1   The significance of CD163 expressing macrophages in asthma

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3 Short title: CD163 and macrophages in asthma

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21

26

27   **Keywords;** CD163, asthma, macrophage, chronic obstructive pulmonary disease, COPD,

28   airway hyper-responsiveness

29

30   **Abbreviations;** AB-PAS: Alcian blue-periodic acid-Schiff, ACh: Acetylcholine, ACO:

31   Asthma-COPD overlap, AHR: Airway hyper-responsiveness, BALF: Bronchoalveolar lavage

32   fluid, COPD: Chronic obstructive pulmonary disease, HDM: House dust mite, HE:

33   Hematoxylin and eosin, ICS: Inhaled corticosteroid, IHC: Immunohistochemistry, KO:

34   Knockout, OCS: Oral corticosteroid, OVA: Ovalbumin, SD: Standard deviation, SEM:

35   Standard error of the mean, WT: Wild-type

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39   The significance of CD163 expressing macrophages in asthma

40

41   **Introduction**

42   Asthma is characterized clinically by airflow limitation and airway hyper-responsiveness

43   (AHR) induced by chronic lower airways inflammation. The inflammatory process in allergic

44   asthma is initiated by T-helper 2 (Th2) CD4 + cells, which produce a repertoire of cytokines,

45   including IL-4, IL-5, IL-9, and IL-13. These cytokines play a critical role in IgE production,

46   airway eosinophilia, and goblet cell hyperplasia [1]. The histopathologic characteristics of lungs

47   in fatal cases of asthma include severe airway remodeling accompanied by hypertrophy of the

48   smooth muscle, airway wall edema, hyperplasia, and hypertrophy of goblet cells, mucous plugs,

49   and massive pulmonary inflammation [2]. The asthma mortality rates were maintained at a high

50   level of 0.62 per 100,000 in late 1980s until the widespread use of inhaled corticosteroid (ICS)

51   therapy. Next, the asthma mortality rates reduced to a level of 0.23 per 100,000 in 2000s [3]. The

52   burden of asthma continues to be quite high and Japan continues to have the highest asthma-

53   related mortality among developed nations; the age-standardized asthma mortality rate in Japan

54   was 9.34 per million across all ages in the period 2001–2010 [4].

55   Macrophages can be broadly classified into 2 major subtypes: classically activated phenotypes

56   cells (M1) and alternatively activated cells (M2). M1 macrophages, induced by IFN-γ and

57   lipopolysaccharide (LPS), upregulate the expression of genes involved in pathogen clearance

58   and drive inflammation in response to intracellular pathogens. In contrast, M2 macrophages,

59   induced by IL-4 and IL-13, up-regulate the expression of genes involved in wound healing,

60   clearance of dead and dying cells and tissues, and are involved in anti-inflammatory responses

61   [5-7]. These two macrophage states mirror the Th1-Th2 polarization of T cells [8-10]. In the lung

62   tissues in asthma patients to protect tissue from injury by microorganisms and to repair the

63   tissue, macrophages appear to be less prone to polarization toward either the M1 or M2

64   phenotypes [11].

65   Macrophage activation is extremely complex and heterogeneous to be divided into 2 subtypes;

66   recent researchers often use the term "M1-like/M2-like" in the phenotype of macrophage

67   activation. CD163, a scavenger-receptor specifically expressed on macrophages, is known as

68   a marker for tissue repair/protumor/M2-like macrophages [12, 13]. CD163 is known to be secreted

69   from activated macrophages by shedding with proteases, and the plasma level of soluble

70   CD163 is reportedly increased under inflammatory conditions in several diseases, including in

71   lung diseases [14-16].

72   Asthma is characterized by chronic airway inflammation and eosinophilic inflammation that

73   are induced by Th2 cell-mediated immune response. The presence of a correlation between

74   M2-like macrophages and Th2 inflammation is well known [17]. However, the distribution and

75   roles of CD163 in asthma remains unclear. Therefore, we examined the distribution and roles

76   of CD163 using human lung specimens and mouse models.

77

Methods

Patients

80 We study carefully excluded smokers and ex-smokers and analyzed the nonsmokers to

81 exclude those with chronic obstructive pulmonary disease (COPD). Nine patients diagnosed

82 with asthma had been treated at the Kurume University Hospital (Kurume, Japan) and the

83 Fukuoka National Hospital (Fukuoka, Japan). All patients were diagnosed by physicians.

84 Medical records showed that all patients had histories of transient paroxysmal and repeated

85 wheezes and dyspnea with spontaneous improvement, or use of bronchodilators or oral

86 corticosteroids. These asthma patients were nonsmokers and had died between 1973 and 1999.

87 Their lung tissues were obtained at autopsy examination. As materials for comparison, the

88 normal lung tissues were obtained as controls from 8 nonsmokers (2 men and 6 women; age

89 range, 55–73 years), all of whom underwent surgical resection for lung cancer at the Kurume

90 University Hospital from 2000 to 2005. No significant difference was observed in the ages of

91 patients with fatal asthma and the non-asthma control subjects. However, samples of patients

92 with asthma after 1999 and control samples between 1973 and 1999 could not be obtained from

93 the two hospitals.

94 Sample collection and all procedures were approved by the ethics committees of the Kurume

95 University in accordance with the ethical standards of the Declaration of Helsinki of 1975.

96

Histology

98   The lung tissues were fixed with 10% formalin and embedded in paraffin wax, as reported

99   previously [18]. Paraffin-embedded lung tissues (1–3 in numbers) were obtained from each

100   patient. Sequential sections were made from each paraffin-embedded lung tissue. Sections (4-

101   μm thick) were serially cut, placed on poly-L-lysine-coated slides, and incubated overnight at

102   55°C–60°C, as described previously [2].

103

Immunohistochemical Staining for Human Subjects

105   For the blockade of endogenous peroxidase activity, 4-μm thick deparaffinized sections were

106   incubated with 1% H_2O_2 for 30 min. To detect the macrophages, the tissues were reacted

107   overnight at 4°C with anti-human CD68 (mouse monoclonal, clone PG-M1, Agilent   Technol,

108   Santa Clara, CA, USA) and anti-human CD163 (mouse monoclonal, clone 10D6, Leica

109   Biosystems, Nussloch, Germany) antibodies, and control nonimmune mouse IgG (DAKO) was

110   used as the negative control, as described previously [19]. Then, the samples were washed

111   extensively and incubated further with appropriate horseradish peroxidase-conjugated

112   secondary antibodies (Nichirei, Tokyo, Japan) for 1 h at the room temperature. After the

113   removal of unreacted secondary antibodies, the samples were incubated with 3,3'-

114   diaminobenzidine-4HCl (Agilent, Tokyo, Japan)-H_2O_2 solution to visualize immunolabeling.

