

27 **ABSTRACT**

28 Angiotensin-(1-7) [Ang-(1-7)]/Mas receptor pathway is currently recognized as a
29 counterbalancing mechanism of the renin-angiotensin system in different
30 pathophysiological conditions. We have previously described that treatment with Ang-(1-7)
31 attenuates lung inflammation and remodeling in an experimental model of asthma. In the
32 present study, we investigated whether lack of Mas receptor could alter the inflammatory
33 response in a model of chronic allergic lung inflammation induced by ovalbumin (OVA).
34 MasWT and MasKO mice were subjected to 4 doses of OVA (20µg/mice, i.p.) with 14 days
35 interval. At the 21st day, nebulization with OVA (1%) was started, 3 times/week until the
36 46th day. Control groups received saline (0.9%, i.p.) and were nebulized with saline (0.9%).
37 MasWT-OVA developed a modest inflammatory response and minor pulmonary
38 remodeling to OVA challenge. Strikingly, MasKO-OVA presented a significant increase in
39 inflammatory cell infiltrate, extracellular matrix deposition, increase in thickening of the
40 alveolar parenchyma, increase in thickening of the smooth muscle layer of the pulmonary
41 arterioles, increase in pro-inflammatory cytokine and chemokines levels in the lungs,
42 characteristic of chronic asthma. Additionally, MasKO-OVA presented an increase in
43 ERK1/2 phosphorylation in comparison to MasWT-OVA. Furthermore, MasKO-OVA
44 showed a worse performance in a test of maximum physical exercise in comparison to
45 MasWT-OVA. Our study shows that effects triggered by Mas receptor are important to
46 attenuate the inflammatory and remodeling processes in a model of allergic lung
47 inflammation in mice. Our data indicate that impairment of Ang-(1-7)/Mas receptor
48 pathway may lead to worsening of the pathophysiological changes of asthma.

49

50 **Keywords:** asthma; airway remodeling, pulmonary remodeling, Mas receptor and
51 Angiotensin-(1-7)

52 INTRODUCTION

53 Angiotensin-(1-7) [Ang-(1-7)] presents an essential role not only in the homeostasis
54 of the blood pressure, but also in counterbalancing the effects induced by Ang II in different
55 pathophysiology processes (25, 29). Ang-(1-7) is produced in the circulation and in
56 different tissues mainly by the action of angiotensin converting enzyme 2 (ACE2) (5, 30)
57 and the majority of its biological actions are mediated through the G-coupled Mas receptor
58 (26). At the present, the activation of the Ang-(1-7)/Mas receptor pathway is associated
59 with anti-inflammatory and anti-remodeling effects in different experimental pathological
60 conditions, such as cardiovascular disease (2, 18, 25, 29), arthritis (4), brain stroke (10, 22)
61 and lung fibrosis (19, 28). Recently, we (17, 24) and others (6) showed that Ang-(1-7)
62 treatment can attenuate inflammation and lung remodeling in a murine model of asthma.

63 Asthma is characterized by airway hyperresponsiveness and pulmonary remodeling,
64 in which immune, inflammatory and structural cells play important pathophysiological role
65 (7). Experimental models of allergic asthma are characterized by the predominance of
66 eosinophils, increased secretion of Th2 cytokines, such as IL-4, IL-5, IL-13, TNF α
67 chemokines, such as CCL2/MCP1 and CCL5/RANTES and, more adverse, remodeling in
68 large and small airways in the lung parenchyma and in the pulmonary arteries (17, 24, 31,
69 32). The alarming increase in the incidence of asthma is a major global health problem, not
70 only in the occidental world, but also in developing countries (23). Globally, approximately
71 300 million people have asthma, with 250,000 annual deaths attributed to the disease.
72 Recent studies suggest there will be a further increase of 100 million asthmatics by 2025
73 (23).

74 Two recent studies performed in our lab showed that treatment with Ang-(1-7) (17)
75 or the Mas receptor agonist, AVE 0991 (24), in an experimental model of chronic allergic
76 asthma could prevent leucocytes migration to the lung and significantly attenuate the levels

77 of different cytokines (IL-4, IL-5, TNF α) and chemokines (CCL2, CCL5, GMCSF) in the
78 lung. In addition, these treatments attenuated the thickness of alveolar parenchyma,
79 pulmonary arterioles and airway, decreased collagen deposition and airway
80 hyperresponsiveness (17). Furthermore, results from these studies suggested that the
81 protective effect of Ang-(1-7) in chronic allergic lung inflammation and remodeling can be
82 dependent on a reduction in the phosphorylation of ERK1/2 (17). These data are in keeping
83 with those of El-Hashim et al (6) which showed, in a short-term model of allergic lung
84 inflammation, Ang-(1-7) attenuated the increase in the total inflammatory cells infiltration
85 in the lung, decreased lung fibrosis and attenuated the phosphorylation of ERK1/2 and I κ B-
86 α in the lung. Additionally these authors showed that the MAS receptor antagonist, A779,
87 reversed these effects (6).

88 The effects mediated by Ang-(1-7) in the prevention and treatment of asthma in
89 mice are promising and may lead to new pharmacological strategies to suppress the Th2
90 inflammatory response. However, more experimental studies are needed to identify the key
91 mechanisms triggered by Ang-(1-7) leading to anti-inflammatory and anti-fibrotic effects in
92 asthma. In the present study, we investigated whether lack of receptor Mas could alter
93 inflammation and lung remodeling in a model of chronic allergic lung inflammation in
94 mice.

95

96 MATERIALS AND METHODS

97 *Animals.* FVB/N wild type (WT) and Mas receptor knockout (MasKO) mice were
98 separated in aleatory manner into different groups: Control MasKO (MasKO-CTRL; n=16);
99 MasKO, sensitized with OVA (MasKO-OVA; n=16); WT control mice (MasWT-CTRL;
100 n=16) and WT sensitized with OVA (MasWT-OVA; n=16). The animals were housed
101 under a 12-h light-dark cycle (light: 6 a.m. to 6 p.m.) with free access to standard chow and

102 water. All groups were performed at the same time. Animals were obtained from the
103 transgenic animal facility of the Laboratory of Hypertension, Federal University of Minas
104 Gerais, Brazil. The present study was approved by the Ethics Committee for Animal
105 Experimentation (CEUA) of the Federal University of Minas Gerais, Belo Horizonte, MG,
106 Brazil (protocol #309/2013).

107 *Ovalbumin immunization and challenge.* Sensitization was made by 4 injections of
108 OVA (20 µg/ mouse, i.p., Sigma, St. Louis, MO, USA) at 14 days intervals (Days 0, 14, 28
109 and 42nd). Beginning on the 21st day, sensitized mice received OVA challenge by
110 nebulization (1% OVA, during 30 min, 3 times/ week for 4 weeks). Nebulization was
111 performed in an acrylic box (30 cm x 15 cm x 20 cm) coupled to an ultrasonic nebulizer
112 (Soniclear; Sao Paulo, SP, Brazil) in groups of 5 animals for 4 weeks. The CTRL groups
113 received saline (NaCl 0.9%; 0.5 ml/ mouse. i.p.) and were challenged with saline at the
114 same time points. OVA was diluted in sterile isotonic saline and no adjuvant was used
115 (17,24).

