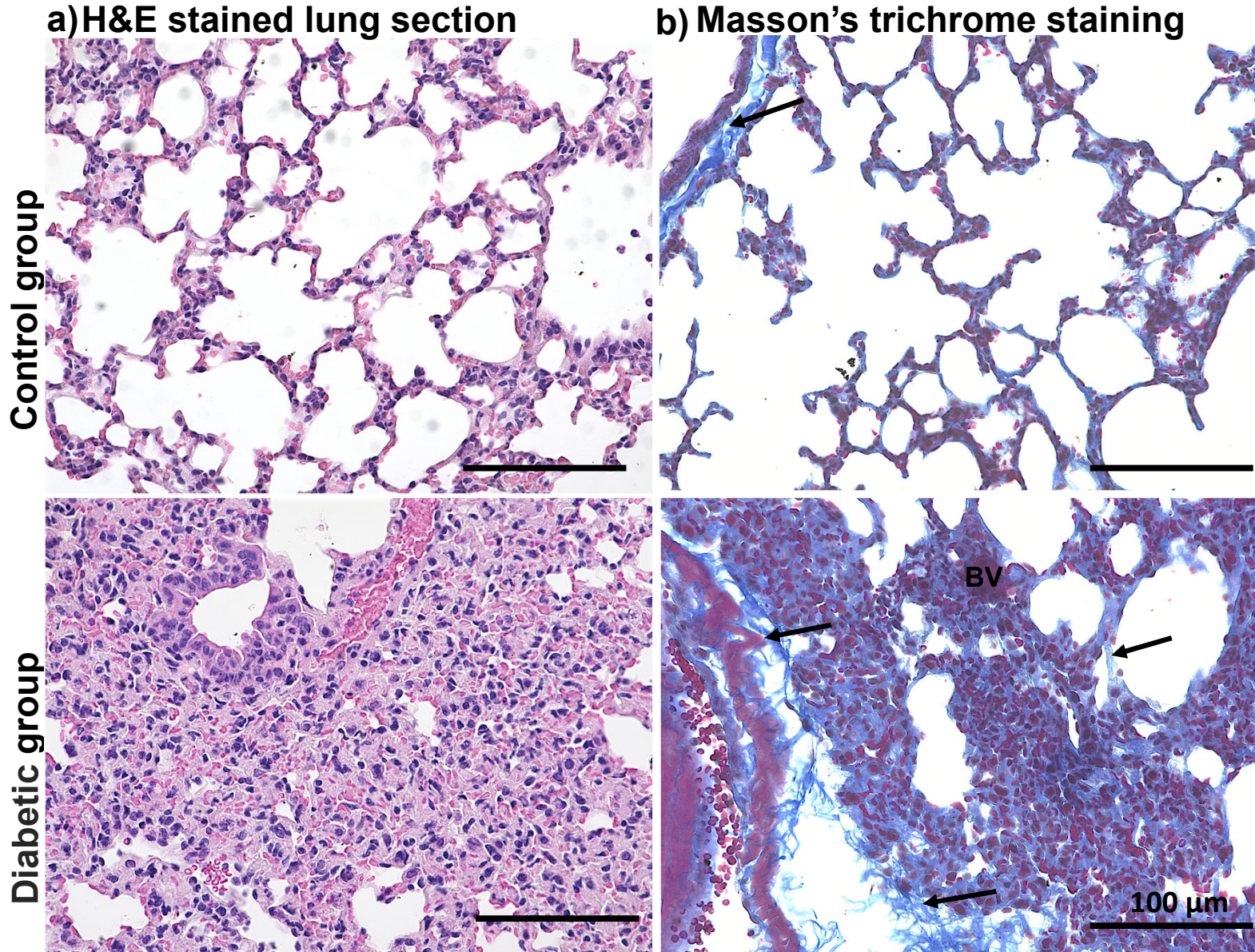


b) Ratio of lymphoid clusters area/ total MFT area (μm^2)