115 Then, some sections were counterstained with hematoxylin and eosin and mounted with a

116 coverslip, as described previously [20].

117

118 **Quantitative Assessment of CD68+ and CD163+ Macrophages in the Lung Tissue**

119 The quantitative assessment of macrophages was performed as reported previously, with

120 minor modification [21]. Initially, nine square fields in which small-airway inflammation

121 appeared most severe were selected (hot spots) [22]. The numbers of CD68-positive cells, as

122 macrophages, in the interstitial lung tissues were counted within these nine square fields and

123 expressed in number per millimeter square. The total numbers of macrophages in asthma

124 patients and controls were expressed as mean ± standard error of the mean (SEM) cells/mm^2.

125 Then, the CD163+ cells were counted in the same fields as CD68+ cells. Two pathologists

126 examined these sections independently in a blinded manner, without prior knowledge of the

127 patients' clinical status.

128

129 **Study Design for Mouse Asthma Model**

130 Balb/c wild-type (WT) mice were purchased from Charles River Japan (Yokohama, Japan).

131 CD163-deficient (knockout, KO) mice in the C57BL/6N background were obtained from the

132 Knockout Mouse Project [23]. The CD163KO mice were backcrossed to the Balb/c strain for

133 more than 7 generations. All mice used in this study were of female gender aged 6–8 weeks.

134 The mice were bred under specific pathogen-free conditions and provided with standardized

135 diets at the animal facilities (Kurume University Animal Center). Less than 6 mice were bred

136 in one cage to minimize animal suffering and distress. The method of euthanasia was cervical

137 dislocation or exsanguination in unconscious mice under anesthesia. The Committee for Ethics

138 of Animal Experiments, Kurume University approved all procedures (approval no. 050-058,

139 March 30, 2016), and animal care was provided in accordance with the procedures outlined in

140 the "principle of laboratory animal care" (National Institutes of Health Publication No.86-23,

141 revised 1985). The experimental procedure (Fig 1) has been described previously [24, 25]. Balb/c

142 CD163 KO mice and control Balb/c WT mice (6–9 mice per group) were treated twice with an

143 intraperitoneal injection of 10 μg sterile chicken ovalbumin (OVA, grade V, Sigma-Aldrich

144 Chemical, St. Louis, MO) emulsified with 4 mg of sterile aluminum hydroxide (Alu-Gel-S

145 Suspension, Serva Electrophoresis GmbH, Heidelberg, Germany) in a total volume of 200 μL.

146 The injections were administered at days 0 and 5. These mice were challenged for 20 min with

147 0.9% saline (Group 1) or with 5% OVA in 0.9% saline (Group 2) administered via the airways

148 using an ultrasonic nebulizer (Omron NE-U07, Tokyo, Japan) in a closed box. We performed

149 histological analysis to find bronchoalveolar lavage fluid (BALF) and AHR in all groups, as

150 reported previously [24, 25]. We conducted the experiments twice each and combined the results.

151

152 **Histological Analysis in Mice**

153 For the histological analysis, the mice were euthanized via intraperitoneal injection of

154 pentobarbital sodium (2.5–5 mg per mouse). After the thorax was opened, the trachea was

155 dissected free from the underlying soft tissues, and a 0.8-mm tube was inserted through a small

156 incision in the trachea. The lung tissues were immediately fixed by intratracheal instillation of

157 10% buffered formalin (pH 7.40) for 15–20 min at a constant pressure of 25-cm H_2O. After

158 gross examination, the extracted tissues were placed into 10% buffered formalin and further

159 fixed for at least 24 h. Sections (4-μm thick) were cut from paraffin-embedded tissues, placed

160 on poly-l-lysine–coated slides, and then incubated overnight at 55°C–60°C. The deparaffinized

161 sections were stained with hematoxylin and eosin and alcian blue-periodic acid-Schiff (AB-

162 PAS), as described previously [24, 26].

163

Immunohistochemical Staining for Mice

165 We performed immunohistochemical staining of Iba1 in lung macrophages and CD163 as

166 M2-like macrophages in the lung of mice. Anti-mouse Iba1 (rabbit polyclonal, WAKO, Tokyo,

167 Japan) and anti-mouse CD163 (rabbit polyclonal, CosmoBio, Tokyo, Japan) antibodies were

168 used as the first antibody for immunohistochemistry (IHC), and IHC were performed as

169 described above.

170

Analysis of BALF Obtained from Mice

172   The trachea was inserted with a tubing adaptor, and the lungs were washed thrice with 3 mL

173   saline. Aliquots of the cells were centrifuged onto glass slides, dried in air, and stained with

174   May–Grunwald–Giemsa stain. The total cell and the differential cell counts were obtained from

175   cytospin preparations, dried in air, and stained with May–Grunwald–Giemsa stain. The cell

176   populations were counted, and the absolute number of cell populations were then calculated,

177   as reported previously [24, 25]. The supernatants of BALF was kept at −30ºC for measurements.

178

179   **Measurement of IL-4, -5, -13, Eotaxin, and IFN-γ in the Supernatants of BALF**

180   The levels of IL-4, -5, -13, eotaxin, and IFN-γ in the supernatants of BALF were measured

181   using commercially available ELISA Kits (ThermoFisher, Waltham, Massachusetts, USA).

182

183   **Assessment of AHR**

184   AHR to aerosolized acetylcholine (ACh; Sigma-Aldrich Japan, Tokyo, Japan) was tested 24

185   h after OVA or saline challenge, as described previously [24, 25]. Briefly, under mechanical

186   ventilation (150 breaths/min, tidal volume 10 mL/kg, and positive end-expiratory pressure 2

187   cmH$_2$O) (Buxco FinePointe RC; Data Science International, MN) after anesthetization and

188   intratracheal intubation via tracheotomy, mean airway resistance of mice was measured

189   automatically after inhalation of 0.9% saline at the baseline, followed by increasing doses of

190   aerosolized ACh (doubling doses from 0 to 160 mg/mL in 20 μL) via a nebulizer (inhalation

191   for 30 s and response for 3 min) [25].

192

Statistical Analysis

194   All data were expressed as means ± SEM. Differences between 2 and 3 or more groups were

195   compared by Tukey–Kramer test and the analysis of variance with the Tukey Honestly

196   Significant Difference Test, respectively. Differences at $P < 0.05$ were considered to be

197   statistically significant. JMP 12.2.0 (SAS Institute Japan, Tokyo, Japan) was used for statistical

198   analysis.