116 *Incremental Exercise Test.* In order to assess the animal performance for physical
117 exercise, on the 47th day of the protocol, animals were subjected to an evaluation of their
118 functional capacity by a swimming incremental exercise test, as described by Almeida et al
119 (1). Briefly, all animals were adapted to a water pool (20 cm deep and water temperature
120 kept approximately at 28°C with a thermostat), for 5 minutes per day, for 5 consecutive days
121 with no overload (41st to 46th day). On the day of the test, mice were placed to swim with a
122 progressive load, which consisted in weights corresponding to 2% of body weight that were
123 added every 3 minutes to the mouse tail until maximal voluntary fatigue was achieved.
124 Maximal voluntary fatigue was considered when mice failed to rise to the surface of the
125 water to breathe within a seven-seconds period (1).

126 At the 49th day of the protocol, mice (n=10) were anesthetized with a mixture of
127 ketamine (0.5 mg.Kg⁻¹, i.p.) and xylazine (0.43 mg.Kg⁻¹, i.p.) and subjected to a midline
128 incision in the neck, in order to expose and isolate the trachea. The carotid artery was cut
129 and after the cardiorespiratory arrest, the trachea was then immediately clamped and the
130 lungs were collected in functional residual capacity. In some animals, the left lung and right
131 ventricle were collected for morphometric analysis and the right lung was removed, quickly
132 snapped frozen in dry ice and kept at -80°C until assayed, as previously described (17, 24).

133 *Bronchoalveolar lavage (BAL).* In different groups of animals (n=6 each), on the
134 49th day of the protocol, mice (MasKO-CTRL; MasKO-OVA; MasWT-CTRL and MasWT-
135 OVA) were killed by anesthetic overdose and the trachea of each animal was exposed and
136 cannulated with a polypropylene catheter of 1.7 mm. Airways were washed with 2 ml of
137 ice-cold PBS, BAL was collected and centrifuged. The pellet was resuspended in PBS/
138 BSA 3%. The trachea was clamped and the lungs were collected in functional residual
139 capacity, snapped frozen in dry ice and kept at -80°C until assayed. Total number of
140 leukocytes were counted using a Neubauer chamber after cells being stained with Turk's
141 solution. Differential leukocyte counts were performed on cytocentrifuge preparations
142 (Shandon Cytospin III,), stained with May-Grünwald-Giemsa using standard
143 morphological criteria to identify cell types.

144 *Morphometric analysis.* The lung was fixed in formalin and embedded in paraffin.
145 Sections (4 µm) were stained with hematoxylin and eosin for measurement of thickness of
146 alveolar parenchyma, airway epithelial wall and peribronchial arterial wall. Adjacent
147 sections were stained with Gomori's trichrome to evaluate extracellular matrix deposition in
148 the lungs. The method used for these measurements was detailed in previous studies (17,
149 24). For morphological analysis, both the experimenter and the reader/ interpreter were
150 blinded to the groups.

151 *Interleukin measurements in the lung.* Kits for multiple cytokines (Multiplex
152 cytokine analysis technology; Luminex®, Austin, TX, USA) were used for evaluation of
153 IL-13, TNF- α , CCL2/MCP-1 and CCL5/RANTES, following the instructions of the
154 manufacturer.

155 *ERK1/2 protein measurement by Western blotting.* Total protein was extracted from
156 lung tissue (n=4 from each group), and 50 μ g of total protein were applied to polyacrylamide
157 gel/SDS 10% and transferred to nitrocellulose membranes. The membranes were incubated
158 with primary antibody for the total or phosphorylated ERK1/2 [rabbit p44/42 Mark
159 (ERK1/2), 1:500; Cell Signaling (6)] followed by a fluorescent secondary antibody
160 [IRDye® 680 conjugated goat (polyclonal) anti-rabbit IgG (H+L), 1:10,000; Li-Color
161 Biosciences]. Levels were normalized to β -actin in the same sample. The ratio phospho-
162 ERK1/2 to total-ERK1/2 was made in each sample. Staining was visualized and quantified
163 on a LI-COR Odyssey Scanner (Lincoln, NE, USA).

164 *Statistical analyses.* All results are expressed as mean $\pm$ SEM. Comparisons among
165 groups were performed by two-way ANOVA followed by the Bonferroni post-hoc test. All
166 analysis and graphics were made with the GraphPad Prism software (version 5.0; GraphPad
167 Software, Inc., La Jolla, CA, USA). The level of significance was set at $p < 0.05$.

168

169 **RESULTS**

170 *Aerobic Exercise Capacity.* It is well known that changes in pulmonary function
171 may influence the functional capacity and exercise tolerance in patients with asthma or
172 animals subjected to a model of allergic lung injury (33). In the Figure 1, we observe that
173 OVA-challenge significantly reduced aerobic exercise capacity in MasWT mice (570 ± 6 s
174 vs 666 ± 13 s, in MasWT-CTRL) and in MasKO mice (438 ± 22 s vs 695 ± 23 s, in MasKO-
175 CTRL). In addition, mice with deletion of Mas receptor sensitized/challenged with OVA

176 had a worse performance compared with the wild type mice challenged with OVA. These
177 results suggest that chronic OVA sensitization might have induced inspiratory muscle
178 weakness culminating with inability to maintain adequate respiratory function during
179 physical stress test. This alteration is further exacerbated in animals with deletion of the
180 Mas receptor.

181 *Total inflammatory cells infiltration in the lung.* The increase in eosinophils
182 infiltration in the lung of asthmatic patients or experimental models of asthma are associated
183 with epithelial damage, goblet cell hyperplasia, smooth muscle hypertrophy and airway
184 hyperresponsiveness (7, 32). Here, we investigated the effect of genetic deletion of Mas
185 receptor on eosinophilic inflammation in the lung of mice subjected the model of chronic
186 asthma. First, pulmonary inflammation was assessed by quantification of total cells (Fig.
187 2A) and differential cells (Fig. 2B and 2C) in the BAL. The challenge with OVA induced
188 an increase in the number of total cells in BAL in MasWT and MasKO mice compared to
189 their respective controls (Fig. 2A). However, while in MasKO-OVA 62% of the total
190 leukocytes were eosinophils, in MasWT-OVA only 16% were eosinophils (Fig. 2B),
191 showing marked difference in the allergic response of these mice.

192 It has been shown that inflammation in asthmatic mice is not restricted to the large
193 airways, extending to the small airways, parenchyma and pulmonary vessels (11, 24).
194 Challenge with OVA in MasWT and MasKO groups increased total inflammatory cells in
195 the lung compared with the respective control groups (Fig. 3). However, animals MasKO-
196 OVA presented significant greater score of inflammatory cells infiltrate in comparison to
197 the MasWT-OVA group.