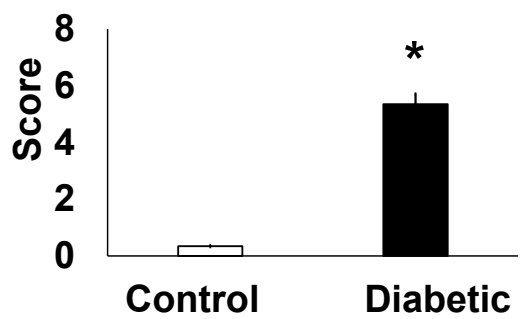


c) Spleen/ B.W. ratios

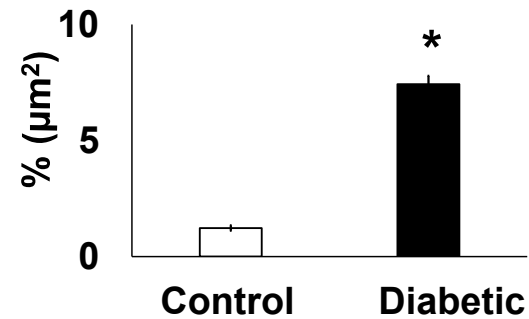




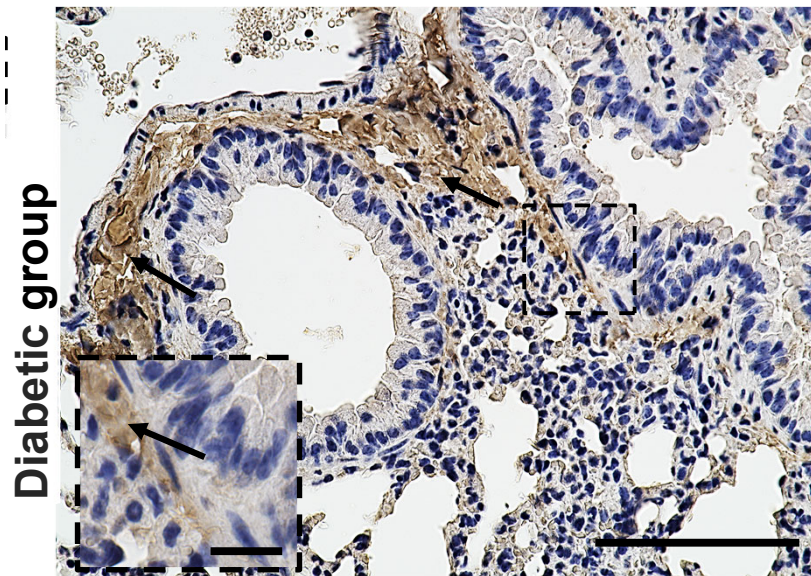
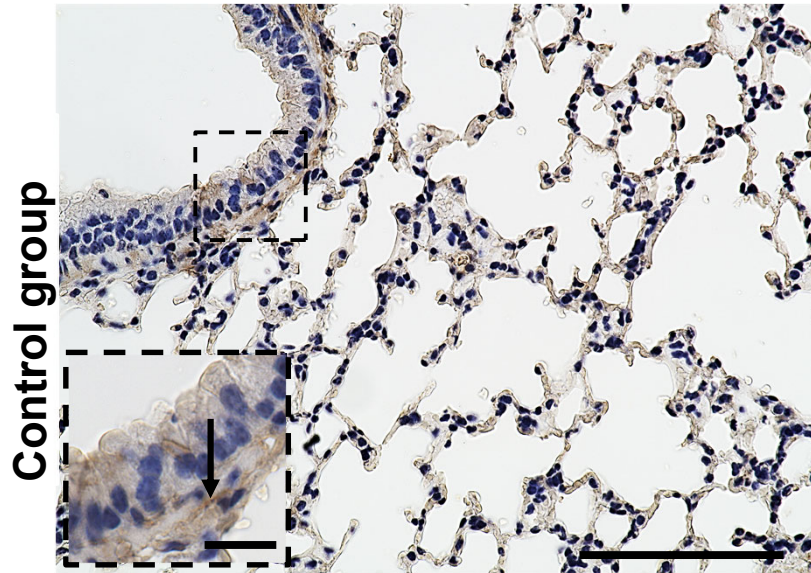
c) Lung injury score



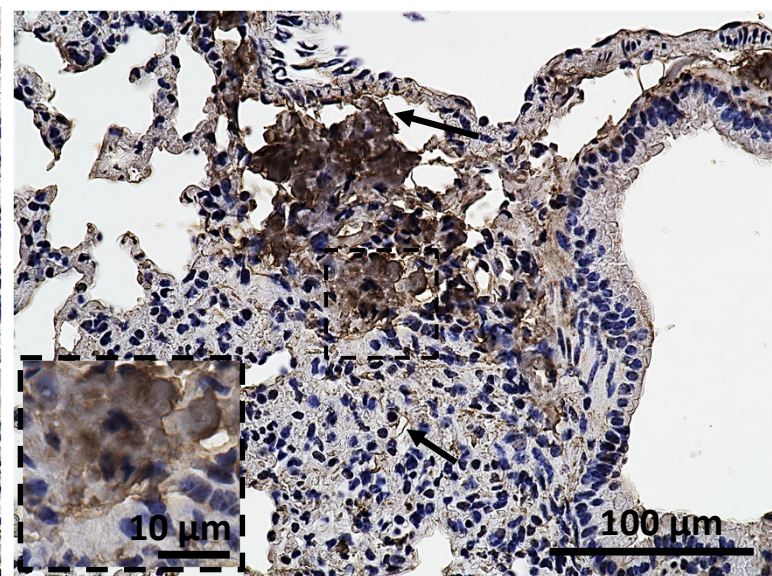
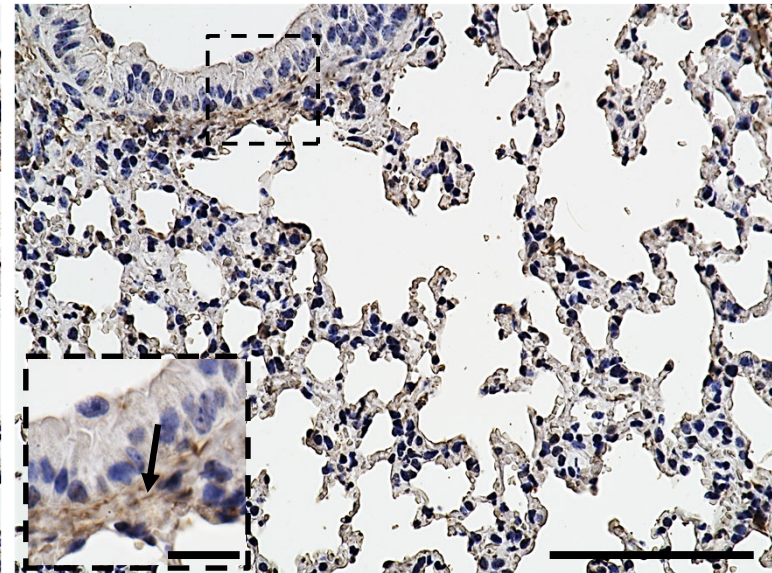
d) Percentage of aniline blue+ area