199

Results

Clinical Findings

202   All 9 patients with fatal asthma were nonsmokers, and none had COPD. Their ages ranged

203   from 5 to 79 years (Table 1). Five of the 9 patients died within 24 h of the onset of asthma

204   attack. The duration of disease in these patients varied widely from 2 to 50 years; 7 patients

205   had asthma for >6 years. Six patients had been treated with systemic corticosteroid, and no

206   patient received ICS. Three patients died without treatment with systemic corticosteroid or ICS

207   (Table 2).

208

Increased Number of CD68+ and CD163+ Macrophages in Patients with Fatal Asthma

210   The representative examples of the histology of the lung tissues obtained from 2 patients with

211   fatal asthma are shown (Fig 2A). The control subject was a 48-year-old man. Case 1 patient

212   was a 5-year-old boy (Patient #1 in Table 1), and Case 2 was a 32-year-old man (Patient #2 in

213   Table 1). Immunostaining to detect CD68 and CD163 was successful in 40-year-old specimens

214   of formalin-preserved lung tissues. We performed heat-induced and proteolysis-induced

215   antigen retrieval as described previously [19]. This indicated that CD68 and CD163 antigens on

216   the macrophage surface were intact in the formalin-preserved tissues. Immunostaining for

217   CD68 was performed using pathological sections to examine the distribution of lung

218   macrophages. The number of CD68+ macrophages were increased in patients with fatal asthma.

219   It is well known that CD163 is specifically expressed on macrophages and are useful as M2-

220   like macrophage markers. Therefore, we performed immunostaining of CD163 using serial

221   sections. Similar to the number of CD68+ macrophages, the numbers of CD163+ macrophages

222   were significantly increased in the patients with fatal asthma as compared with that in the non-

223   asthma control subjects (Fig 2B). There were no significant differences in the background and

224   cell counts between males and females in the control group.

225

226   **CD163+ Cells are increased in the Lungs of OVA/OVA-WT Mice**

227   Immunohistochemical staining of Iba1 revealed that the numbers of Iba1+ cells were

228   increased in the lungs of OVA-sensitized and OVA-challenged (OVA/OVA-) WT mice as

229 compare with that those in the lungs of OVA-sensitized and saline-challenged (OVA/saline–)

230 WT mice. We performed immunostaining of CD163 using serial sections. Similar to the

231 numbers of Iba1+ cells, the numbers of CD163+ cells increased in OVA/OVA-WT mice as

232 compare with that in OVA/saline-WT mice (Fig 3).

233

234 **Decreased Airway Inflammation in CD163 KO Mice Mouse Asthma Model**

235 Histological examination by hematoxylin eosin and alcian blue-periodic acid-Schiff staining

236 revealed decreased airway inflammation and mucous cell metaplasia in the lungs of

237 OVA/OVA-CD163 KO mice compared with the lungs of OVA/OVA-WT mice. In contrast,

238 airway inflammation and mucous cell metaplasia were not observed in the lungs of

239 OVA/saline-CD163 KO mice and WT mice (Fig 4A and B). The cells in BALF with May–

240 Grunwald–Giemsa stain showed decreased eosinophils in OVA/OVA-CD163 KO mice as

241 compared to that in OVA/OVA-WT mice (Fig 4C). BALF analysis revealed that the number

242 (mean ± SEM, x 10^4 cells/mL) of total cells in OVA/OVA-CD163KO mice was significantly

243 lower than that in OVA/OVA-WT mice. The number of eosinophils, lymphocyte, neutrophils

244 and macrophages in OVA/OVA-CD163KO mice was significantly lower than that in

245 OVA/OVA-WT mice (Fig 4D).

246

247 **Decreased Levels of IFN-γ and IL-5 in the BALF of OVA/OVA-CD163KO Mice**

248   We analyzed the protein levels of IL-4, IL-5, IL-13, IFN-γ, and eotaxin in the BALF. The

249   concentrations of IFN-γ and IL-5 was significantly decreased in the BALF of OVA/OVA-

250   CD163KO mice as compared to that in OVA/OVA-WT mice. In contrast, no significant

251   difference was noted in the concentrations of IL-4, IL-13, and eotaxin between OVA/OVA-

252   CD163KO mice and OVA/OVA-WT mice (Fig 5).

253

254   **AHR Was Suppressed in OVA/OVA-CD163KO Mice**

255   We investigated AHR in OVA/OVA-CD163KO and WT mice on day 19. We found that AHR

256   was increased in OVA/OVA-WT mice as compared to that in OVA/saline-WT mice. In contrast,

257   AHR was suppressed in OVA/OVA-CD163KO mice as compared to that in OVA/OVA-WT

258   mice (Fig 6).

259

260   **Discussion**

261   M2-like macrophages are associated with Th2 inflammation, and CD163 is known as a marker

262   for M2-like macrophage [17]. In the present study, CD163 was strongly expressed on

263   macrophages in the lungs of all patients with fatal asthma. In the mouse models of asthma,

264   AHR and the numbers of total cells and eosinophils in the BALF were significantly decreased

265   in the OVA/OVA-CD163KO mice when compared with that in the control WT mice.

266   Several studies have examined CD163 in asthmatic patients. Sputum macrophages from mild

267   and moderate asthma patients expressed less cell-surface CD163 than macrophages from

268   healthy individuals [27]. On the other hand, the concentration of sputum-soluble CD163

269   (sCD163) was significantly greater in mild asthma patients than in healthy controls [16, 28], and

270   the sputum sCD163 level was significantly greater in patients with severe asthma as compared

271   to that in patients with mild/moderate asthma [16]. Our present results showed that patients with

272   fatal asthma expressed more CD163-positive macrophages in the lung tissues. It has been a

273   long time since we collected lung tissues from patients with fatal asthma. Presently, asthma

274   patients rarely die from asthma attack because of the widespread awareness of the use of ICS;

275   therefore, it is difficult to collect specimens from the lungs of patients with fatal asthma now.

276   If possible, it is desirable to examine fresh lung tissues in the future. In this study, we examined

277   only the lungs of patients with fatal asthma, and it is desirable to examine the lungs of patients

278   with mild or moderate asthma in the future.