198 *Cytokines concentrations in the lung.* Figure 4 shows the concentration of
199 interleukin IL-13, TNF α , CCL2/MCP-1 and CCL5/RANTES in the lung. OVA challenge
200 did not significantly alter interleukin (IL-13 and TNF α) or CCL5/RANTES in Mas WT

201 mice compared to the MasWT-CTRL. On the other hand, OVA challenge in MasKO mice
202 induced a significant increase in IL-13, TNF α , CCL2/MCP-1 and CCL5/RANTES in
203 comparison to the MasKO-CTRL and a significant increase in IL-13, CCL2/MCP-1 and
204 CCL5/RANTES in comparison to the MasWT-OVA.

205 *Evaluation of lung remodeling.* There was no significant difference between
206 MasWT-CTRL and MasWT-OVA with respect of thickness of alveolar parenchyma (Fig.
207 5A, 5B and 5E). The lungs of animals with deletion of the Mas receptor showed greater
208 thickening of the alveolar parenchyma. In addition, the MasKO-OVA group exhibited a
209 large interalveolar interstitial thickening, resulting mainly from the accumulation of
210 inflammatory cells (Fig. 5D and 5E). These results suggest that OVA challenge in mice
211 with deletion of the Mas receptor produces increase in inflammatory cells infiltration and
212 increase in alveolar wall thickness in mice with a background that seems to be less effective
213 to induce asthma.

214 MasWT mice challenged with OVA showed no significant difference in the
215 proportion of peribronchiolar extracellular matrix deposition and epithelial thickness
216 compared to control mice (Fig. 5A, 5B, 5F and 5G). However, sensitization with OVA, in
217 MasKO group, increased the epithelial thickness and peribronchiolar extracellular matrix
218 deposition in comparison to other groups (Fig. 5D, 5F and 5G). Again, FVB/N mice, seems
219 to be less sensitive to develop of asthma, at least using the protocol of the present study.
220 However, lack of the Mas receptor resulted in intense increase in extracellular matrix
221 deposition and thickness of the bronchioles epithelial. In addition, chronic OVA exposure
222 significantly increased smooth muscle layer in vessels around airway wall, in both MasWT
223 and MasKO groups in comparison with their respective controls (Fig. 5 A-D and 5H).
224 However, in mice with deletion of the Mas receptor exposed to OVA,

225 hyperplasia/hypertrophy of vascular smooth muscle were greater than that observed in with
226 WT mice (Fig. 5B, 5D and 5H).

227 *ERK1/2 in the lung.* In evaluating the degree of phosphorylation of ERK1/2 in mice
228 subjected to experimental model of asthma, we observed that the ratio of phospho to total
229 ERK1/2 in MasWT-OVA group was not different from MasWT-CTRL group. However,
230 MasKO-OVA mice presented an increase in the ratio of phospho to total ERK1/2 in the
231 lung in comparison to MasKO-CTRL (Fig. 6).

232

233 **DISCUSSION**

234 In the present study, we showed that Mas is an important receptor to trigger anti-
235 inflammatory mechanisms in a murine model of asthma. This study indicates that
236 impairment in Ang-(1-7)/receptor pathway may lead to deterioration of the
237 pathophysiologic progress of asthma. We showed that FVB/N mice with genetic deletion of
238 the receptor Mas subjected to a model chronic allergic lung inflammation presented a worse
239 tolerance towards physical exercise. Additionally, MasKO-OVA mice presented a
240 significant increase in the number of eosinophils in BAL and inflammatory cell infiltrate in
241 the lung, an increased deposition of the extracellular matrix in the airways, an increased
242 thickening of the alveolar parenchyma and smooth muscle layer of the pulmonary arterioles.
243 Furthermore, in MasKO-OVA there was an increase in ERK1/2 phosphorylation and an
244 increase in pro-inflammatory cytokine (IL13) and chemokines (CCL2/MCP-1 and CCL5/
245 RANTES) in the lungs. The majority of these effects were greater in MasKO-OVA than
246 those observed in MasWT-OVA.

247 Ang-(1-7) acting through Mas receptor has been described as an important pathway
248 of the renin-angiotensin system (RAS) involved in attenuating inflammation and fibrosis, in
249 different pathophysiological conditions (4, 13, 19, 28, 29). Recently, we (17, 24) and others

(6) have shown that administration of Ang-(1-7) promotes attenuation of the pulmonary inflammation and tissue remodeling. The present work advanced these results by showing that lack of Mas receptor resulted in an intense degree of lung inflammation and remodeling in a mice strain (FVB/N) less sensitive to experimental model of asthma. Indeed, FVB/N-WT mice presented an attenuated response to OVA challenge compared to the response observed in Balb/C mice subjected to the same protocol in a previous study in our laboratory (17). For example, asthmatic Balb/C mice presented increased index of peribronchiolar matrix deposition (5.64 ± 0.76) and an increased score of inflammatory cell infiltrate (2.275 ± 0.18) compared to the control group (1.33 ± 0.17 and 0.15 ± 0.07 , respectively). However, the index of matrix deposition (1.92 ± 0.15) and the score of cell infiltrate (1.78 ± 0.18) were lower in asthmatic FVB/N-WT, although significantly different from CTRL-FVB/N-WT. These data indicate that FVB/N background is less susceptible to the protocol used to induce asthma in the present study (3, 11). Strikingly, however, lack of Mas resulted in an intense inflammatory response. Asthmatic FVB/N-MasKO mice presented a higher score of cell infiltration (2.76 ± 0.06) in comparison with asthmatic Balb/C or FVB/N-WT and not significantly different from the peribronchiolar matrix deposition index (3.78 ± 0.24) of Balb/C mice. Therefore, deletion of Mas receptor induces worsening of the development of chronic allergic lung inflammation in mice.

In previous studies, we showed that treatment with Ang-(1-7) or AVE0991, a Mas receptor agonist, decreased inflammatory cell infiltration, collagen deposition and remodeling in the lung parenchyma of OVA-sensitized and challenged Balb/C mice (17, 24). In addition, Ang-(1-7) or AVE 0991 inhibited the vascular remodeling and right ventricular hypertrophy (17, 24). Furthermore, the results in these studies suggested that Ang-(1-7) protective effects in the lung could be mediated by bronchial cells and are dependent on the reduction in the phosphorylation of ERK1/2 and a decrease in the levels of

275 cytokines and chemokines in the lung (17). Other studies are in keeping with these
276 observations and, indicate that Ang-(1-7) protection against pulmonary fibrosis (19) and
277 asthma (6) can be mediated by down-regulation of the MAPK/NF- κ B pathway.