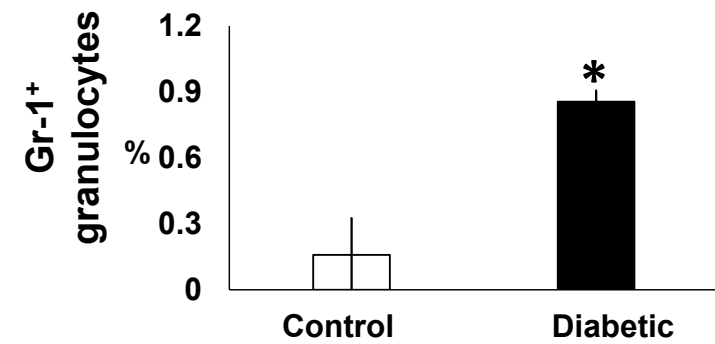
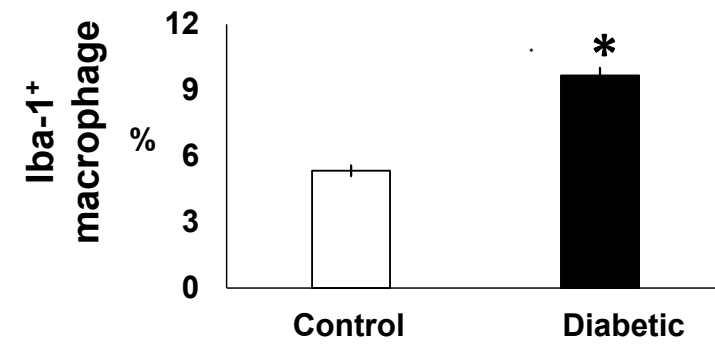
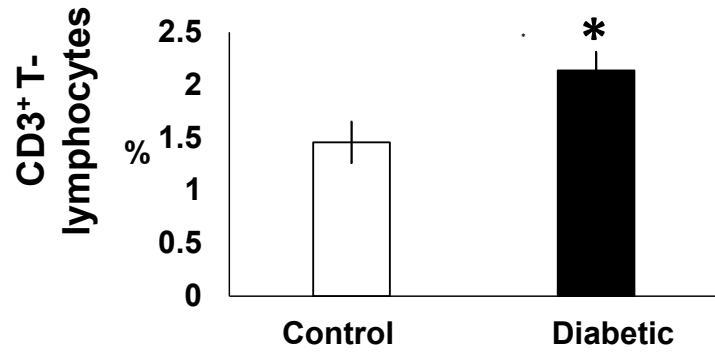
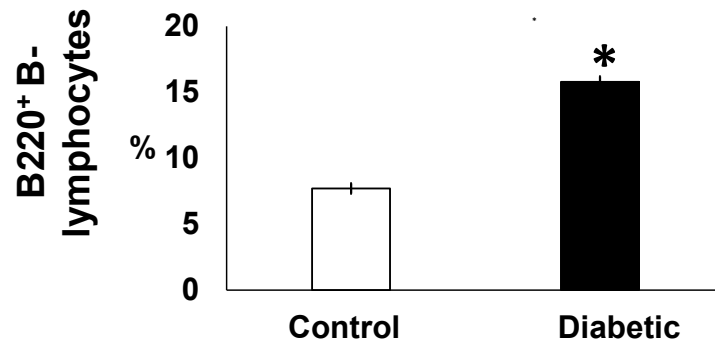
a) Collagen I immunostaining



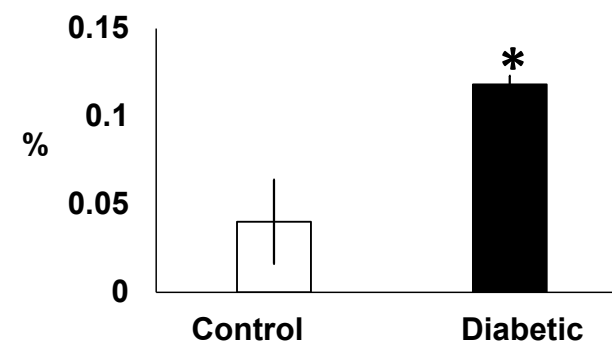
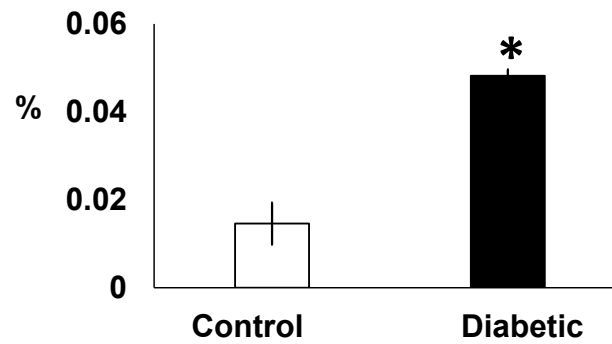
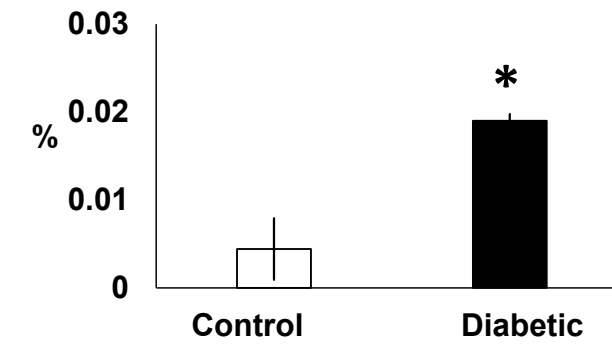
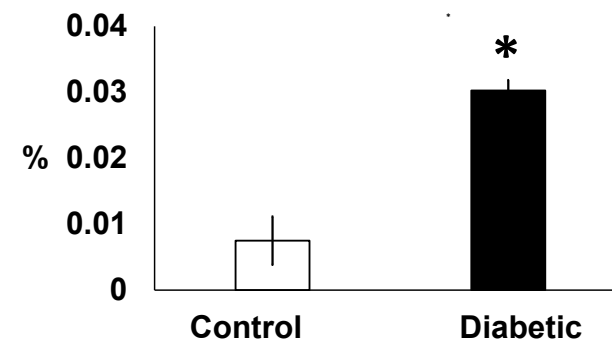
b) Col3A1 immunostaining