279   Regarding animal experimentation, in previous researches, the total cells and eosinophils in

280   BALF were significantly increased in HDM and Der p1-challenged-CD163-deficient mice, and

281   the lung histology similarly revealed an increase in the peri-bronchial inflammatory cell

282   infiltrates in HDM- and Der p1-challenged CD163-deficient mice as compared to that in WT

283   mice [29]. In contrast, our present results showed that the numbers of total cells and eosinophils

284   in the BALF were significantly decreased in OVA/OVA-CD163KO Balb/c mice. On the other

285   hand, neither HDM-challenged CD163-deficient mice nor WT mice developed methacholine-

286 induced increases in AHR, which likely reflects the C57BL/6 genetic background [29]. In the

287 present study, AHR was increased in OVA/OVA-WT Balb/c mice as compared to that in

288 OVA/saline-WT Balb/c mice, and it was suppressed in OVA/OVA-CD163KO Balb/c mice as

289 compared to that in OVA/OVA-WT Balb/c mice. This difference may be attributed to the

290 difference in the mice background or antigen used to establish allergy mouse model. Regarding

291 AHR, OVA-sensitized and challenged model may reflect the asthmatic conditions more

292 accurately in Balb/c mice.

293   In this study, the number of total cells in BALF in OVA/Saline-CD163KO mice was

294 significantly lower than that in OVA/Saline-WT mice. CD163KO mice are complete knockouts

295 of the CD163 gene lack CD163 expression on macrophages. Therefore, the number of total

296 cells, particularly macrophages, may not have increased in OVA/Saline-CD163KO mice.

297 Further analysis is needed to test this possibility.

298   M2-like macrophages are conventionally considered to display anti-inflammatory properties;

299 recently, they were subdivided further into several subtypes. Some subtypes of M2-like

300 macrophages are believed to be activated by Th2 cytokines (IL-4 and IL-13) and known to

301 induce allergic immune responses, such as eosinophilic inflammation [17, 30]. Previous studies

302 have found that M2-like macrophages correlate significantly with the percent of eosinophils in

303 BALF of HDM-induced asthma model [31].The present study demonstrated that the numbers of

304 eosinophils and IL-5 in the BALF are significantly increased in OVA/OVA-WT mice. However,

305 they are decreased in OVA/OVA-CD163KO mice. A previous study showed that the intranasal

306 administration of anti-IL-5 antibody inhibited the development of eosinophilic lung

307 inflammation and AHR in OVA-sensitized/challenged Balb/c mice [32]. The reduction of IL-5

308 may suppress eosinophilic inflammation and AHR in OVA-OVA CD163KO mice. IFN-γ in the

309 BALF are significantly increased in OVA/OVA-WT mice and decreased in OVA/OVA-

310 CD163KO mice. A previous study showed that the IFN-γ expression in the lungs and AHR was

311 induced in Balb/c mice by the transfer of activated eosinophils and that IFN-γ-deficient

312 eosinophils or eosinophils treated with a blocking anti-IFN-γ receptor antibody failed to induce

313 AHR in mice [33]. The reduction of IFN-γ may also contribute to suppress AHR in OVA-OVA-

314 CD163KO mice.

315 Presently, asthma-COPD overlap (ACO) is gained increasing recognition [34]. Previously, we

316 reported that CD163-positive macrophages are expressed on alveolar macrophages in the lungs

317 of severe COPD patients [20]. This common feature of asthma and COPD patients suggests that

318 CD163 may be involved in the development of ACO.

319 Our study had several limitations. First, our study did not investigate the correlation

320 between the number of CD163+ macrophages and AHR in patients with mild or moderate

321 asthma. Second, the incidence and severity of asthma has been reported to be greater in women

322 than in men [35] and in female mice compared with male mice [36, 37]. We performed experiments

323 investigating airway inflammation in the murine asthma model using male and female mice.

324 However, male mice did not exhibit airway inflammation via using our methods [24, 25]. Male

325 mice have been shown to produce lower amounts of Th2 cytokines and specific IgE, and have

326 fewer lung lymphocytes after OVA sensitization and challenge in the asthma model [37].

327 Castrated mice have been found to have increased OVA-induced eosinophil and lymphocyte

328 infiltration in BALF [38]. Therefore, we included only female mice in the present study. Third,

329 no significant difference was noted in the protein levels of IL-10 in BALF between OVA/OVA-

330 CD163KO mice and OVA/OVA-WT mice, and IL-33 was not detected in BALF using ELISA

331 (data not shown), although a previous study reported that IL-33 released from airway epithelial

332 cells after antigen challenge can modulate M2 macrophage polarization through ST2 [39]. This

333 discrepancy may be due to differences between in vivo and in vitro studies.

334 The present study showed that the numbers of CD163-positive macrophages were

335 significantly increased in the lungs of patients with fatal asthma. Furthermore, AHR was

336 suppressed and the number of total cells, eosinophils, and neutrophils of BALF were decreased

337 in OVA/OVA-CD163 KO mice as compared to that in OVA/OVA-WT mice. Our result suggests

338 that the inhibition of CD163 can improve eosinophilic inflammation and suppress AHR. Thus,

339 CD163 or CD163-associated macrophage activation may play important roles in airway

340 inflammation and AHR in asthma.

341

342 **Acknowledgments**

346

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446

Tables

Table 1. Characteristics of 9 nonsmokers with fatal asthma

Patient number	Age	Sex	Year at autopsy	Therapy						Duration from onset
				OCS	ICS	β2-agonist	Theophylline	LTRA	Ventilation	
1	5	M	1977	-	-	-	-	-	+	36 h
2	32	M	1973	+	-	-	+	-	-	75 min
3	67	M	1980	-	-	-	-	-	-	5 h
4	44	M	1981	+	-	-	-	-	-	< 24 h
5	75	M	1982	+	-	-	-	-	-	20 min
6	57	M	1986	+	-	-	-	-	-	unknown
7	16	F	1984	-	-	+	+	-	-	6 h
8	79	F	1986	+	-	-	-	-	-	7 days
9	67	F	1999	+	-	-	-	-	+	26 days

Abbreviations: ICS, inhaled corticosteroid; LTRA, leukotriene receptor antagonist; M, male;

OCS, oral corticosteroid.

455 **Table 2. Characteristics of patients with fatal asthma and control patients**

	Control	Asthma death
Patients (male/female), n	8 (2/6)	9 (6/3)
Age (y), mean±SD	62.6 ± 7.9	49.11±25.0
Body mass index (kg/m^2), mean ± SD	24.5 ± 3.1	21.49±2.49
VC (% predicted), mean ± SD	122.3 ± 13.3	ND
FEV$_1$ (% predicted), mean ± SD	113.0 ± 11.9	ND
FEV$_1$/FVC (%), mean ± SD	75.1 ± 5.8	ND

456 Abbreviations: FEV$_1$, forced expiration in 1 second; FVC, forced vital capacity; ND, not done;

457 SD, standard deviation.

458

459 **Figure Legends**

460 **Figure 1. Study design for a mouse asthma model.**

461 Ovalbumin (OVA)-sensitized mice injected intraperitoneally with OVA on days 0 and 5 and

462 saline-challenged (Group 1) or OVA-challenged (Group 2) on day 18, as reported previously [8].