278 In the present study, we showed a significant increase in phospho-ERK1/2, IL-13,
279 CCL2/MCP-1 and CCL5/ RANTES in the lung of MasKO-OVA mice, associated with
280 intense pulmonary inflammatory cell infiltration and remodeling. A large number of studies
281 have demonstrated that IL-13 is implicated in the pathogenesis of lung inflammation and
282 airway remodeling, which are characteristic of asthma (9, 15, 16, 21, 36). These studies
283 demonstrated that IL-13 is a potent stimulator of eosinophils, lymphocytes and macrophage
284 migration to the site of injury and stimulates chemokines, such CCL2/MCP-1 and
285 CCL5/RANTES (9, 12, 15, 16). Consequently, the increased level of IL-13 induces mucus
286 production, lung remodeling and airway hyperresponsiveness (12, 15, 16, 29). Lee et al.
287 (12) demonstrated that activation of ERK1/2 MAPK pathway plays a critical role in
288 mediating IL-13-induced inflammation, stimulation of chemokines (MIP-1 α /CCL-3, MIP-
289 1 β /CCL-4, MIP-2/CXCL1 and RANTES/CCL5) and lung remodeling. In addition, Zhu et
290 al (36) suggested that MCP-1 mediated signaling via CCR2 plays a key role in lung
291 inflammation and fibrosis induced by IL-13. These data suggest that these inflammatory
292 mediators are important targets for further investigation designed to identify new agents,
293 which can block the increase in IL-13, CCL2/MCP-1 and CCL5/RANTES and the
294 phosphorylation of ERK1/2.

295 The treatment with Ang-(1-7) decreased phospho-ERK1/2 in short (6) or long-term
296 models the asthma (17). Additionally, Ang-(1-7) decreased the expression of chemokines,
297 CCL2/MCP-1 and CCL5/RANTES, in the lung of OVA-challenged mice (17). These
298 results were associated with reduced lung inflammation and remodeling (17). Interestingly,
299 our data showing that OVA-challenged MasKO mice presented increased levels of IL-13,

300 CCL2/MCP-1, CCL5/RANTES, p-ERK1/2 and inflammatory cell infiltration and airway
301 remodeling are in agreement with these previous results. Altogether, these data suggest that
302 Ang-(1-7)/Mas receptor pathway is an important target for modulating the release of
303 inflammatory mediators involved in the pathogenesis of asthma. We cannot rule out,
304 however, that other intracellular mechanisms, cytokines and chemokines are involved in
305 mediating the anti-inflammatory effects of Ang-(1-7) in asthma.

306 The bronchial epithelial cells are the first barrier of defense against antigens and are
307 also a source of inflammatory mediators which are responsible for neutralizing antigen
308 invasion (14, 34). During allergic pulmonary inflammation, bronchial epithelial cells
309 produce a variety of inflammatory mediators and proteins of the extracellular matrix which
310 contribute to the induction of the inflammation and, later on, with lung remodeling, which
311 are the key feature of asthma (14, 34). In addition to IL-13, the chemokines, eotaxin-
312 CCL11, MCP-1, IL-4, IL-5, IL-8, IL-17 are examples of mediators produced by epithelial
313 cells and leukocytes in chronic allergic asthma (8). In our previous study, we showed that
314 Ang-(1-7) treatment prevents the increase in IL5, GM-CSF, CCL2/MCP-1, CCL5/RANTES
315 in OVA-challenged mice (17) and these alterations in cytokines/chemokines were
316 accompanied with attenuation of inflammation in the lung. On the other hand, in asthmatic
317 patients, IL-8 is an important protein for the activation and chemo-attraction of neutrophils
318 during airway inflammation in severe asthma (35). Patients with severe asthma exhibit
319 increased IL-8 in the sputum correlated with the increase in neutrophils in the lung (35). In
320 addition, neutrophils and IL-8 are inflammatory components that differentiate between
321 controlled and uncontrolled asthma (8, 35). IL-8, however, is not expressed in mice (20).
322 Therefore, further studies are needed to investigate whether the effect of Ang-(1-7)/Mas
323 receptor in the production of pro-inflammatory mediators by human lung cells, eosinophils

324 and neutrophils and their intracellular mechanisms are comparable to those described in
325 mice.

326 In the present study, we showed the importance of Mas receptor on an
327 immunological response caused by chronic challenge with ovalbumin. Interestingly,
328 FVB/N-WT mice challenged with OVA showed a modest inflammatory response, while,
329 genetic deletion of the Mas receptor removed this protection. Indeed, chronic allergic
330 pulmonary inflammation is aggravated in Mas receptor KO mice. These animals presented
331 increased infiltration of inflammatory cells, increased levels of IL-13, CCL2/MCP-1, and
332 CCL5/RANTES, greater deposition of extracellular matrix in the lung, possibly by the
333 activation of ERK1/2 signaling by IL-13. In addition, these lung alterations were associated
334 with worsening tolerance to exercise. These data indicate that pulmonary inflammation and
335 remodeling caused by OVA challenge in mice with genetic deletion of the Mas receptor
336 promotes deterioration of lung function and consequently worsening tolerance to aerobic
337 capacity.

338

339 In conclusion, our findings show that Mas receptor-induced effects are important
340 counterbalancing mechanisms of the RAS for attenuating the inflammatory process in
341 asthma. Moreover, impairment of the Ang-(1-7)/Mas receptor pathway may lead to the
342 deterioration of the pathophysiology of asthma.

343

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353 REFERENCES

- 354 1. Almeida PW, Gomes-Filho A, Ferreira AJ, Rodrigues CE, Dias-Peixoto MF,
 355 Russo RC, Teixeira MM, Cassali GD, Ferreira E, Santos IC, Garcia AM, Silami-
 356 Garcia E, Wisløff U, Pussieldi GA. Swim training suppresses tumor growth in mice.
 357 *J Appl Physiol* 107: 261-265, 2009.
- 358 2. Almeida PW, Melo MB, Lima RF, Gavioli M, Santiago NM, Greco L, Jesus IC,
 359 Nocchi E, Parreira A, Alves MN, Mitraud L, Resende RR, Campagnole-Santos
 360 MJ, Santos RA, Guatimosim S. Beneficial effects of angiotensin-(1-7) against
 361 deoxycorticosterone acetate-induced diastolic dysfunction occur independently of
 362 changes in blood pressure. *Hypertension* 66(2): 389-395, 2015.
- 363 3 Conrad ML, Yildirim AO, Sonar SS, Kılıc A, Sudowew S, Lunowz M, Teich R,
 364 Renz H, Garn H. Comparison of adjuvant and adjuvant-free murine experimental
 365 asthma models. *Clin Exper Allergy* 39: 1246–1254, 2009.
- 366 4. da Silveira KD, Coelho FM, Vieira AT, Sachs D, Barroso LC, Costa VV, Bretas
 367 TL, Bader M, de Sousa LP, da Silva TA, dos Santos RA, Simões e Silva AC,
 368 Teixeira MM. Anti-inflammatory effects of the activation of the angiotensin-(1-7)
 369 receptor, MAS, in experimental models of arthritis. *J Immunol* 185: 5569-5576, 2010.
- 370 5. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan
 371 M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel
 372 angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin
 373 I to angiotensin 1-9. *Circ Res* 87: E1-9, 2000.
- 374 6. El-Hashim AZ, Renno WM, Raghupathy R, Abduo HT, Akhtar S, Benter IF.
 375 Angiotensin-(1-7) inhibits allergic inflammation, via the MAS1 receptor, through
 376 suppression of ERK1/2- and NF-κB-dependent pathways. *Br J Pharmacol* 166: 1964-
 377 1976, 2012.

