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Astragalin inhibits allergic inflammation and airway thickening in ovalbumin-challenged mice

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Running Title: Blockade of airway thickening and inflammation by astragalin

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ABSTRACT

Lung inflammation and oxidative stress are the major contributors to developing obstructive pulmonary diseases. Macrophages are involved in pulmonary inflammation and alveolar damage in emphysema. Astragalin is an anti-inflammatory flavonoid present in persimmon leaves and green tea seeds. This study elucidated that astragalin inhibited inflammatory cell infiltration induced by 20 μ M H₂O₂, and blocked airway thickening and alveolar emphysema induced by 20 μ g ovalbumin (OVA) in mice. OVA induced mouse pulmonary MCP-1, and H₂O₂ enhanced the expression of MCP-1/ICAM-1/ α v integrin in bronchial airway epithelial BEAS-2B cells. Such induction was inhibited by supplying 10-20 mg/kg astragalin to OVA-challenged mice and 1-20 μ M astragalin to oxidant-stimulated cells. Oral administration of 20 mg/kg astragalin reduced the induction of F4/80/CD68/D11b in airways of mice challenged to OVA. Additionally, emphysema tissue damage was observed in OVA-exposed alveoli. The mast cell recruitment in the airway subepithelium was blocked by supplementing astragalin to OVA-challenged mice. Orally-treating 20 mg/kg astragalin reduced α -SMA induction in inflammation-occurring airways and appeared to reverse airway thickening and constriction induced by OVA episode. These results revealed that astragalin may improve airway thickening and alveolar destruction with blockade of allergic inflammation in airways. Therefore, astragalin may be a therapeutic agent antagonizing asthma and obstructive pulmonary diseases.

Key Words: Airway inflammation; airway smooth muscle; astragalin; macrophages; mast cells; neutrophils; oxidative stress

28 INTRODUCTION

29

30 Chronic obstructive pulmonary disease (COPD) may be associated with pro-inflammatory
31 responses of the lung to toxic materials and oxidants.^{(1),(2),(3)} Chronic inflammation in the airways is
32 primarily characterized by hypersecretion of mucus and stenosis of the smaller airways.^{(1),(4),(5)}
33 Airway narrowing takes place due to inflammation, and abnormal inflammatory pathways result in a
34 loss of alveolar integrity and are observed under clinical conditions of idiopathic pulmonary fibrosis
35 (IPF) and emphysema.^{(6),(7)} The responsible inflammatory cells include neutrophil granulocytes and
36 macrophages, and inflammatory responses are brought on by various mediators derived from
37 these cells such as reactive oxygen species (ROS), chemotactic factors, and proteinases.^{(3),(8),(9)}
38 The bronchiolar epithelium forming the interface between the airway milieu and the internal setting
39 induces the expression of interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1 that
40 can be responsible for the neutrophil chemotactic activity of sputum.⁽⁹⁾ In addition, ROS promotes
41 the induction of IL-1 β and tumor necrosis factor (TNF)- α from macrophages, alveolar and bronchial
42 epithelial cells, which may excite macrophages to produce matrix metalloproteinase-9, and
43 bronchial epithelial cells to generate extracellular matrix (ECM) proteins.⁽⁹⁾ However, the pathology
44 and the underlying mechanisms in airway inflammation have been poorly investigated.

45 Squamous metaplasia and increased deposition of subepithelial ECM are associated with
46 fibrosis and thickening of the airway wall. Investigations on the mechanisms contributing to small
47 airway destruction and structural changes in ECM will reveal key airway remodeling processes of
48 diverse cells implicated in COPD.^{(10),(11)} In obstructive pulmonary diseases, neutrophils,
49 macrophages, and lymphocytes are salient.^{(8),(9)} In addition, bronchial epithelial cells can
50 coordinate immune and inflammatory responses leading to chronic pulmonary inflammation and
51 lung tissue damage.⁽¹²⁾ Moreover, pulmonary mesenchymal cells including airway smooth muscle
52 cells and lung fibroblasts react to inflammatory mediators and produce their own mediators.⁽¹³⁾
53 These airway processes by airway resident cells may represent major therapeutic targets in
54 asthma, IPF and COPD. Inhaled corticosteroids and β 2 agonists are the core therapeutic options

that effectively alleviate these pulmonary diseases.^{(14),(15)} Specifically antagonizing inflammation and targeting immunomodulatory function in airways may improve therapeutic control of chronic airway diseases. There are novel anti-inflammatory tactics to the controlling of obstructive diseases, including a use of phosphodiesterase inhibitors and statins in clinical trials.^{(16),(17)} These drugs can manipulate pulmonary obstructive disease-specific mechanistic actions of inflammation, hypersecretion and tissue destruction. Nevertheless, undesirable effects may be generated in patients with long-term treatments of inhaled corticosteroids and long-acting β 2-agonists for COPD.⁽¹⁸⁾ Therefore, new therapeutic strategies should be developed for pulmonary inflammation to alleviate worsening rates and all-cause mortality.^{(19),(20),(21)}

Astragalin (Figure 1A), kaempferol-3-O-glucoside, is naturally present in red wine, persimmon leaves and green tea seeds as food components, and exerts anti-inflammatory features in lipopolysaccharide (LPS)-mediated mastitis.⁽²²⁾ Astragalin can enhance survival from lethal endotoxemia and reduce acute lung injury in a murine asthma model.⁽²³⁾ In addition, this compound inhibits ovalbumin (OVA)-induced allergic inflammation and eosinophilia in lung tissues.⁽²⁴⁾ Our recent studies also showed that astragalin antagonized oxidative stress-induced eosinophilia and epithelial apoptosis and OVA-induced bronchial fibrosis.^{(25),(26)} However, the inhibition of pulmonary smooth muscle thickening and emphysema by astragalin is not yet elucidated. The current study elucidated that astragalin blocked oxidant- and OVA-induced recruitment of macrophages/neutrophils/mast cells, and that this compound blunted smooth muscle hypertrophy leading to emphysema in OVA-challenged mice.