463 **Figure 2. Histological analysis of the lung tissues collected from patients who died of**

464 **asthma.**

465 **(A) Immunostaining of the lung tissue samples with CD68 and CD163 from control**

466 **subjects who were nonsmoker and patients who died of asthma (left: ×400 and right: ×40).**

467 Scale bar = 20 μm in the panel left and 100 μm in the right.

468 **(B) The numbers of CD68- and CD163-positive cells in control nonsmokers and patients**

469 **who died of asthma.** *: $p < 0.05$.

470

471 **Figure 3. Immunostaining of lung tissue samples with Ibal and CD163 from OVA-**

472 **sensitized and saline-challenged (OVA/saline–) WT mice and OVA-sensitized and OVA-**

473 **challenged (OVA/OVA-) WT mice (left: ×400 and right: ×40).** Scale bar = 20 μm in the left

474 panel and 100 μm in the right panel. Arrows indicated each positive cell

475

476 **Figure 4. Airway inflammation in the CD163 KO mice mouse asthma model.**

477 **(A) Histological tissues of airway inflammation in OVA-sensitized mice. (×200)**

478 Scale bar = 50 μm. Arrows indicated peribronchial proliferation of inflammatory cells. (HE staining)

479 **(B) Histological tissues of mucous cell metaplasia in OVA-sensitized mice (×400).**

480 Scale bar = 20 μm. Arrows indicated mucous cell metaplasia. (HE and AB-PAS staining)

481 **(C) Eosinophils in BALFs decreased in CD163KO mice in a mouse asthma model**.

482 **(D) The number of total cell, eosinophils, Lymphocyte, Neutrophil, and Macrophages in**

483 **BALFs decreased in CD 163 KO mice in a mouse asthma model.** The cell populations in

484 the BALFs (n = 15–18 per each group) *: $p < 0.05$

485

486 **Figure 5. IFN-γ and IL-5 in the BALFs decreased in CD 163 KO mice in a mouse asthma**

487 **model.**

488 The concentrations of IFN-γ, IL-5, IL-4, IL-13, and eotaxin in the BALFs were measured by

489 specific ELISA kits (n = 15–18 per each group) *: $p < 0.05$

490

491 **Figure 6. Airway hyper-responsiveness**

492 The data were expressed as airway resistance changes from the baseline in response to 8

493 different doses of Ach (n = 10–12 per each group), as described previously[17, 18]. *: $p < 0.05$

494

495   Figure.1
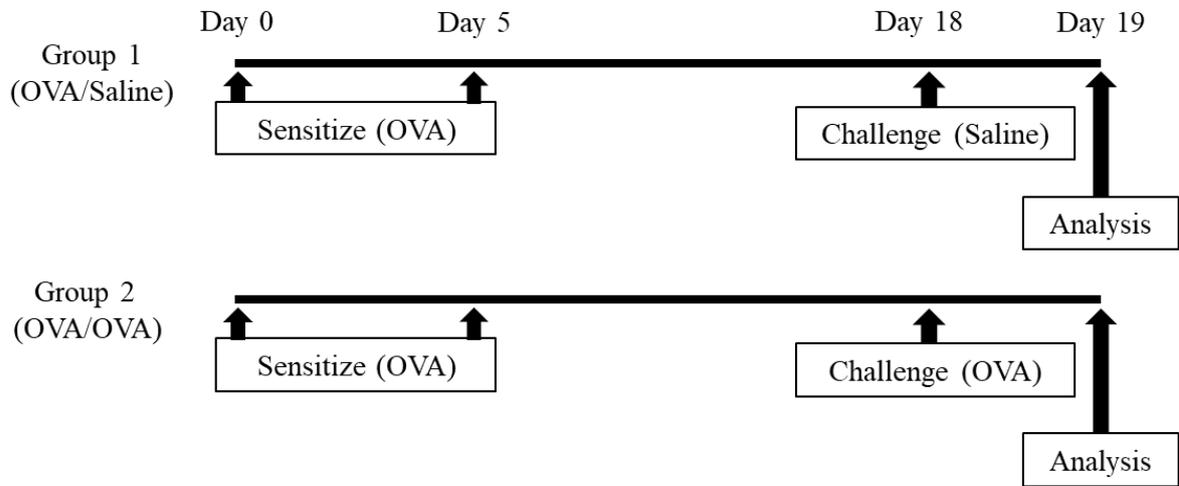
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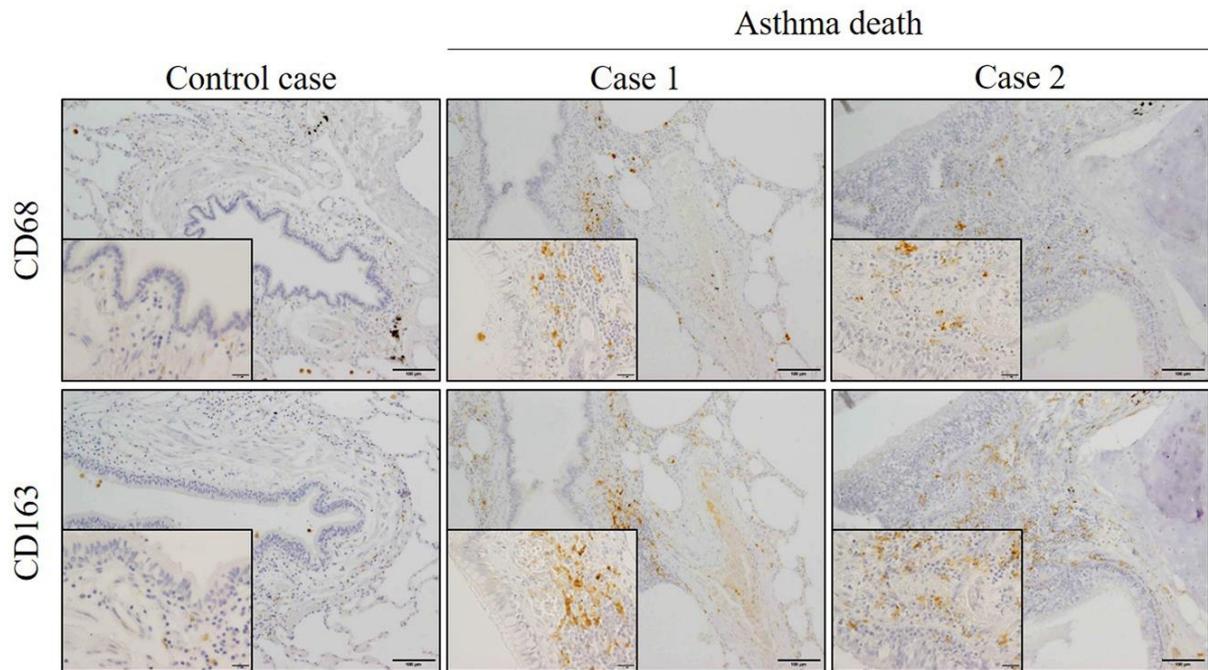
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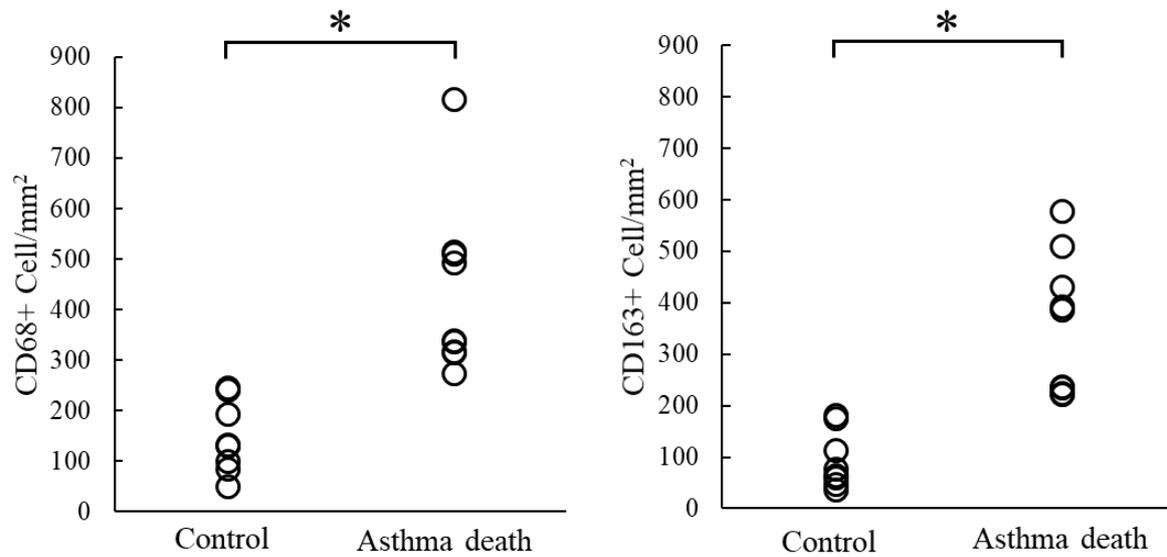
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509   Figure.4A