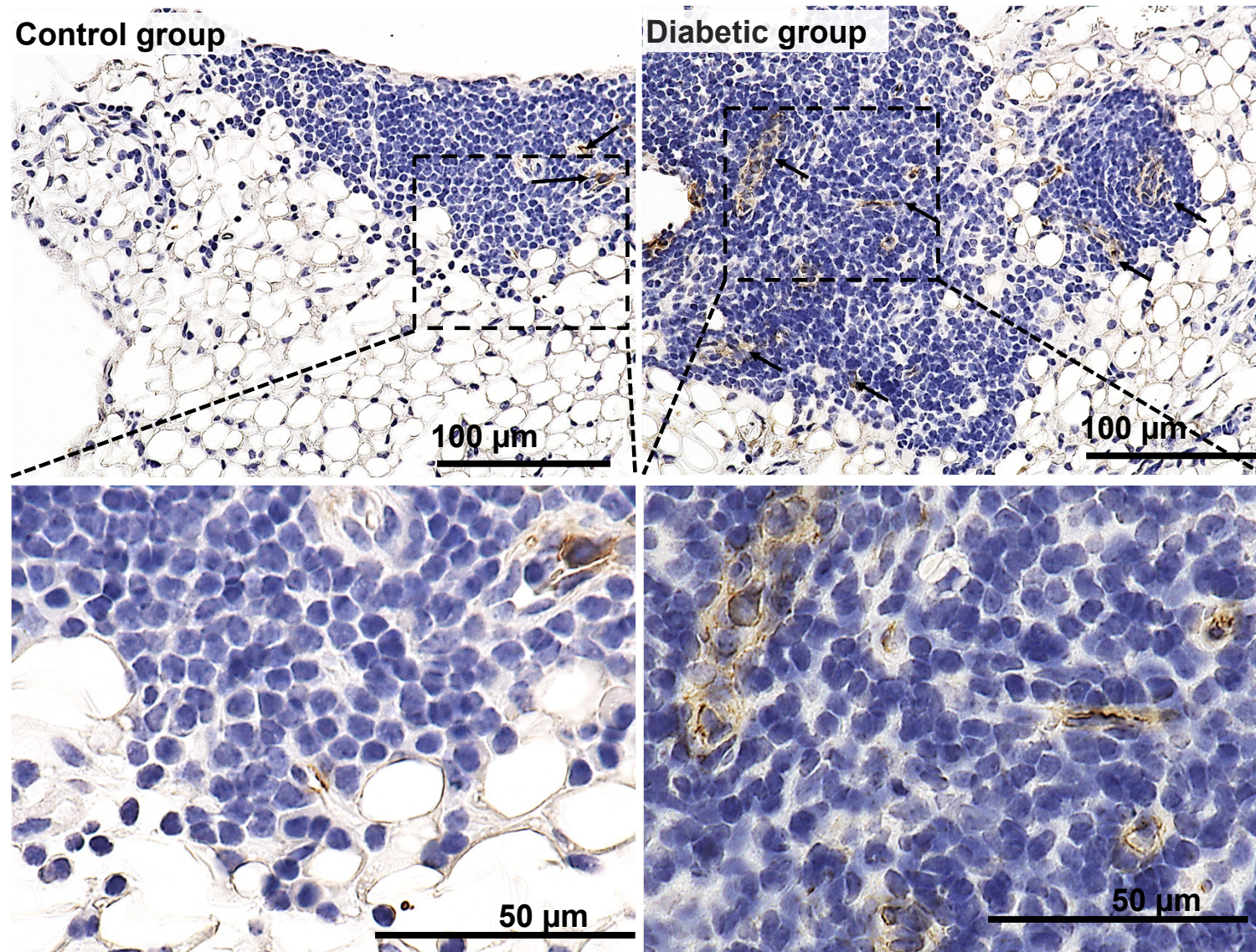
a) Immune cells positive area ratio in MFALCs (μm^2)



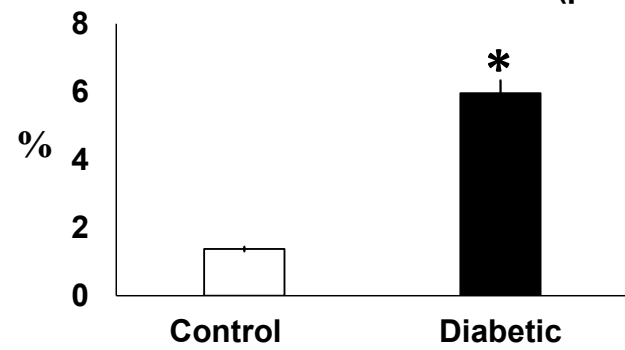
b) Immune cells positive index ratio in the lung (μm^2)



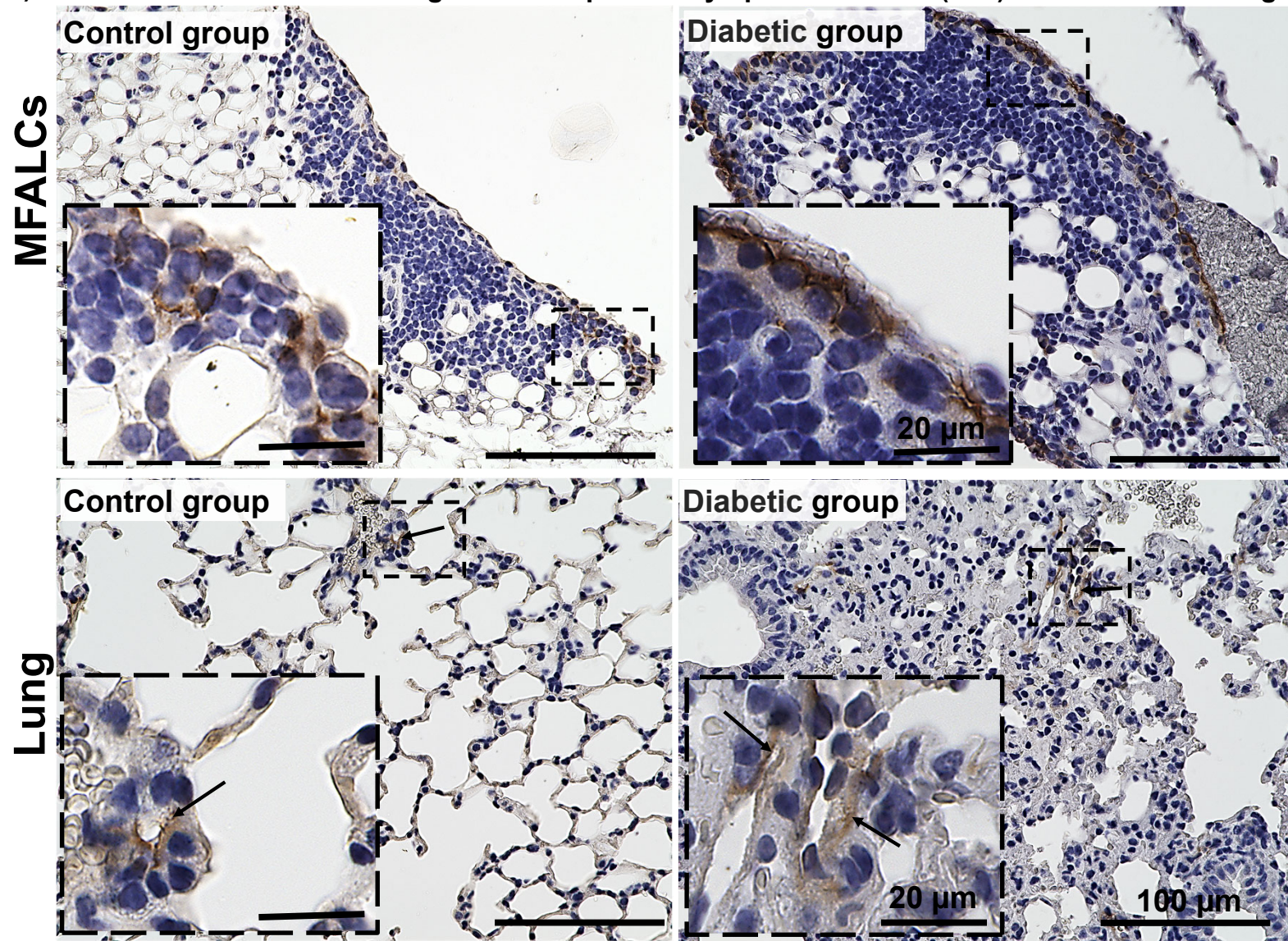
a) Immunohistochemical staining of PNA^d positive high endothelial venules in MFALCs and lung