82 MATERIALS and METHODS

83

84 Chemicals

85 M199, human epidermal growth factor (EGF), hydrocortisone, human insulin,
86 apotransferrin, H₂O₂ and toluidine blue O were obtained from the Sigma-Aldrich Chemical (St.
87 Louis, MO, USA), unless specifically mentioned elsewhere. Fetal bovine serum (FBS), penicillin-
88 streptomycin and trypsin-EDTA were purchased from the Lonza (Walkersville, MD, USA). OVA was
89 obtained from the Sigma-Aldrich Chemical and Imject Alum obtained from the Thermo Fisher
90 Scientific (Rockford, IL, USA). Antibodies of intracellular adhesion molecule (ICAM)-1, α v integrin
91 and CD68 were supplied by the Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies of MCP-1,
92 F4/80 and CD11b were provided by Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-
93 conjugated goat anti-rabbit IgG, donkey anti-goat IgG and goat anti-mouse IgG were provided by
94 the Jackson Immuno-Research Laboratories (West Grove, PA, USA). Essential fatty acid free
95 bovine serum albumin (BSA) and skim milk were supplied by Becton Dickinson Company (Sparks,
96 MD, USA). 4',6-Diamidino-2-phenylindole (DAPI) was obtained from Santa Cruz Biotechnology.
97 Astragalin was dissolved in dimethyl sulfoxide (DMSO) for live culture with cells; a final culture
98 concentration of DMSO was <0.5%.

99

100 BEAS-2B cell culture

101 Human bronchial airway epithelial cell line BEAS-2B cells (American Type Culture
102 Collection, Manassas, VA, USA) were incubated in 25 mM HEPES-buffered M199 containing 10%
103 FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin supplemented with 2.5 μ g/ml
104 insulin, 361 ng/ml hydrocortisone, 2.5 μ g/ml apotransferrin and 20 ng/ml EGF. BEAS-2B cells were
105 sustained in 90-95% confluence at 37°C in an atmosphere of 5% CO₂. BEAS-2B cells were
106 cultured with 1-20 μ M astragalin for 30 min and continuously stimulated with 20 μ M H₂O₂ for up to
107 72 h. H₂O₂-untreated cells were also cultured under same H₂O₂ protocols.

108

109 **Mouse asthma model**

110 Six week-old male BALB/c mice (Hallym University Breeding Center for Laboratory
111 Animals) were used in the current study. Mice were kept on a 12 h light/12 h dark cycle at $23 \pm 1^\circ\text{C}$
112 with $50 \pm 10\%$ relative humidity under specific pathogen-free circumstance, fed a non-purified diet
113 (Table 1, RodFeedTM, DBL, Umsung, Korea) and were supplied with water *ad libitum* at the animal
114 facility of Hallym University. Mice were acclimatized for 1 week before commencing the
115 experiments. Mice were divided into four subgroups ($n = 9$ for each subgroup). Chronic allergic
116 asthma was induced in murine models as described in previous studies.^{(25),(26)} Mice were sensitized
117 with 20 μg OVA dissolved in 30 μl phosphate buffered saline (PBS) with 50 μl Imject Alum through
118 subcutaneously injecting twice on day 0 and day 14. For the dietary interventions, 0.1 ml astragalin
119 solution (containing 10 or 20 mg/kg BW) was administrated via oral gavage to OVA-sensitized mice
120 1 h before challenge. On the day 28, 29 and 30, 5% OVA inhalation to mice was performed for 20
121 min in a plastic chamber linked to an ultrasonic nebulizer (Clenny² Aerosol, Medel, Italy). Control
122 mice were sensitized and challenged with PBS as the OVA vehicle. Twenty four hours after the
123 latest provocation (day 30), all mice were killed with an anesthetic (0.3 g/kg avertin and 8 $\mu\text{l/kg}$ tert-
124 amyl alcohol). The right lungs were kept at -80°C until used for Western blotting, and the left lungs
125 were preserved and fixed in 4% paraformaldehyde and then used for immunohistochemical
126 analyses.

127 The current study was approved by the Hallym University Institutional Review Board and
128 Committee on Animal Experimentation (hallym R2014-6). This study was performed in compliance
129 with the University's Guidelines for the Care and Use of Laboratory Animals. There were no dead
130 mice observed and no evident signs of lethargy were detected during the experimental period.

131

132 **Western blot analysis**

133 BALB/c lung tissue extracts and whole BEAS-2B cell lysates were prepared in 1 mM Tris-
134 HCl (pH 6.8) lysis buffer containing 10% sodium dodecyl sulfate (SDS), 1% glycerophosphate, 0.1
135 mM Na_3VO_4 , 0.5 mM NaF and protease inhibitor cocktail. Tissue extracts or cell lysates containing

equal amounts of proteins for the protein expression, and equal volume of culture media for the protein secretion were electrophoresed on 6-15% SDS-PAGE and transferred onto a nitrocellulose membrane. Blocking a nonspecific binding was performed using either 3% fatty acid-free BSA or 5% non-fat dry skim milk for 3 h. The membrane was incubated with a specific primary antibody of MCP-1, ICAM-1, α v integrin or α -smooth muscle actin (α -SMA) overnight at 4°C. The membrane was then applied to a secondary antibody conjugated to HRP for 1 h. Following triple washing, the target protein was determined using the Immobilon Western Chemiluminescent HRP substrate (Millipore Corp., Billerica, MA, USA) and the Agfa medical X-ray film blue (Agfa HealthCare NV, Mortsel, Belgium). Incubation with β -actin antibody was conducted for the comparative control.

Immunohistochemical staining

Paraffin-embedded tissue sections (5 μ m thick) of airways and lungs were deparaffinized and hydrated to conduct immunohistochemical analyses. The sections were pre-incubated in a boiling sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for antigen retrieval. The tissues were blocked with 5% BSA in PBS for 1 h. A specific primary antibody against F4/80, CD68 CD11b or α -SMA was incubated overnight with the sectioned tissues. Subsequently, the tissue sections were incubated for 1 h with FITC-conjugated IgG or Cy3-conjugated IgG (Rockland, Pottstown, PA, USA). For the nuclear counter-staining, the fluorescent DAPI was applied for 10 min. For the detection of α -SMA in bronchial tissues, chromogenic substrate detection kits (Dako, Carpinteria, CA, USA) of 3,3'-diaminobenzidine (DAB) and 3-amino-9-ethylcarbazol were employed. Counter-staining was carried out with hematoxylin. Stained tissues were mounted on slides using mounting medium (Vector Laboratories, Burlingame, CA, USA). Images of each slide were obtained with an optical microscope Axioimager system (Zeiss, Gottingen, Germany).