OVA/Saline   OVA/OVA

WT

CD163 KO
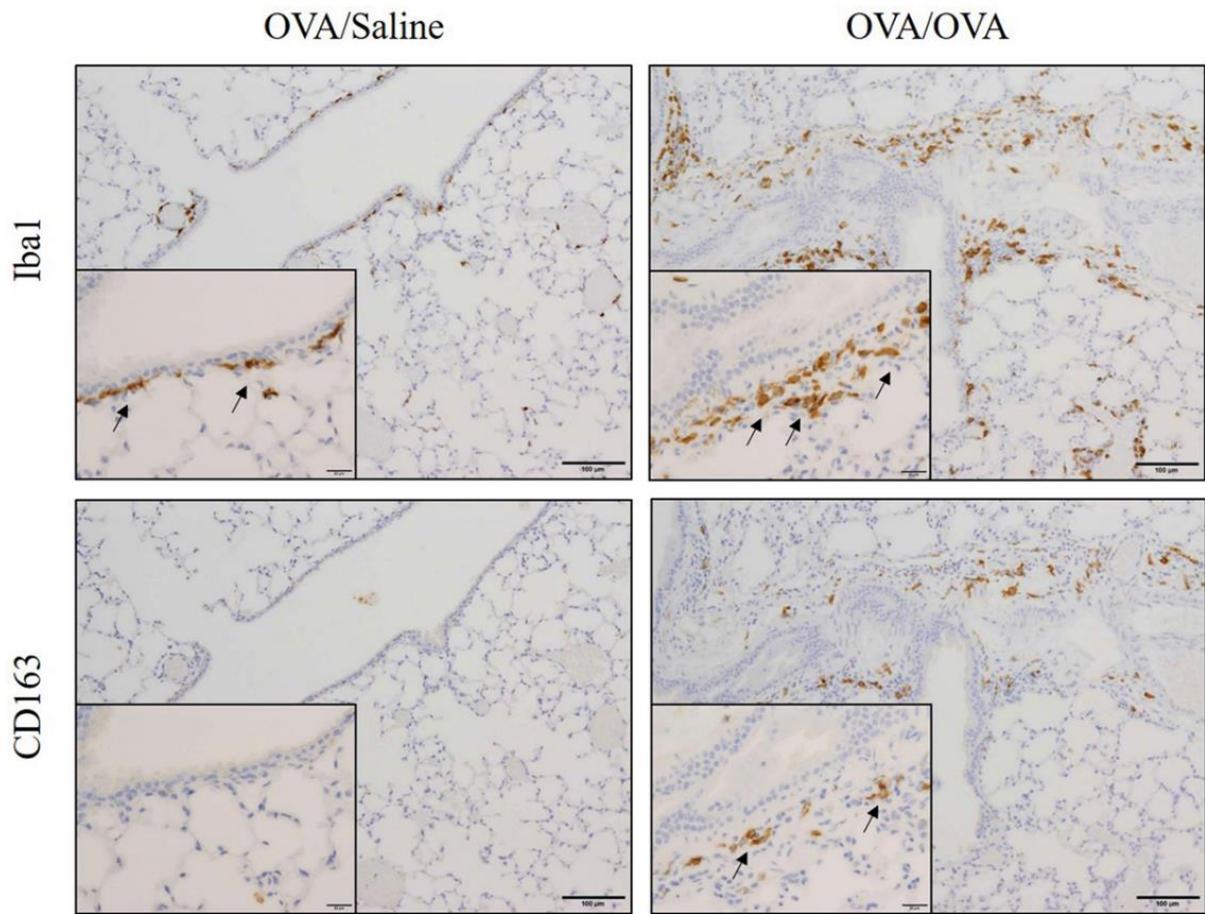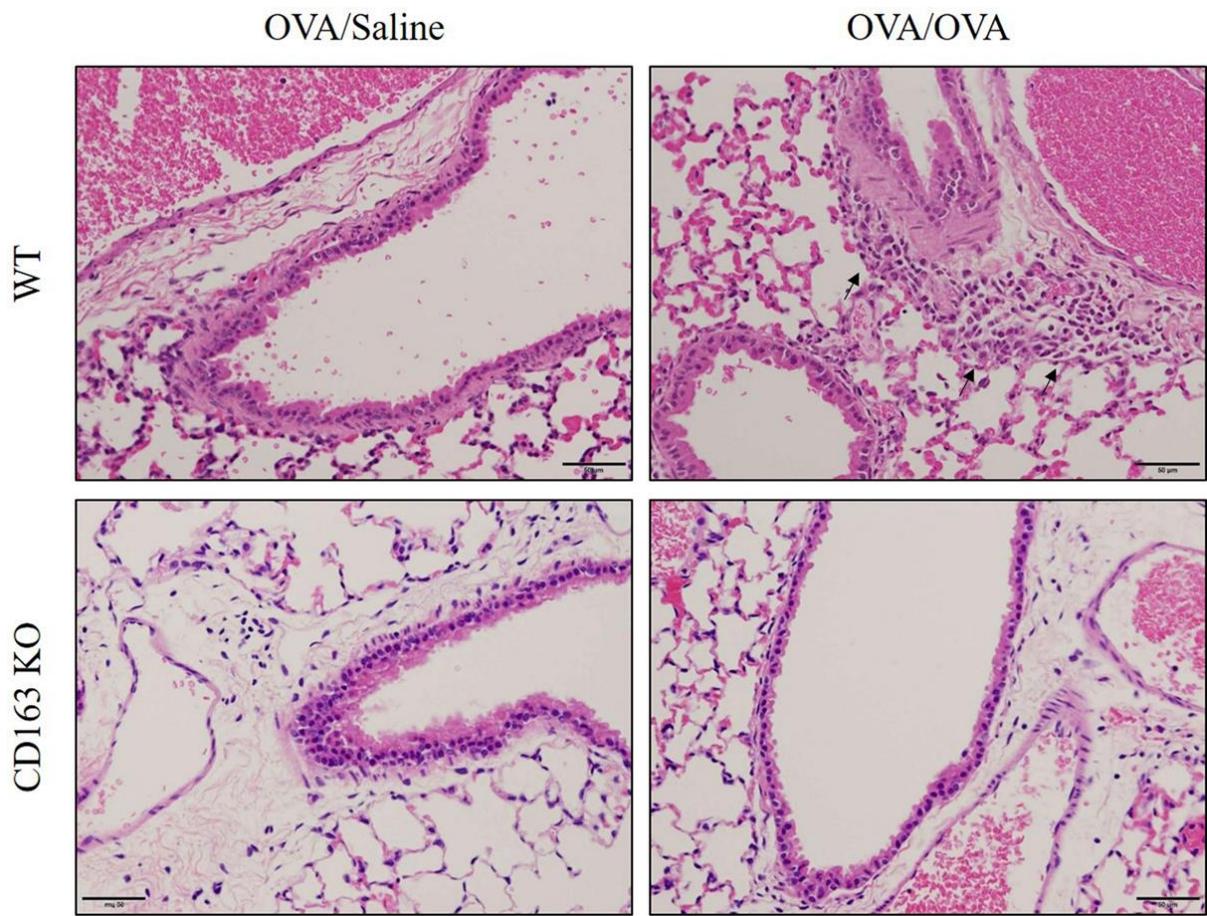
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512   Figure.4B