- 378 7. **Holgate ST.** Asthma: a simple concept but in reality a complex disease. *Eur J Clin*
379 *Invest* 41: 1339-1352, 2011.
- 380 8. **Hosoki K, Ying S, Corrigan C, Qi H, Kurosky A, Jennings K, Sun Q, Boldogh I,**
381 **Sur S.** Analysis of a panel of 48 cytokines in BAL fluids specifically identifies IL-8
382 levels as the only cytokine that distinguishes controlled asthma from uncontrolled
383 asthma, and correlates inversely with FEV1. *PLoS One* 10(5): e0126035, 2015.
- 384 9. **Ingram JL, Kraft M.** IL-13 in asthma and allergic disease: asthma phenotypes and
385 targeted therapies. *J Allergy Clin Immunol* 130(4): 829-842, 2012.
- 386 10. **Jiang T, Gao L, Lu J, Zhang YD.** ACE2-Ang-(1-7)-Mas Axis in Brain: A Potential
387 Target for Prevention and Treatment of Ischemic Stroke. *Curr Neuroparmacol* 11:
388 209-217, 2013.
- 389 11. **Kumar RK, Herbert C, Foster PS.** The "Classical" Ovalbumin Challenge Model of
390 Asthma in Mice. *Current Drug Targets* 9: 485-494, 2008.
- 391 12. **Lee PJ, Zhang X, Shan P, Ma B, Lee CG, Homer RJ, Zhu Z, Rincon M, Mossman**
392 **BT, Elias JA.** ERK1/2 mitogen-activated protein kinase selectively mediates IL-13-
393 induced lung inflammation and remodeling in vivo. *J Clin Invest* 116: 163-173, 2006.
- 394 13. **Liang B, Wang X, Zhang N, Yang H, Bai R, Liu M, Bian Y, Xiao C, Yang Z.**
395 Angiotensin-(1-7) Attenuates Angiotensin II-Induced ICAM-1, VCAM-1, and MCP-1
396 Expression via the MAS Receptor Through Suppression of P38 and NF- κ B Pathways
397 in HUVECs. *Cell Physiol Biochem* 35: 2472-2482, 2015.
- 398 14. **Lin TY, Venkatesan N, Nishioka M, Kyoh S, Al-Alwan L, Baglole CJ, Eidelman**
399 **DH, Ludwig MS, Hamid Q.** Monocyte-derived fibrocytes induce an inflammatory
400 phenotype in airway smooth muscle cells. *Clin Exp Allergy* 44(11): 1347–1360, 2014.

- 401 15. **Ma B, Zhu Z, Homer RJ, Gerard C, Strieter R, Elias JA.** The C10/CCL6
402 Chemokine and CCR1 Play Critical Roles in the Pathogenesis of IL-13-Induced
403 Inflammation and Remodeling. *J Immunol* 172: 1872-1881, 2004.
- 404 16. **Ma B, Liu W, Homer RJ, Lee PJ, Coyle AJ, Lora JM, Lee CG, Elias JA.** Role of
405 CCR5 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol*
406 176: 4968-4978, 2006.
- 407 17. **Magalhães GS, Rodrigues-Machado MG, Motta-Santos D, Silva AR, Caliari MV,**
408 **Prata LO, Abreu SC, Rocco PR, Barcelos LS, Santos RA, Campagnole-Santos**
409 **MJ.** Angiotensin-(1-7) attenuates airway remodelling and hyperresponsiveness in a
410 model of chronic allergic lung inflammation. *Br J Pharmacol* 172: 2330-2342, 2015.
- 411 18. **Marques FD, Ferreira AJ, Sinisterra RD, Jacoby BA, Sousa FB, Caliari MV, Silva**
412 **GA, Melo MB, Nadu AP, Souza LE, Irigoyen MC, Almeida AP, Santos RA.** An
413 oral formulation of angiotensin-(1-7) produces cardioprotective effects in infarcted and
414 isoproterenol-treated rats. *Hypertension* 57: 477-483, 2011.
- 415 19. **Meng Y, Yu CH, Li W, Li T, Luo W, Huang S, Wu PS, Cai SX, Li X.** Angiotensin-
416 converting enzyme 2/angiotensin-(1-7)/Mas axis protects against lung fibrosis by
417 inhibiting the MAPK/NF- κ B pathway. *Am J Respir Cell Mol Biol* 50: 723-736, 2014.
- 418 20. **Modi WS, Yoshimura T.** Isolation of novel GRO genes and a phylogenetic analysis of
419 the CXC chemokine subfamily in mammals. *Mol Biol Evol* 16(2): 180-93, 1999.
- 420 21. **Rael EL, Lockey RF.** Interleukin-13 signaling and its role in asthma. *World Allergy*
421 *Organ J* 4: 54-64, 2011.
- 422 22. **Regenhardt RW, Desland F, Mecca AP, Pioquinto DJ, Afzal A, Mocco J, ,**
423 **Sumners C.** Anti-inflammatory effects of angiotensin-(1-7) in ischemic stroke.
424 *Neuropharmacology* 71: 154-163, 2013.

- 425 23. **Rincon M, Irvin CG.** Role of IL-6 in asthma and other inflammatory pulmonary
426 diseases. *Int J Biol Sci* 8: 1281-1290, 2012.
- 427 24. **Rodrigues-Machado MG, Magalhães GS, Cardoso JA, Kangussu LM, Murari A,**
428 **Caliari MV, Oliveira ML, Cara DC, Noviello ML, Marques FD, Pereira JM,**
429 **Lautner RQ, Santos RA, Campagnole-Santos MJ.** AVE 0991, a non-peptide mimic
430 of angiotensin-(1-7) effects, attenuates pulmonary remodelling in a model of chronic
431 asthma. *Br J Pharmacol* 170: 835-846, 2013.
- 432 25 **Santos RA.** Angiotensin-(1-7). *Hypertension* 63: 1138-1147, 2014.
- 433 26. **Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I,**
434 **Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS,**
435 **Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T.** Angiotensin-(1-7) is
436 an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S*
437 *A* 100: 8258-8263, 2003.
- 438 27. **Seumois G, Chavez L, Gerasimova A, Lienhard M, Omran N, Kalinke L,**
439 **Vedanayagam M, Ganesan AP, Chawla A, Djukanović R, Ansel KM, Peters B,**
440 **Rao A, Vijayanand P.** Epigenomic analysis of primary human T cells reveals
441 enhancers associated with TH2 memory cell differentiation and asthma susceptibility.
442 *Nat Immunol* 15: 777-788, 2014.
- 443 28. **Shenoy V, Ferreira AJ, Qi Y, Fraga-Silva RA, Díez-Freire C, Dooies A, Jun JY,**
444 **Sriramula S, Mariappan N, Pourang D, Venugopal CS, Francis J, Reudelhuber T,**
445 **Santos RA, Patel JM, Raizada MK, Katovich MJ.** The angiotensin-converting
446 enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against
447 lung fibrosis and pulmonary hypertension. *Am J Respir Crit Care Med* 182: 1065-1072,
448 2010.