Staining of toluidine blue O and Hematoxylin and eosin stain (H&E)

For the histological analyses, small airway specimens collected at the end of the experiments were fixed in 10% paraformaldehyde. The paraffin-embedded lung specimens were

sectioned at 5 μm thickness, de-paraffinized and stained with toluidine blue O or H&E for 2 min, and quickly dehydrated through 95% absolute alcohol. The stained tissue sections were observed using an optical microscope Axioimager system. Five images were obtained for each section.

Statistical analysis

The results were presented as mean $\pm$ SEM for each treatment group in each experiment. Statistical analyses were conducted using Statistical Analysis Systems statistical software package (SAS Institute, Cary, NC, USA). Significance was determined by one-way analysis of variance, followed by Duncan range test for multiple comparisons. Differences were significant at $P < 0.05$.

189 **RESULTS**

190

191 ***Inhibitory effects of astragalin on induction of MCP-1 and adhesion molecules in OVA-***
 192 ***challenged mouse lungs and H₂O₂-exposed airway epithelial cells***

193 Asthmatic stimuli evoke oxidative stress and inflammatory responses in airways, which are
 194 major features observed in COPD.^{(27),(28)} This study investigated whether OVA induced the
 195 chemotactic factor MCP-1 in whole lung tissues, which was diminished by supplying astragalin.
 196 The MCP-1 production was minimal in the lung tissues of control mice, whereas in OVA-sensitized
 197 the tissue level was markedly enhanced (Figure 1B). However, oral administration of 10 or 20
 198 mg/kg astragalin to OVA-challenged mice attenuated pulmonary MCP-1 induction. When H₂O₂
 199 were solely applied to bronchial epithelial cells, cellular expression and secretion of MCP-1 were
 200 up-regulated (Figure 1C). In contrast, 1-20 μM astragalin diminished oxidant-induced production of
 201 MCP-1 in a dose-dependent fashion. Our previous study found that this OVA challenge increased
 202 ROS production and collagen fiber deposition in mouse airways.⁽²⁵⁾ Accordingly, oxidative stress
 203 can cause the migration and infiltration of monocytes/macrophages by inducing the potent
 204 chemotactic factor of MCP-1 in allergen-exposed airway epithelium.

205 It has been shown that adhesion molecules known as inflammatory target proteins are
 206 related to airway and lung inflammation due to various stimuli.⁽²⁹⁾ The adhesion molecules of ICAM-
 207 1 and αv integrin were highly induced in BEAS-2B cells experienced with 20 μM H₂O₂ for 24 h
 208 (Figure 1D). ICAM-1 and integrins are important players of lung inflammation and remodeling in
 209 airway obstructive diseases,^{(30),(31)} which indicates their potential as pharmacological targets for
 210 preventive and curative purposes. When 1-20 μM astragalin was supplemented to epithelial cells in
 211 the presence of H₂O₂, the induction of these adhesion molecules was dose-dependently inhibited
 212 (Figure 1D).

213

214 ***Inhibition of infiltration of macrophages and neutrophils by astragalin in OVA-stimulated***
 215 ***airways and alveoli***

Macrophages and neutrophils are important in airways and lungs of obstructive pulmonary diseases, while eosinophils and mast cells are activated and infiltrated in asthmatic airways.⁽³²⁾ In severe cases of asthma and COPD, the neutrophil infiltration becomes evident. The airway tissue level of F4/80 (green staining for macrophages) increased in airway subepithelium and wall of animals stimulated with 5% OVA (Figure 2). Similar induction was observed in another macrophage marker CD68 (pink staining) with OVA stimulation. When 10 or 20 mg/kg astragalín was administrated to OVA-challenged mice, the enhanced induction of F4/80 and CD68 was significantly reduced, as confirmed by FITC- or Cy3-conjugated immunohistochemistry staining (Figure 2). It should be noted that in OVA-challenged mice the tissue level of MCP-1 was markedly enhanced (Figure 1B). Therefore, OVA induced the macrophage infiltration in mouse small airways, which was blocked by oral treatment of astragalín.

One investigation shows that CD11b and ICAM-1 are required for pulmonary neutrophil gathering in LPS-induced airway diseases.⁽³⁰⁾ F4/80 and CD11b were highly expressed in alveoli of mice exposed to OVA (Figure 3). Tissue damage seen in emphysema was also observed in OVA-exposed alveoli. However, the immunohistochemical staining showed that the oral treatment with 10 or 20 mg/kg astragalín diminished the elevated induction of F4/80 and CD11b in alveoli of OVA-challenged mice (Figure 3). Accordingly, astragalín may inhibit the infiltration of macrophages and neutrophils in OVA-exposed mouse small airways and alveoli.

Blockade of mast cell recruitment by astragalín in OVA-challenged mouse airways

Mast cell infiltration and mast cell-derived mediator production are associated with airway dysfunction, late phase inflammation and remodeling in asthmatic airways.^{(32),(33)} This study examined mast cell exudates in airway tissues of OVA-challenged mice, confirmed by using toluidine blue staining. There was a marked elevation in the number of mast cells (arrowheads) observed in bronchial airways (Figure 4). Mast cells appeared to be recruited to airway subepithelium, mucous glands and to airway smooth muscle (Figure 4). When 20 mg/kg astragalín was treated to OVA-challenged mice, the mast cell infiltration was abolished (Figure 4).

243

244 ***Suppressive effects of astragalin on airway thickening***

245 Hypertrophy and hyperplasia of airway smooth muscle result in airway wall
246 thickening.^{(34),(35)} In asthmatic airways, mast cells are confined to the airway smooth muscle,
247 indicating direct interaction between mast cells and airway smooth muscle cells.⁽³⁶⁾ This study
248 revealed that the lung tissue level of α -SMA increased in mice sensitized and stimulated with 5%
249 OVA (Figure 5A). When 10 or 20 mg/kg astragalin was administrated to OVA-challenged mice, the
250 enhanced α -SMA induction was significantly reduced. Congruently, there was a heavy brownish
251 staining of α -SMA in the bronchi of OVA-challenged mice (Figure 5B). On the contrary, the
252 treatment of astragalin diminished the subepithelial α -SMA induction by OVA, evidenced by DAB
253 staining (Figure 5B). Astragalin may encumber the increase in airway smooth muscle mass and
254 alleviate the structural changes of bronchial airway wall. It is deemed that mast cells infiltrated in
255 subepithelial airways contribute to the structural changes through direct communication with
256 smooth muscle cells and through their mediator release.