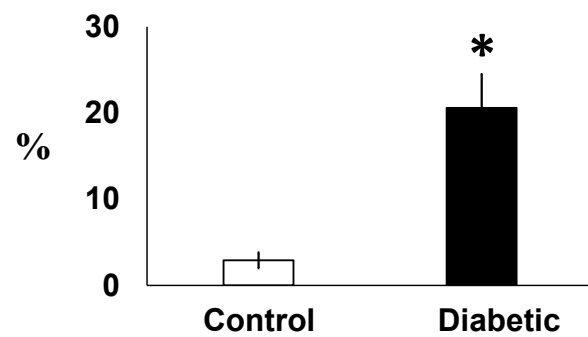
b) Ratio of HEVs area/ cluster area (μm^2)



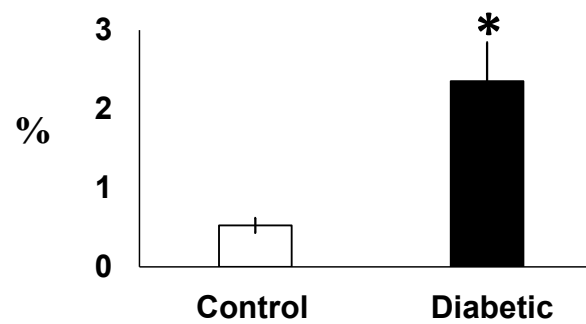
a) Immunohistochemical staining of LYVE-1 positive lymphatic vessels (LVs) in MFALCs and lung



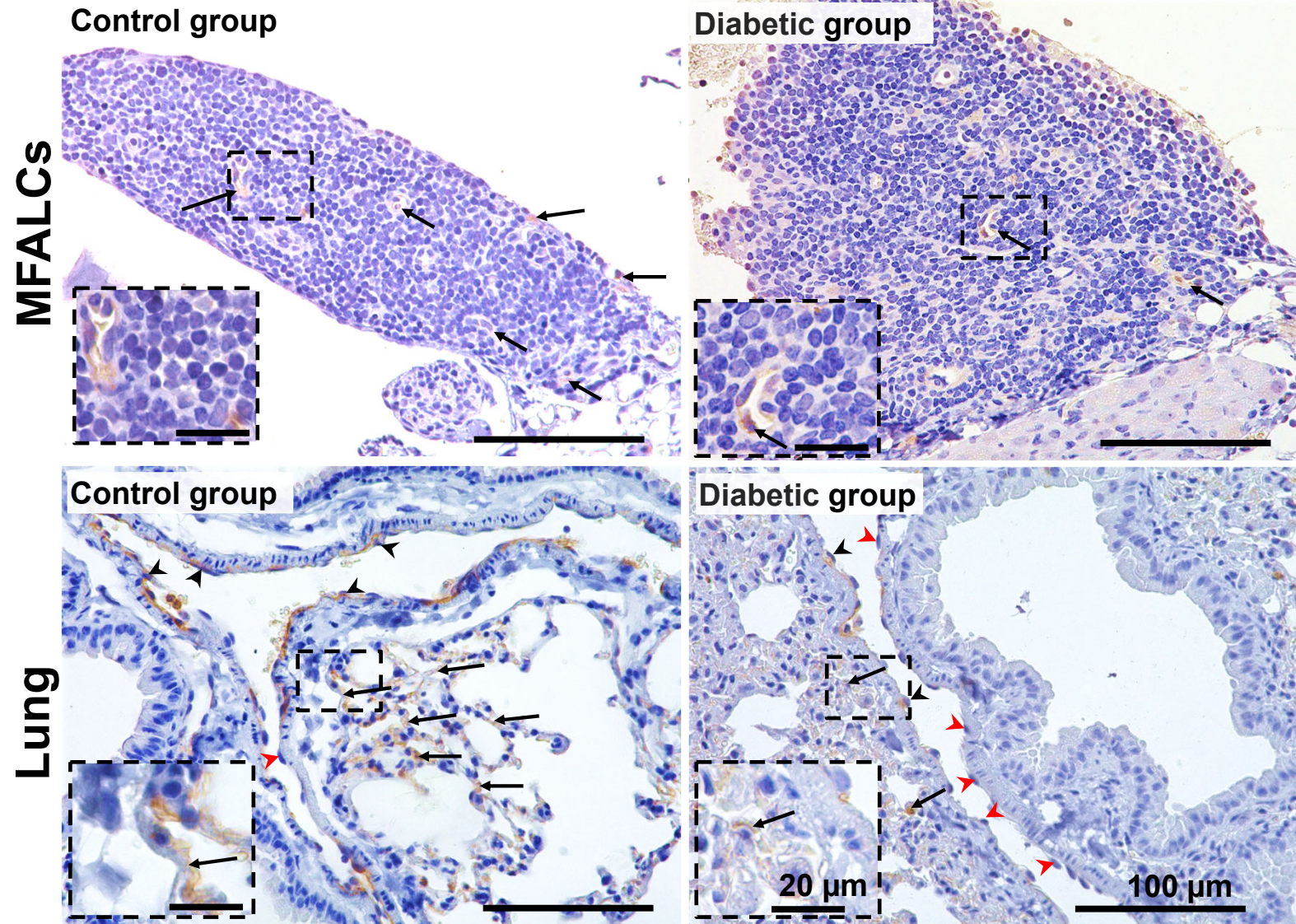
b) Ratio of LYVE-1+ LVs area in MFALCs (μm^2)