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514

515   Figure.4C

OVA/Saline   OVA/OVA

WT

CD163 KO
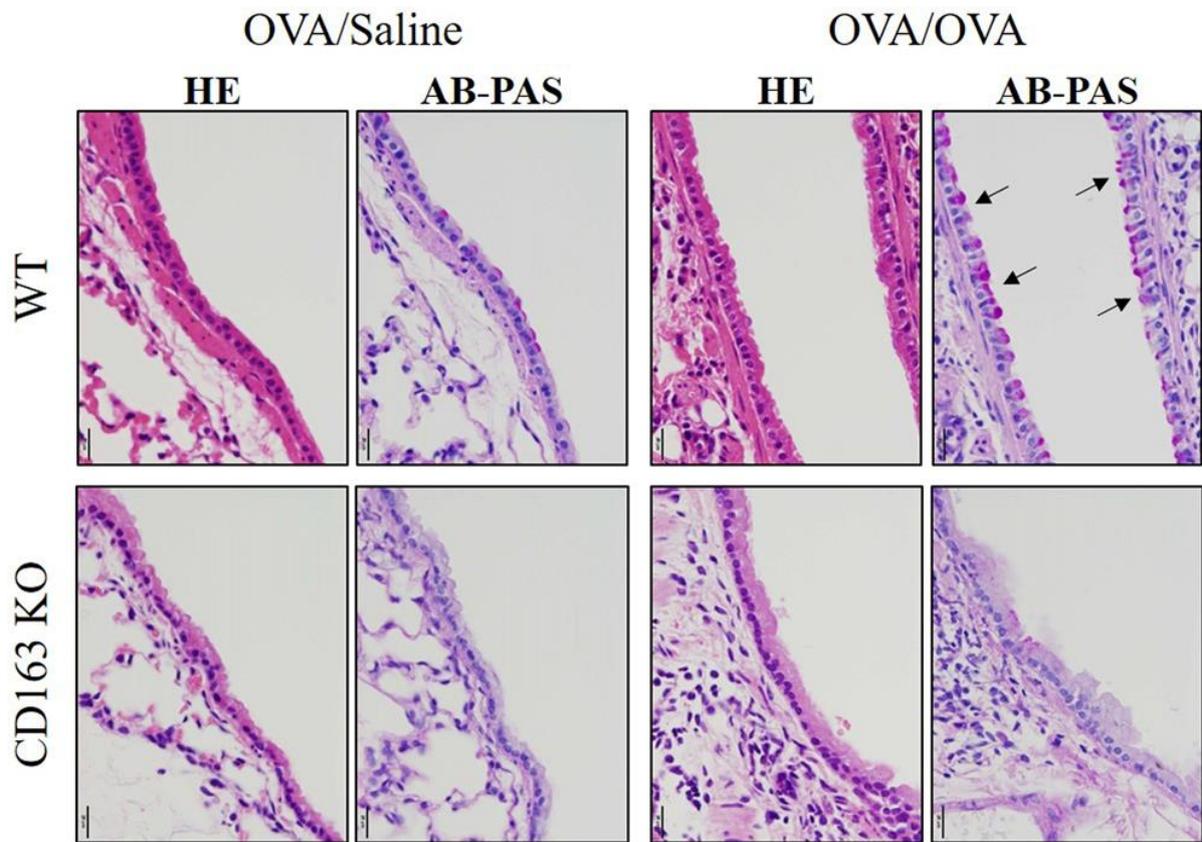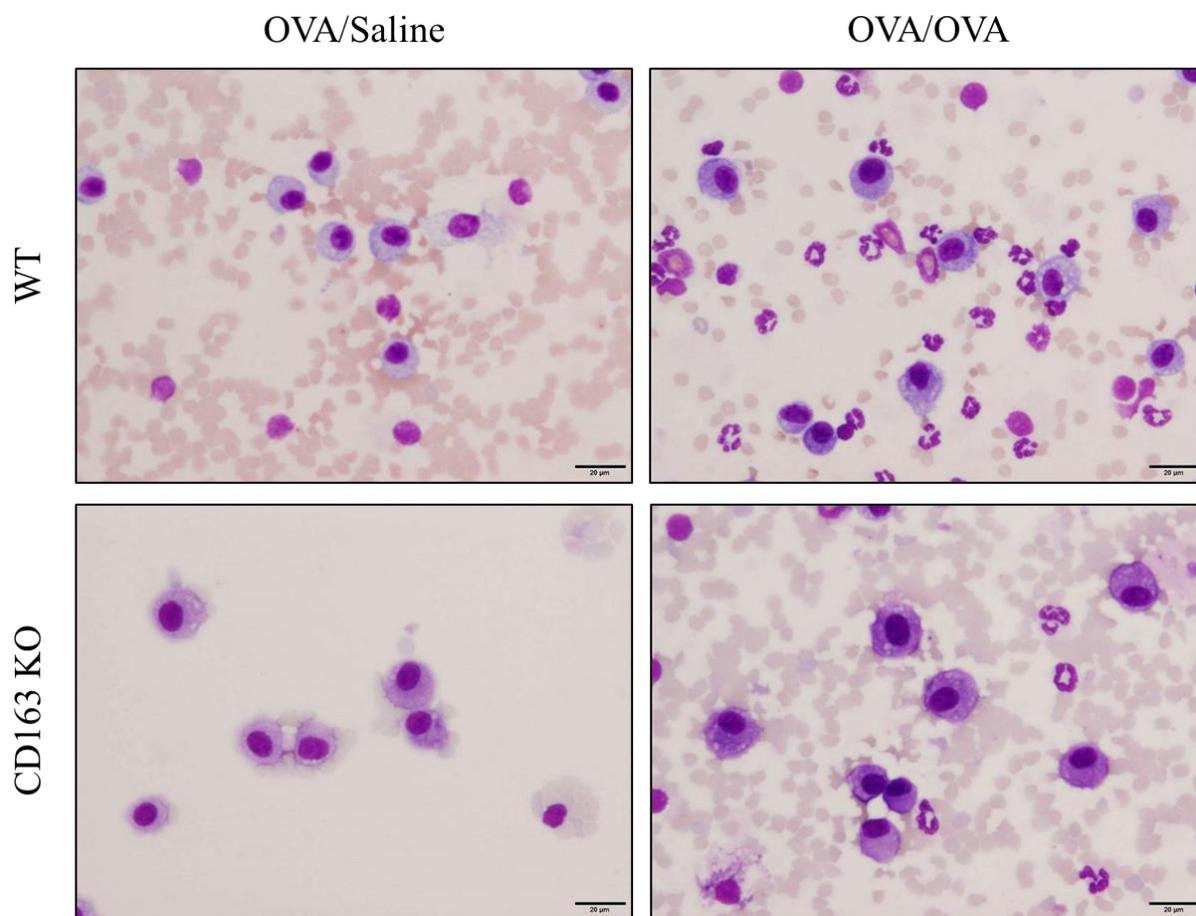
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