257 Whether the increase of airway smooth muscle mass is attributed to the interactions of
258 smooth muscle-mast cells is still not defined.⁽³⁷⁾ This study found that there was a dense reddish α -
259 SMA staining around OVA-challenged small airways with infiltrated macrophages in green color
260 (F4/48 staining), indicating that OVA-induced smooth muscle hypertrophy may be due to airway
261 inflammation by infiltrated macrophages (Figure 6A). It should be noted that α -SMA was highly
262 induced in the pulmonary vessels located near small airways. On the other hand, astragalin
263 reduced the smooth muscle α -SMA and inhibited pulmonary inflammation in small respiratory tracts
264 (Figure 6A). The H&E staining showed that the OVA episode resulted in the thickening and
265 constriction of small airways, which was reversed by treating 10 or 20 mg/kg astragalin to mice
266 (Figure 6B). Additionally, the OVA challenge increased lung destruction ultimately causing
267 emphysema damage, which was blunted by supplying astragalin to mice (Figure 6B).

268

269

DISCUSSION

Nine novel findings were obtained in this study. 1) The oral administration of 10 or 20 mg/kg astragalín to OVA-exposed mice inhibited MCP-1 induction in lung tissues. 2) Non-toxic astragalín at ≤ 20 μ M diminished oxidant-induced cellular production of MCP-1 and cell expression of ICAM-1 and α v integrin. 3) Astragalín decreased the lung tissue levels of F4/80 and CD68 up-regulated in airway epithelium and wall of mice prompted with OVA. 4) Treatment with 10 or 20 mg/kg astragalín diminished the elevated induction of F4/80 and CD11b in alveoli of OVA-exposed mice. 5) Emphysematous tissue damage was also observed in OVA-exposed alveolar wall. 6) When 20 mg/kg astragalín was added to OVA-challenged mice, the mast cell accumulation in small airways was blocked. 7) The mouse lung tissue level of α -SMA enhanced by OVA was reduced by supplying astragalín. 8) Astragalín reduced the smooth muscle mass in small respiratory tracts experiencing inflammation. 9) Oral supplementation of astragalín reversed the OVA episode-induced thickening of small airways. Accordingly, oxidant injury- and asthmatic injury-induced lung inflammation may contribute to elevated airway smooth muscle and airway tissue destruction, ultimately causing small airway thickening and emphysema (Figure 7). However, pre-clinical pharmacokinetic and bioavailability studies showed that the absolute bioavailability of astragalín was very poor, and that astragalín orally administered was rapidly absorbed and eliminated in plasma.⁽³⁸⁾ Further prospective investigation can be needed to confirm and determine consumption levels required to achieve health benefits.

Numerous studies have proposed novel therapeutic strategies for lung diseases related to airway inflammation, fibrosis and emphysema.^{(15),(16),(17),(39)} Inhaled corticosteroids, long-acting β 2 agonists and long-acting muscarinic antagonists have been effective for patients with asthma and/or COPD to effectively alleviate symptoms and to lessen the frequency of exacerbations.^{(15),(40)} However, detrimental effects frequently occur in patients with long-term therapy of inhaled corticosteroids and mistreated bronchodilator therapy.⁽¹⁸⁾ In addition, to better understand specific mechanisms linked to airway inflammation will help to optimize utilization of the currently available

297 interventions for pulmonary disorders. Accordingly, alternative supplements or therapies are
298 currently available as clinical potentials in the setting of lung diseases.⁽³⁹⁾ Amongst potentials that
299 have been known to prevent and treat pulmonary diseases, natural therapeutic compounds have
300 been suggested as agents targeting against airway inflammation.^{(19),(20),(21)} Recently, it was shown
301 that astragalin antagonized allergic inflammation and inhibited oxidative stress-induced airway
302 eosinophilia and epithelial fibrosis^{(24),(25),(26)}

303 Airway inflammation is a key feature in the pathophysiology of inflammatory lung diseases
304 including increased bronchial wall thickness and smooth muscle tone, and damage of parenchymal
305 elastic structures.^{(10),(11),(12),(13)} Inflammatory cell infiltration, cytokine release and airway/lung
306 remodeling play central roles in asthma and obstructive diseases.^{(4),(7)} Proximal airway
307 inflammation is involved in chronic bronchitis, whereas distal and parenchymal inflammation is
308 associated with airflow obstruction and emphysema.^{(6),(11),(17)} The lasting presence of pathological
309 insults results in airway fibrosis, the final generic pathway that causes an irreversible lung
310 dysfunction.⁽⁴¹⁾ Cigarette smoking results in the recruitment of inflammatory cells in the airways and
311 promotes mechanistic actions of innate and adaptive immune systems.^{(42),(43)} This insult exercises
312 direct oxidative modifications on muscle proteins, preceding the characteristic lung changes
313 leading to muscle loss and dysfunction.⁽⁴⁴⁾ Accordingly, pulmonary inflammation and oxidative
314 stress might be the major pathogenic mechanisms known to cause emphysema in obstructive
315 pulmonary diseases. Consistently, our previous study showed that the endotoxin LPS induced
316 epithelial eosinophilia in bronchial epithelial cells via oxidative stress, and that the OVA challenge
317 enhanced ROS-promoted bronchial fibrosis.^{(25),(26)} It is assumed that effective therapeutic
318 antioxidants may mitigate oxygen bursts in respiratory diseases. The current study revealed that
319 astragalin dampened the production of inflammatory mediators of MCP-1, ICAM-1 and α v integrin
320 in bronchial epithelial cells exposed to the oxidant H_2O_2 and inhibited the migration and infiltration
321 of monocytes/macrophages in OVA-exposed mice. One can speculate that the OVA-induced
322 pulmonary inflammation could be attributed to oxidative stress increased by its ROS production,
323 which was suppressed by the antioxidant astragalin.