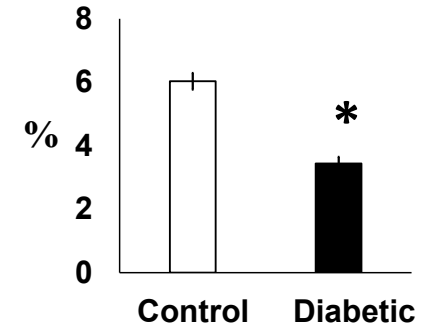
c) Ratio of LYVE-1+ LVs area in Lung (μm^2)



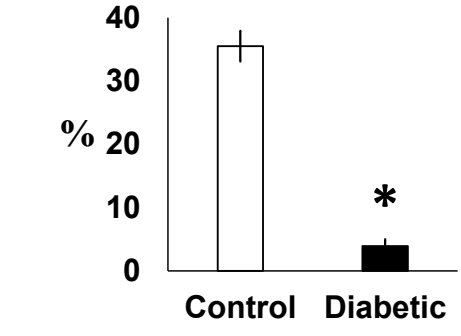
a) Immunohistochemical staining of CD31 positive endothelial cells in MFALCs and lung



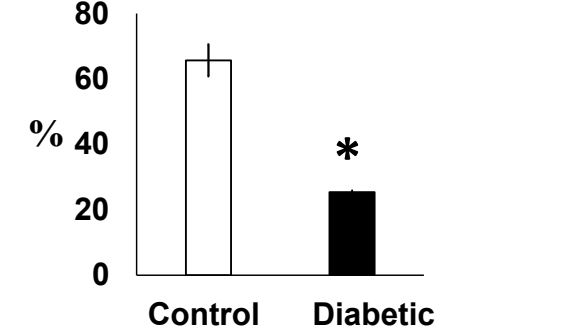
b) Ratio of CD31 area/ MFALCs area (μm^2)



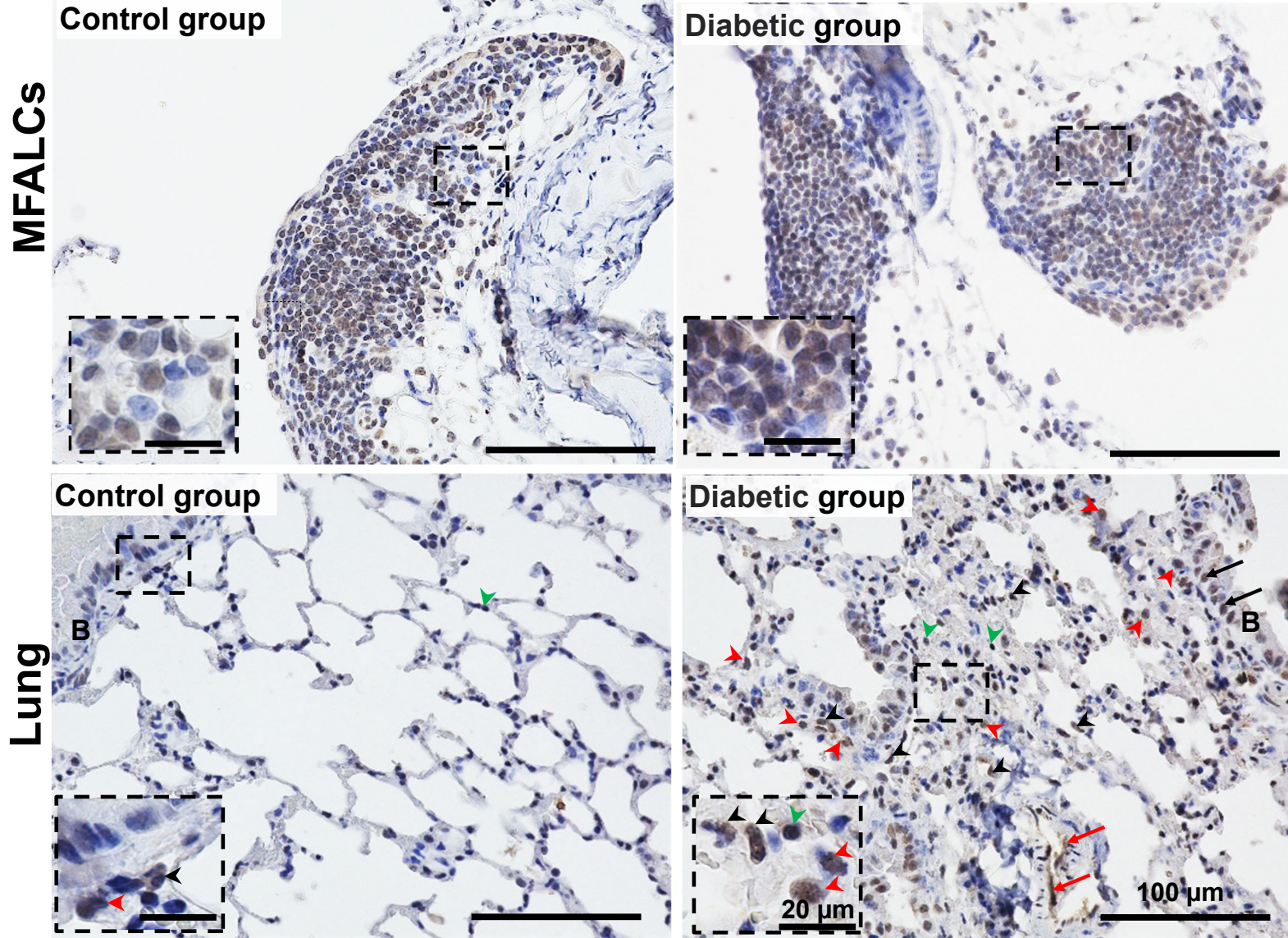
c) Ratio of CD31+ capillary area/ lung area (μm^2)