- 449 29. **Simões e Silva AC, Silveira KD, Ferreira AJ, Teixeira MM.** ACE2, angiotensin-(1-
450 7) and Mas receptor axis in inflammation and fibrosis. *Br J Pharmacol* 169: 477-492,
451 2013.
- 452 30. **Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ.** A human
453 homolog of angiotensin-converting enzyme. Cloning and functional expression as a
454 captopril-insensitive carboxypeptidase. *J. Biol. Chem.* 275: 3238-3242, 2000.
- 455 31. **Vieira RP, Claudino RC, Duarte AC, Santos AB, Perini A, Faria Neto HC, Mauad**
456 **T, Martins MA, Dolhnikoff M, Carvalho CR.** Aerobic exercise decreases chronic
457 allergic lung inflammation and airway remodeling in mice. *Am J Respir Crit Care Med*
458 176: 871-877, 2007.
- 459 32. **Vieira RP, de Andrade VF, Duarte AC, Dos Santos AB, Mauad T, Martins MA,**
460 **Dolhnikoff M, Carvalho CR.** Aerobic conditioning and allergic pulmonary
461 inflammation in mice. II. Effects on lung vascular and parenchymal inflammation and
462 remodeling. *Am J Physiol Lung Cell Mol Physiol* 295: L670-679, 2008.
- 463 33. **Vogiatzis I, Zakynthinos G, Andrianopoulos V.** Mechanisms of physical activity
464 limitation in chronic lung diseases. *Pulm Med* 2012: 634–761, 2012.
- 465 34. **Wong CK, Wang CB, Li ML, Ip WK, Tian YP, Lam CW.** Induction of adhesion
466 molecules upon the interaction between eosinophils and bronchial epithelial cells:
467 involvement of p38 MAPK and NF-kappaB. *Int Immunopharmacol* 6: 1859–1871,
468 2006.
- 469 35. **Yalcin AD, Bisgin A, Gorczynski RM.** IL-8, IL-10, TGF- β , and GCSF levels were
470 increased in severe persistent allergic asthma patients with the anti-IgE treatment.
471 *Mediators Inflamm.* 2012: 720976, 2012.

472 36. **Zhu Z, Bing Ma, Zheng T, Homer RJ, Lee CG, Charo IF, Noble P, Elias JA.** IL-
473 13-Induced Chemokine Responses in the Lung: Role of CCR2 in the Pathogenesis of
474 IL-13-Induced Inflammation and Remodeling. *J Immunol* 168: 2953-2962, 2002.

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482

483

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486

487 **FIGURE LEGENDS**

488 **Figure 1.** Aerobic exercise capacity measured by the total time (sec) spent in the swimming
489 incremental test. OVA challenge significantly reduced aerobic exercise capacity in MasWT
490 and MasKO. MasKO-OVA mice, however, showed a worse performance compared with
491 MasWT-OVA mice (n=6 each group). *p< 0.05 compared to respective control and #p<
492 0.05 compared to MasWT-OVA group (2-way ANOVA followed by Bonferroni test).

493

494 **Figure 2.** Quantification of inflammatory cells in the bronchoalveolar lavage (BAL).
495 Number of total cells (A), mononuclear cells (B) and eosinophils (C) 72 hours after the last
496 challenge with OVA (n=6 each group). *p< 0.05 compared to respective control; #p <0.05
497 compared to MasWT-OVA (2-way ANOVA followed by Bonferroni test).

498

499 **Figure 3.** Histological score of total inflammatory cell infiltrate in different areas of the
500 lung (n=5 each group). OVA challenge significantly increased total inflammatory cells in
501 the lung of WT and KO mice. However, MasKO-OVA mice presented significant higher
502 total cell infiltration in comparison with MasWT-OVA in all areas. *p< 0.05 compared to
503 respective control and #p< 0.05 compared to MasWT-OVA group (2-way ANOVA
504 followed by Bonferroni test).

505

506 **Figure 4.** Concentration of cytokines IL-13, TNF α , CCL2/MCP-1 and CCL5/RANTES in
507 the lung (pg/mg of protein; n=4-5) measured by ELISA assay. MasKO-OVA mice
508 presented higher levels of cytokines in comparison to MasWT-OVA mice. *p< 0.05
509 compared to respective control and #p< 0.05 compared to MasWT-OVA group (2-way
510 ANOVA followed by Bonferroni test).

511 **Figure 5.** Parameters of lung remodeling. Panels A-D present representative
512 photomicrographs of the lung of MasWT-CTRL (A), MasWT-OVA (B), MasKO-CTRL (C)
513 and MasKO-OVA (D) mice showing interalveolar interstitial space (asterisks), airway
514 epithelial thickness and extracellular matrix deposition (arrow). Panels E-H present
515 alveolar thickness (E; mm), indexes of airway epithelial thickness (F) and airway
516 extracellular matrix deposition (G) and vascular wall thickness (H; arrowhead; μm).
517 MasKO-OVA mice presented higher parameters of lung remodeling in comparison to
518 MasWT-OVA. * $p < 0.05$ compared to respective control and # $p < 0.05$ compared to
519 MasWT-OVA (2-way ANOVA followed by Bonferroni test).

520

521 **Figure 6.** ERK1/2 protein levels in the lungs. Densitometric quantification (n=4) by
522 Western blotting, on the left, and representative blots from 2 mice are shown on the right.
523 MasKO-OVA mice presented increased ratio of p-ERK1/2 to t-ERK1/2 in comparison with
524 MAS-KO-CTRL. No change in WT mice was observed. * $p < 0.05$ compared to respective
525 control (2-way ANOVA followed by Bonferroni test).