There is increasing evidence that cytokines, chemokines and adhesion molecules may be the novel promising therapeutic targets in the development of airway inflammation through blocking the recruitment of inflammatory cells.^{(9),(29),(45)} In lung diseases, there is an increased induction of these inflammatory proteins in the respiratory tracts.⁽²⁹⁾ Small molecule inhibitors of chemokine receptors including chemokine CC3 receptor antagonists are in clinical development for treating asthma and COPD.⁽⁴⁵⁾ The current study showed that the infiltration and recruitment of macrophages were observed in OVA-challenged mouse small airways, which was suppressed by treating astragalin to mice. Macrophages are responsible for airway obstruction and alveolar wall damage in pulmonary emphysema.⁽⁴⁶⁾ Also, the oral administration of astragalin attenuated the neutrophilic inflammation and emphysema in alveoli, the major features of COPD. One investigation shows that pulmonary neutrophil recruitment entails CD11b and ICAM-1 proteins in LPS-induced airway diseases.⁽³⁰⁾ Infiltrated neutrophils can induce the pulmonary damage of COPD.⁽³²⁾ In the present study stragalin reduced smooth muscle thickening and alveolar destruction. Collectively, one can speculate that oxidative stress-mediated pulmonary inflammation may be responsible for the OVA-induced smooth muscle thickening and emphysema, which was suppressed by the antioxidant and anti-inflammatory astragalin.

There is growing evidence that mast cells and their mediators can induce hyperplasia and thickening of airway smooth muscle, contributing to the occurrence of multiple pathological properties of chronic asthma and COPD.^{(33),(33)} This study showed that astragalin suppressed the allergic inflammation by OVA through blocking mast cell trafficking in airway smooth muscle and wall. Therefore, astragalin may reduce the smooth muscle mass accumulation and constriction through blocking mast cell recruitment in small respiratory tracts. Kaempferol abrogates airway thickening evoked by allergic reactions in bovine serum albumin-exposed mice.⁽³⁵⁾ In addition, the stilbene resveratrol and the isoflavone genistein may be potent agents treating obstructive pulmonary diseases.⁽²⁰⁾ Despite allergic inflammation, genetic susceptibility, epithelial-mesenchymal transition and mechanical stress can contribute to airway remodeling and fibrosis.^{(39),(47),(48)} Kaempferol suppresses airway epithelial-to-mesenchymal transition-mediated

351 fibrosis in lipopolysaccharide-induced bronchial epithelial cells and OVA-challenged mice.⁽⁴⁷⁾ The
352 flavanone sakuranetin blocks elastase-induced emphysema in mice by influencing
353 metalloproteinases and nuclear factor-κB as well as oxidative stress.⁽⁴⁹⁾

354 In summary, this study investigated that astragalin antagonized oxidative stress-mediated
355 macrophage accumulation and neutrophilic inflammation with concomitant induction of
356 inflammatory F4/80, CD68 and CD11b in OVA-challenged mouse small airways and alveoli. Oral
357 administration of astragalin diminished bronchial thickening and alveolar emphysema in OVA-
358 challenged mice through blocking mast cell infiltration to airway smooth muscle. Although
359 astragalin may serve as a modulator against oxidative stress-mediated inflammatory cell
360 recruitment in airway wall *in vitro*, its dietary *in vivo* role against major chronic diseases of the lung
361 remains unclear. Knowledge of the mechanisms responsible for the regulation of airway
362 inflammation directs to the pharmacological strategy of anti-inflammatory agents such as astragalin
363 in the respiratory diseases. However, pharmacokinetic parameters such as plasma concentration
364 and time profiles and bioavailability after oral administration need to be further investigated.

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539 **FIGURE LEGENDS**

540

541 **Figure 1:** Chemical structure of astragalin (A) and Western blot analysis showing inhibition of
542 airway inflammation by astragalin in OVA-challenged mouse lung tissues (B) and H₂O₂-exposed
543 epithelial cells (C and D). OVA-sensitized mice were orally administrated with 10 or 20 mg/kg
544 astragalin. Tissue extracts were subjected to Western blot analysis with MCP-1 antibody (B).
545 Human BEAS-2B cells were treated with 1-20 μ M astragalin and stimulated with 20 μ M H₂O₂ for 24
546 h. Cell lysates and condition media were prepared for Western blot analysis with a primary
547 antibody against MCP-1, ICAM-1 and α v integrin (C and D). β -Actin protein was used as an
548 internal control. The bar graphs (mean $\pm$ SEM, n = 3) represent quantitative results of the upper
549 bands obtained from a densitometer. Values in bar graphs not sharing a letter indicate significant
550 different at P<0.05.

551

552 **Figure 2:** Suppressive effects of astragalin on macrophage infiltration in OVA-challenged mouse
553 small airways. OVA-sensitized mice were orally administrated with 10 or 20 mg/kg astragalin. The
554 F4/80 localization was identified as FITC-green staining and the CD68 localization confirmed by
555 Cy3-red staining in mouse small airways exposed to OVA. Nuclear staining was done with 4',6-
556 diamidino-2-phenylindole (blue). Each photograph is representative of four mice. Magnification:
557 200-fold. The bar graphs (mean $\pm$ SEM) represent quantitative results of fluorescent staining of
558 FITC or Cy3. Values in bar graphs not sharing a letter indicate significant different at P<0.05.

559

560 **Figure 3:** Immunohistofluorescence analysis showing blockade of cellular infiltration of
561 macrophages and neutrophils by oral administration of astragalin in alveolar tissues of OVA-
562 challenged mice. OVA-sensitized mice were orally administrated with 10 or 20 mg/kg astragalin.
563 The F4/80 localization was identified as FITC-green staining, and CD11b was confirmed by Cy3-
564 red staining in mouse alveoli exposed to OVA. Nuclear staining was done with 4',6-diamidino-2-
565 phenylindole (blue). Each photograph is representative of four mice. Magnification: 400-fold. The

566 bar graphs (mean $\pm$ SEM) represent quantitative results of fluorescent staining of FITC or Cy3.

567 Values in bar graphs not sharing a letter indicate significant different at $P < 0.05$.

568

569 **Figure 4:** Inhibition of allergic inflammation by astragalín in small airways of OVA-challenged mice.

570 OVA-sensitized mice were orally administrated with 10 or 20 mg/kg astragalín. Mast cell deposition

571 in mouse airways was observed by conducting toluidine blue O staining (arrowheads). Each

572 photograph is representative of four mice. Magnification: 200~400-fold. The bar graphs (mean $\pm$

573 SEM) represent infiltrated mast cells counted on randomly selected five fields per mouse. Values in

574 bar graphs not sharing a letter indicate significant different at $P < 0.05$.