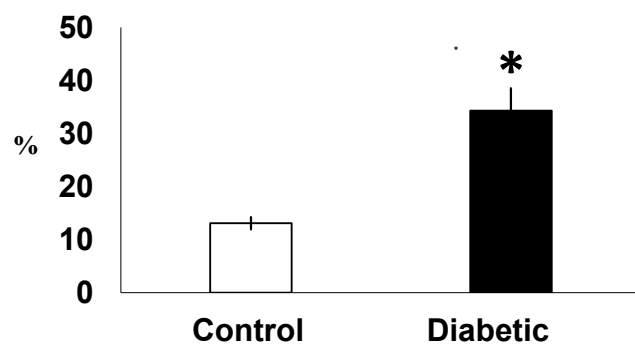
d) Index ratio of CD31+ endothelial cells /blood vessel wall within lung



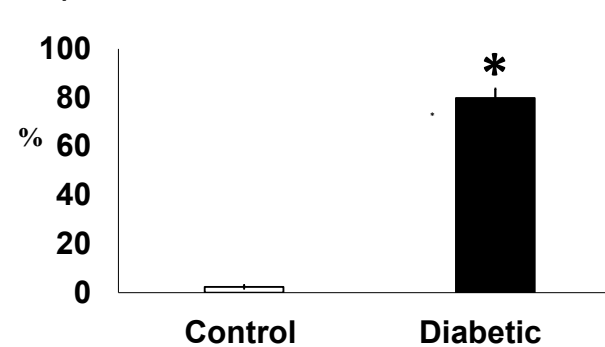
a) Immunohistochemical staining of TNF- α positive endothelial cells in MFALCs and lung



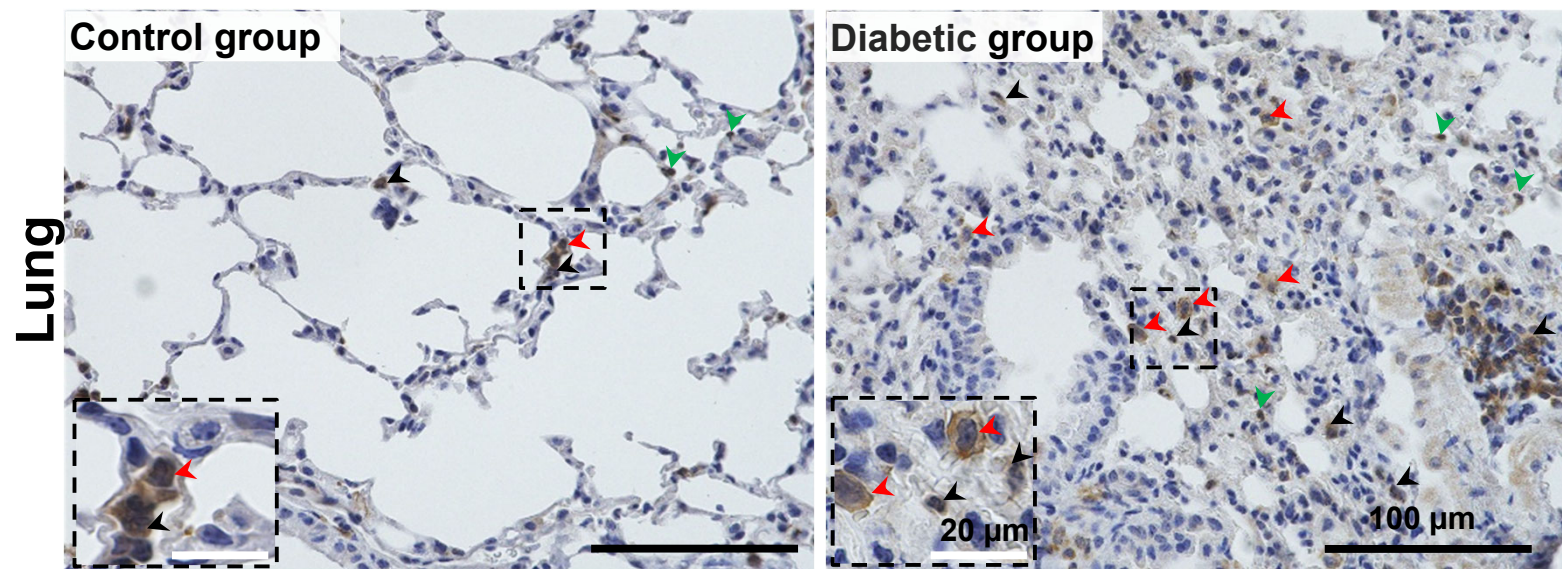
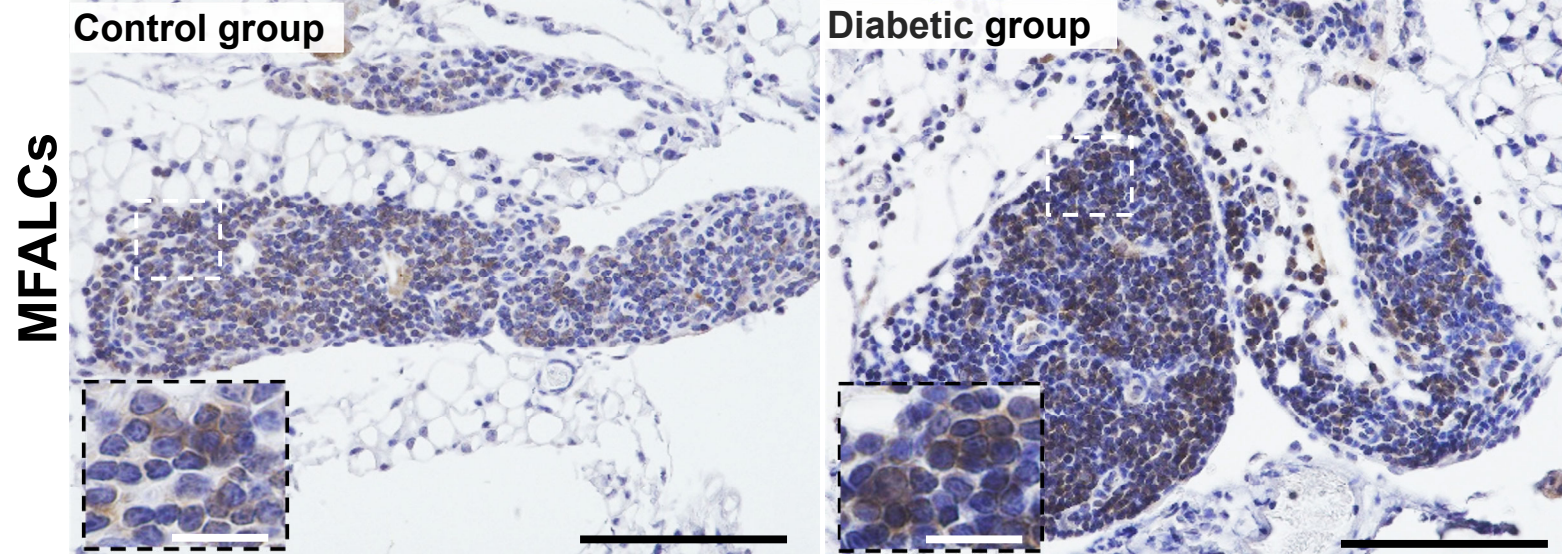
b) TNF- α positive area ratio in MFALCs (μm^2)