526

527

Figure 1

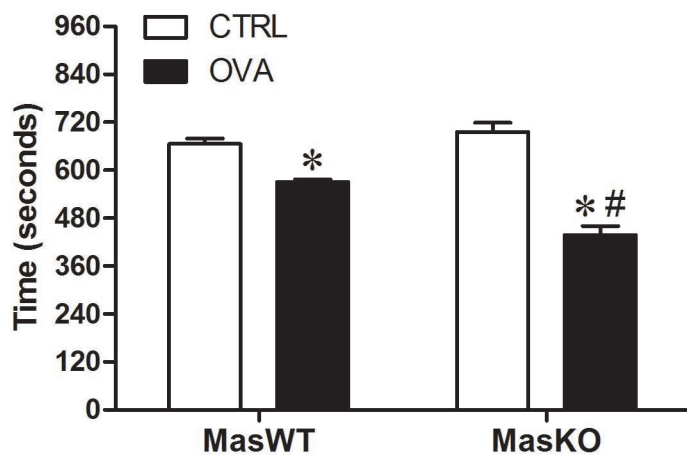


Figure 2

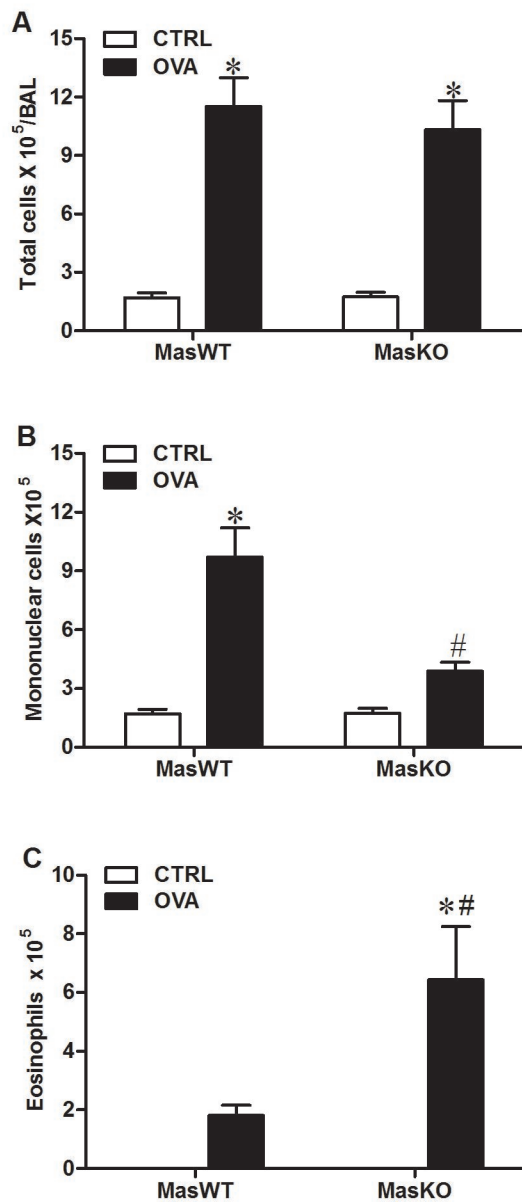


Figure 3

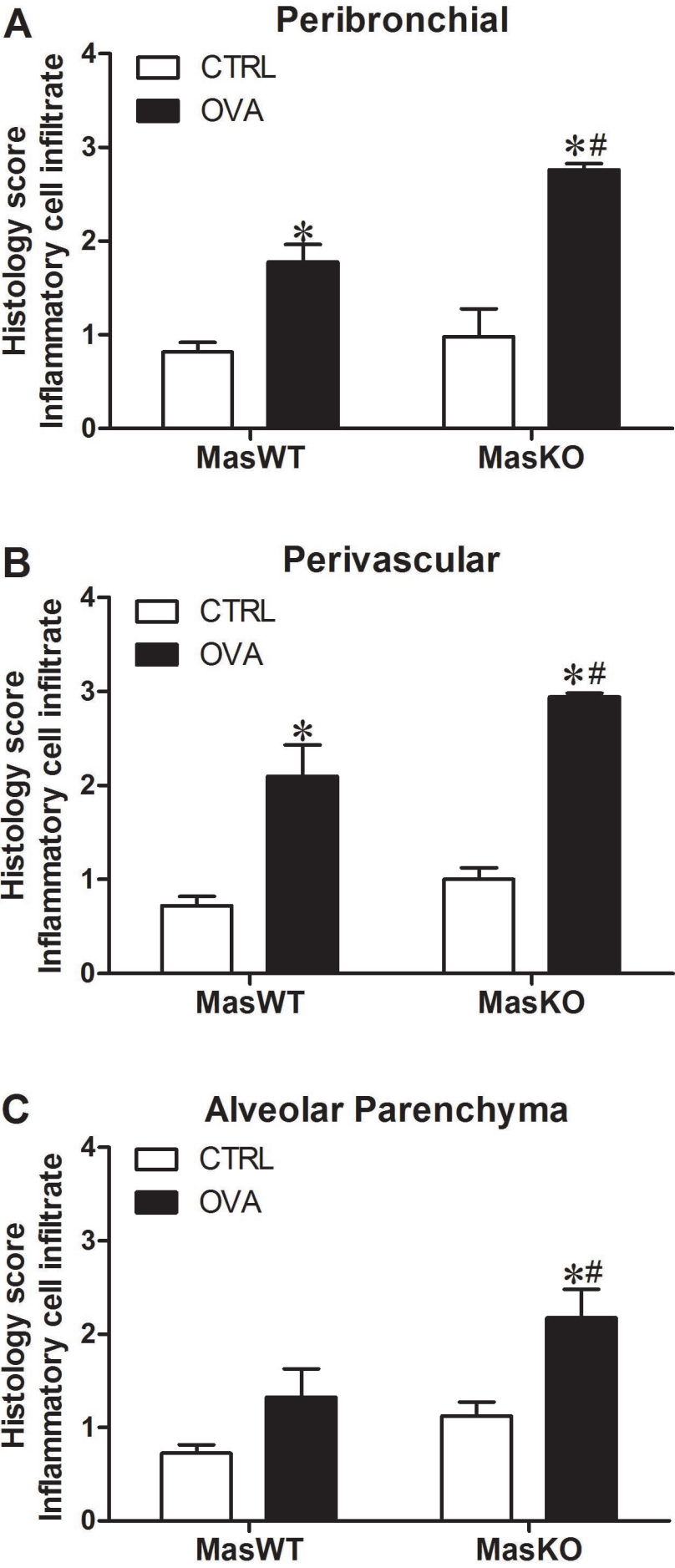
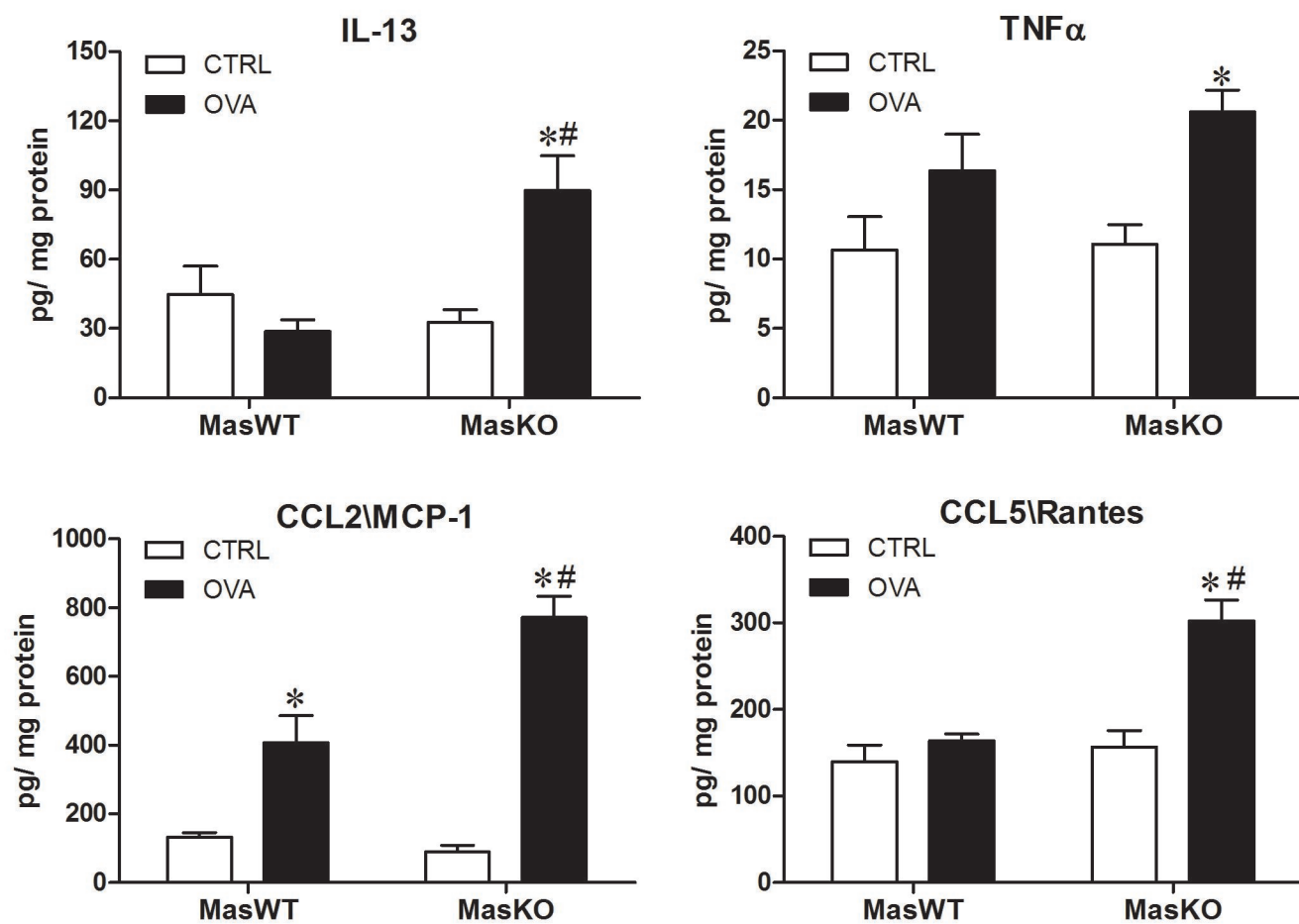