575

576 **Figure 5:** Inhibitory effects of astragalín on α -SMA induction in lung tissues of OVA-exposed mice

577 (A). OVA-sensitized mice were orally administrated with 10 or 20 mg/kg astragalín. Tissue extracts

578 were subjected to Western blot analysis with α -SMA antibody (A). β -Actin protein was used as an

579 internal control. The bar graphs (mean $\pm$ SEM, $n = 3$) represent quantitative results of the left

580 bands obtained from a densitometer. Immunohistofluorescence analysis showing inhibition of

581 tracheal smooth muscle accumulation by astragalín in OVA-challenged mouse trachea (B). The α -

582 SMA induction was visualized with 3,3'-diaminobenzidine (DAB)-conjugated secondary antibody

583 identified as brownish staining, and nuclear counter-staining was done with hematoxylin. Each

584 photograph is representative of four mice. Magnification: 400-fold. The bar graphs (mean $\pm$ SEM)

585 represent quantitative results of DAB staining. Values in bar graphs not sharing a letter indicate

586 significant different at $P < 0.05$.

587

588 **Figure 6:** Inhibitory effects of astragalín on thickening of small airways in OVA-challenged mice.

589 OVA-sensitized mice were orally administrated with 10 or 20 mg/kg astragalín. The F4/80

590 localization was identified as FITC-green staining and α -SMA was visualized with Cy3-red staining

591 (A). Nuclear counter-staining was done with 4',6-diamidino-2-phenylindole (blue). Tissue sections

592 of small airways were stained by using H&E stain (B). Each photograph is representative of four

593 mice. Magnification: 200-fold. A strong thickening in many areas of airway wall was observed.

594

595 **Figure 7:** Schematic diagram showing the inhibitory effects of astragalin on smooth muscle
596 thickening and airway emphysema. As depicted, astragalin blocked airway infiltrations of
597 macrophages, neutrophils and mast cells responsible for pulmonary inflammation. The symbol $\top$
598 indicates sites of inhibition manifested by astragalin.

599

Table 1: Proximate composition (%) of non-purified diets

components	crude protein	crude fat	crude fiber	crude ash	calcium	phosphorus
content (%)	≥20.5%	≥3.5%	≤8.0%	≤8.0%	≥0.5%	≥0.5%