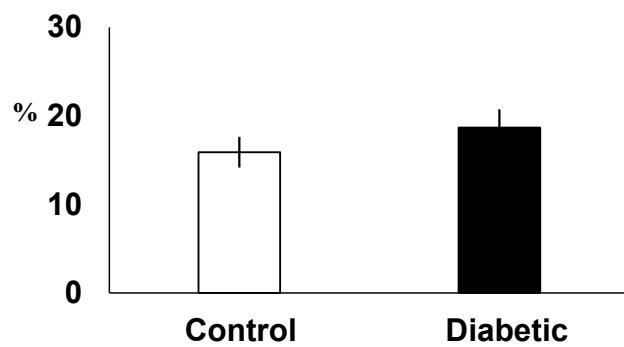
c) TNF- α positive index ratio in lung (μm^2)



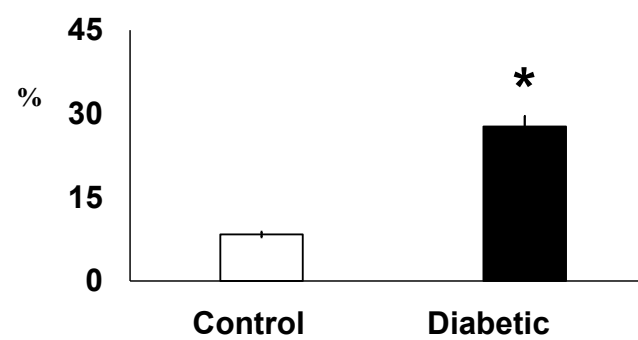
a) Immunohistochemical staining of IL5 positive endothelial cells in MFALCs and lung



b) IL5 positive area ratio in MFALCs (μ m²)

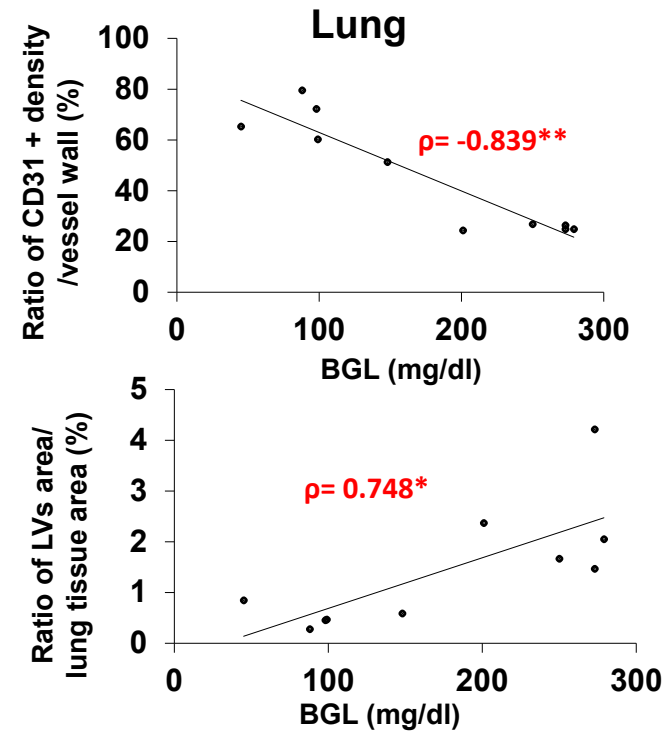
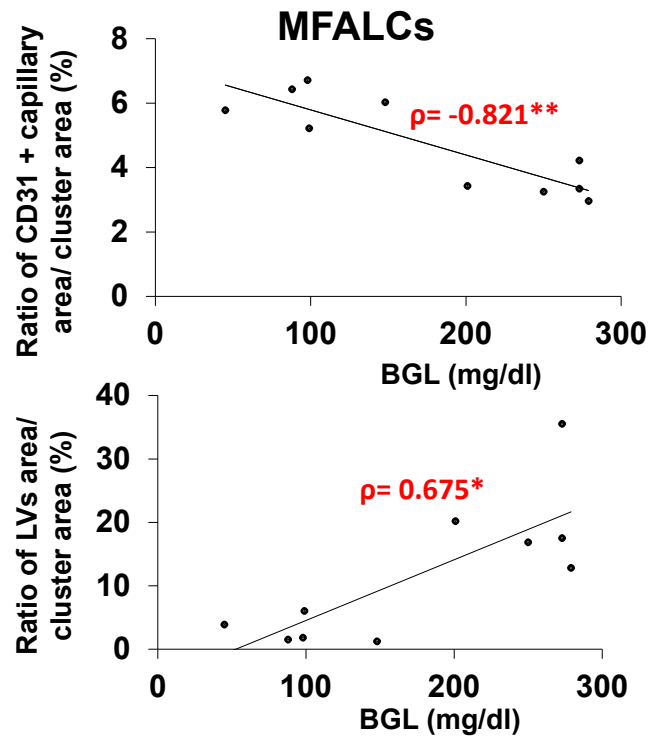


c) IL5 positive index ratio in lung (μ m²)



a) Correlations between blood glucose level (BGL) and expression of vessel markers.

Vessel markers



b) Correlations between blood glucose level and expression of inflammatory markers

Inflammatory markers