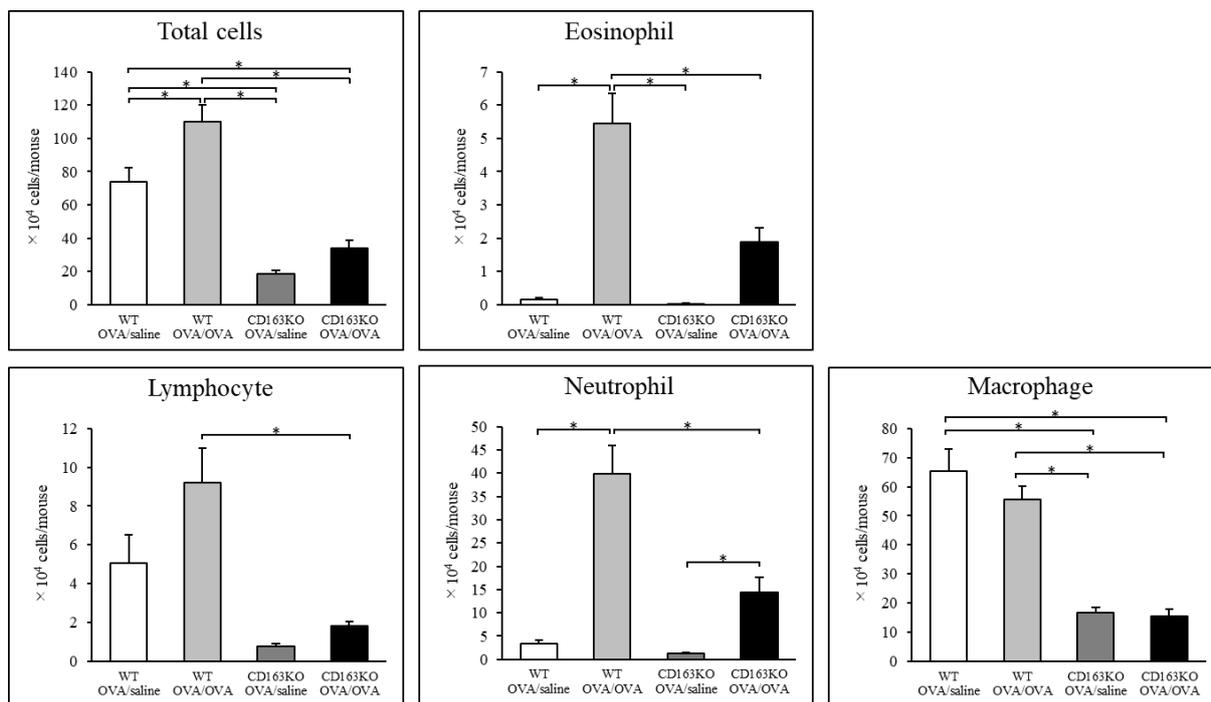
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520

521   Figure.5

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523

524   Figure.6

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