Figure 4



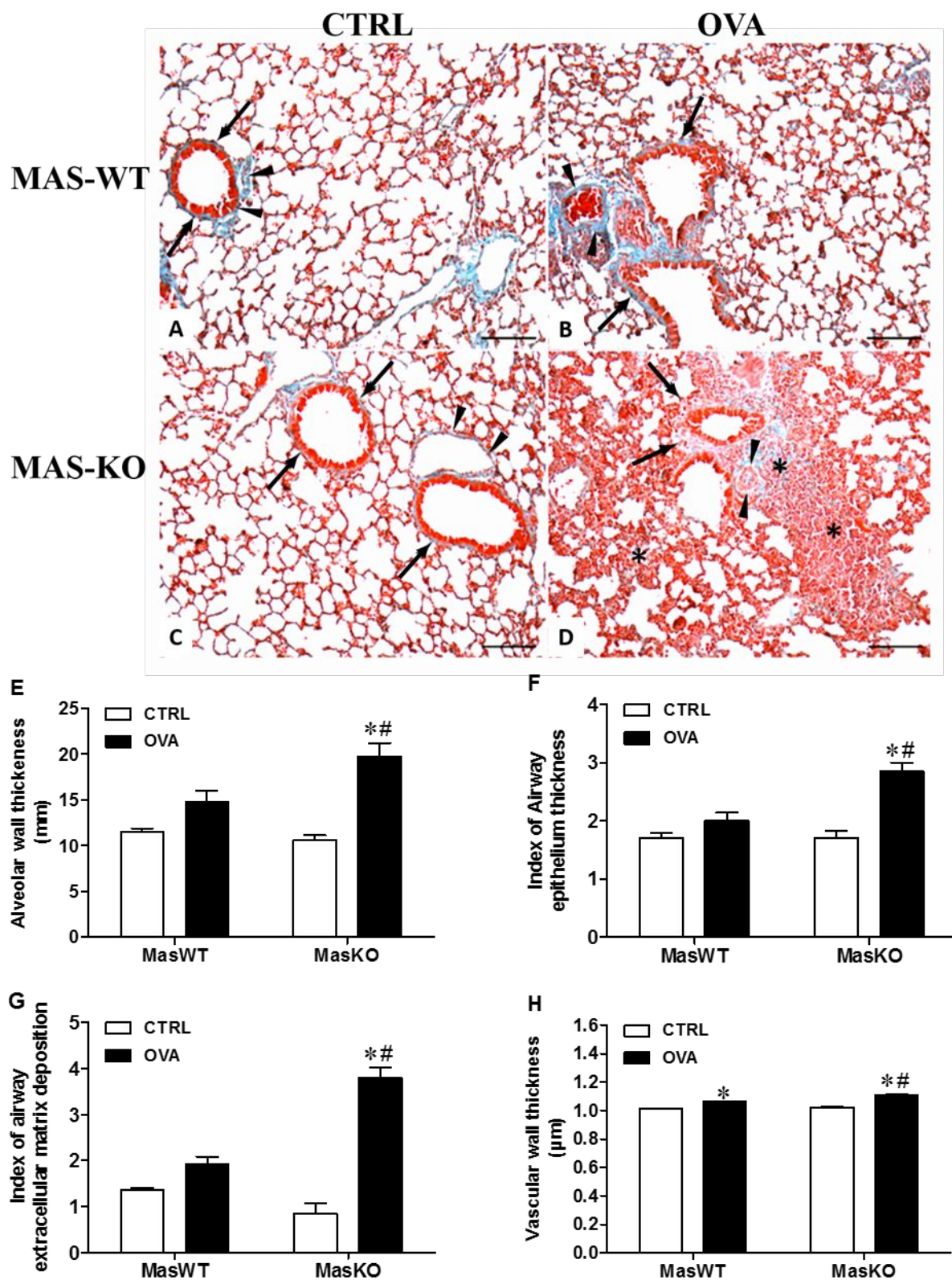


Figure 6