Figure 1

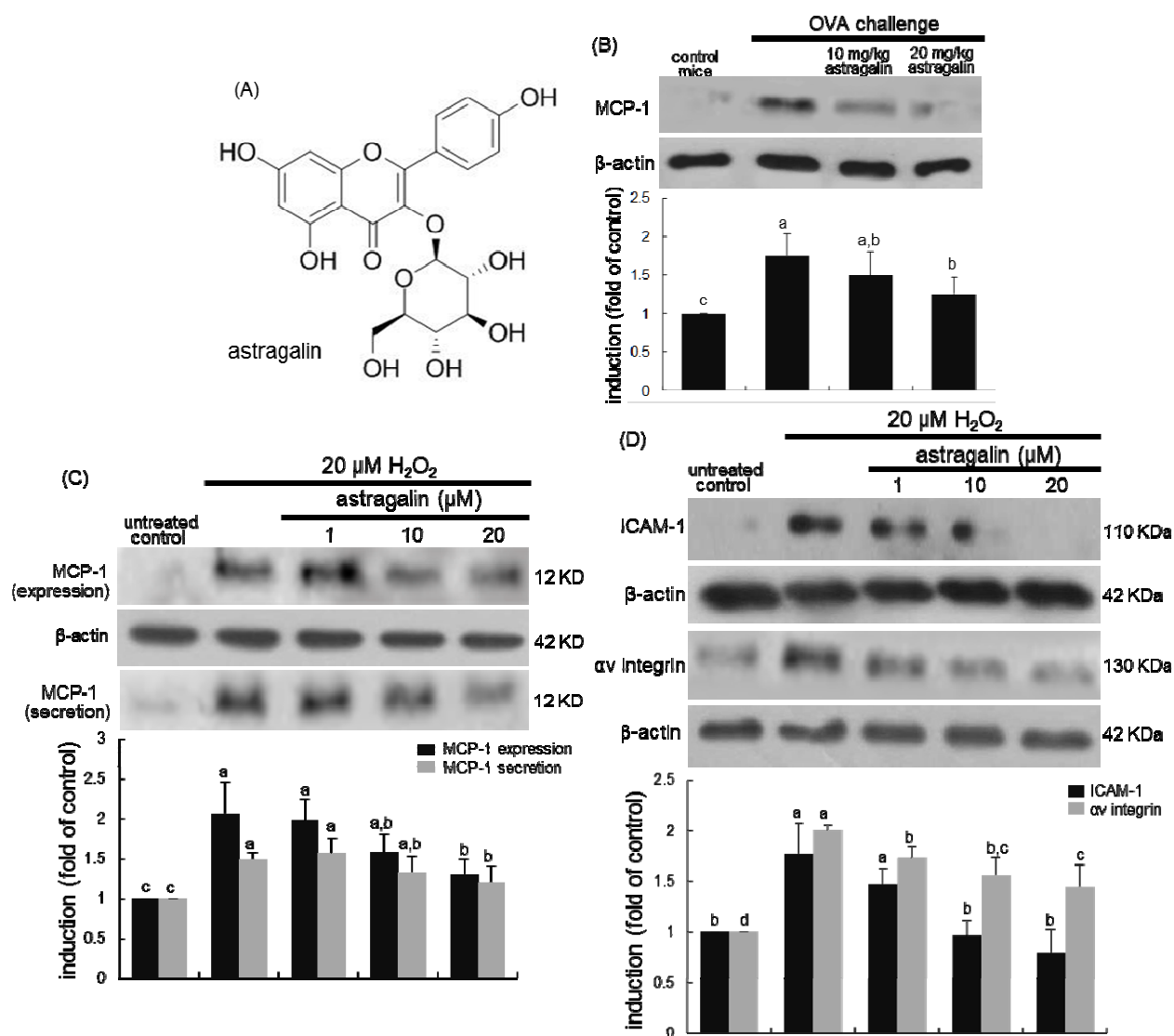


Figure 2

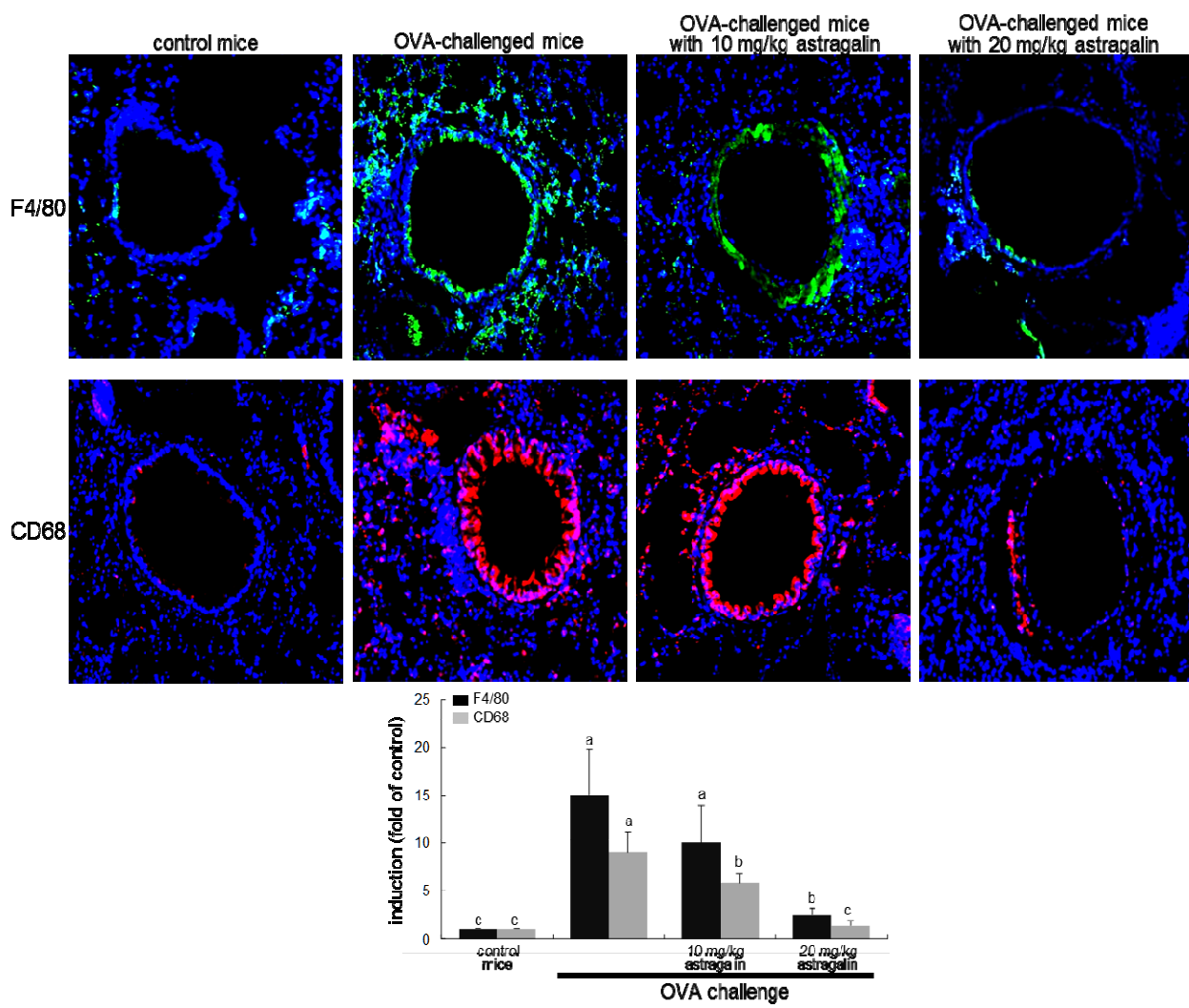


Figure 3

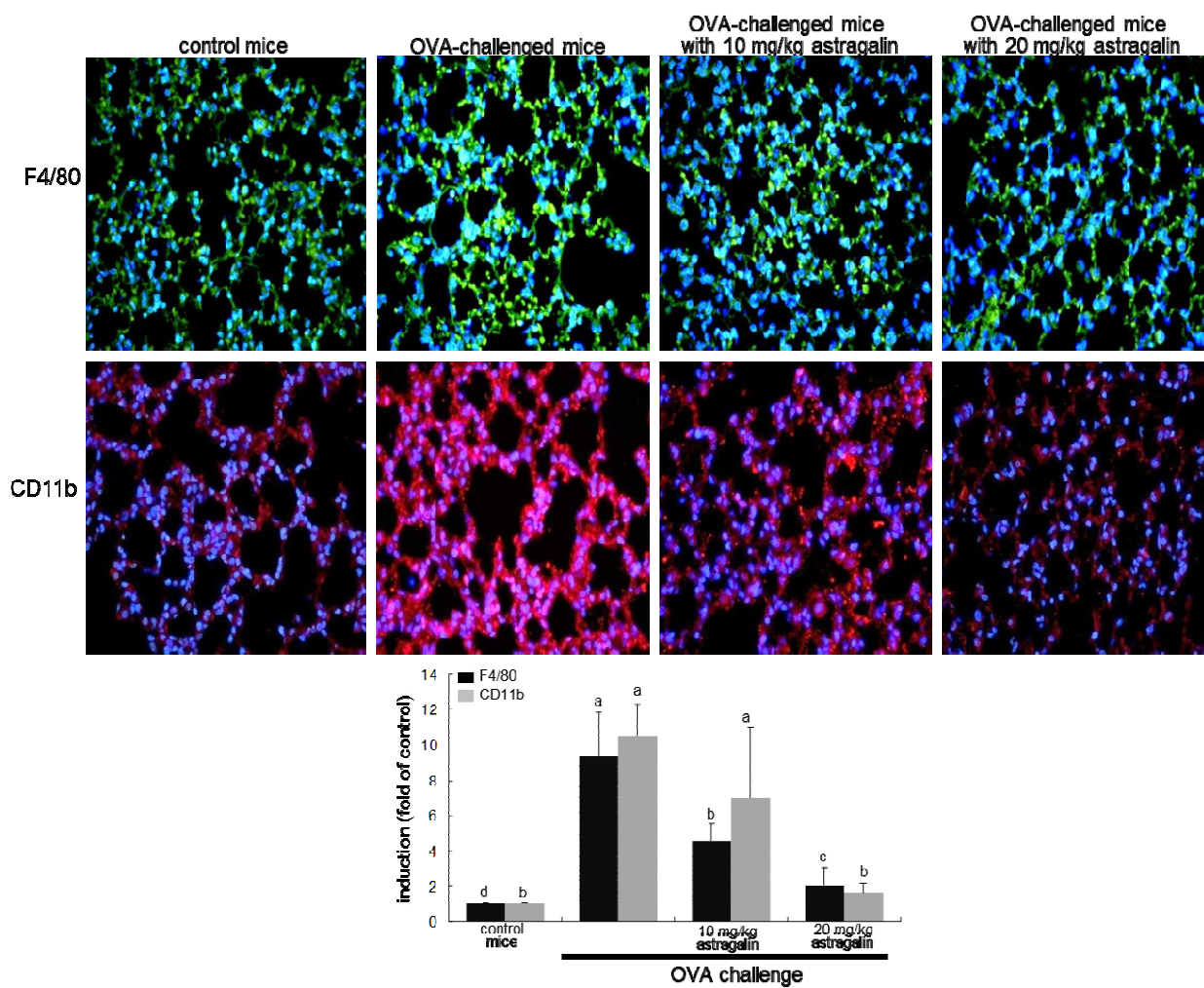


Figure 4

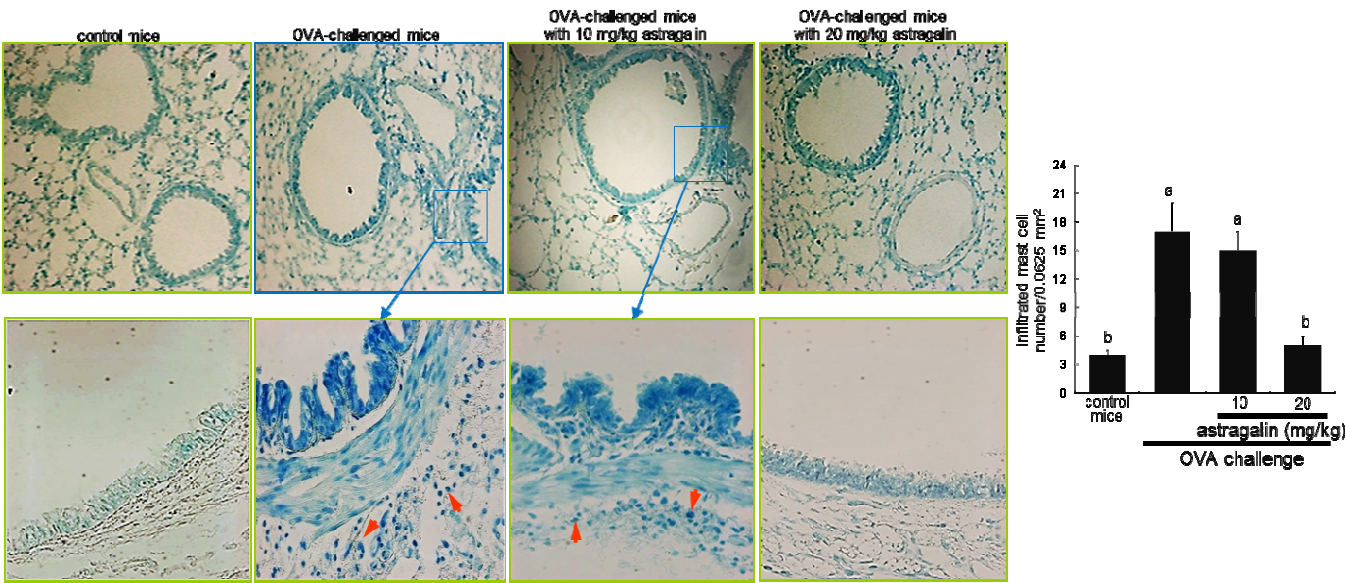


Figure 5

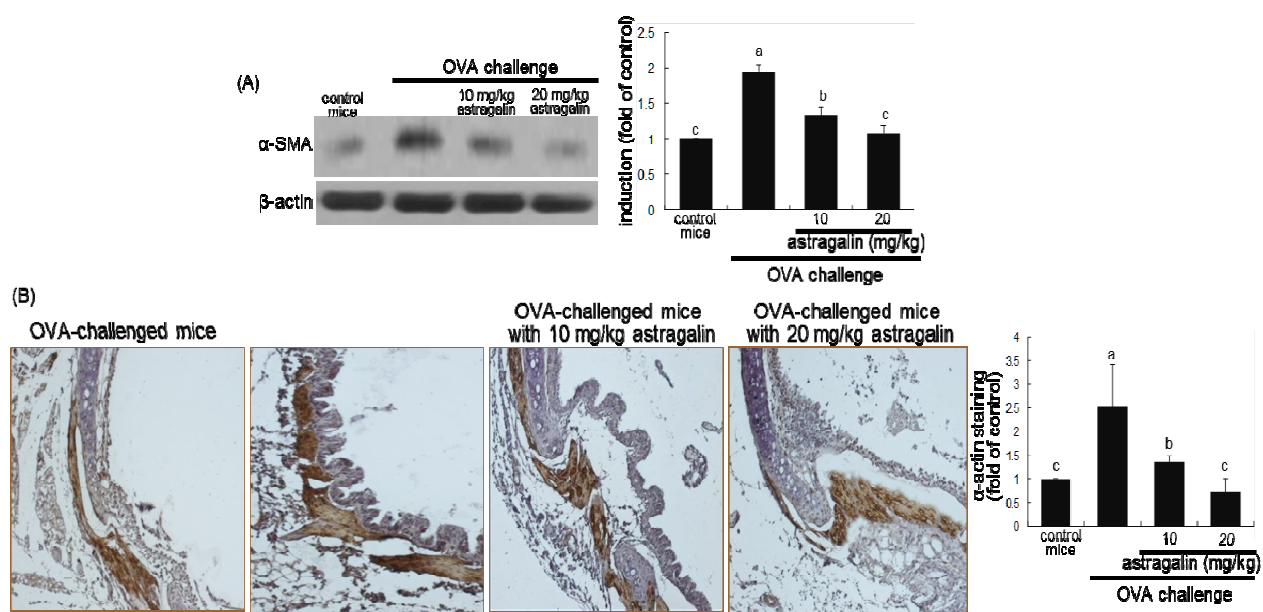


Figure 6

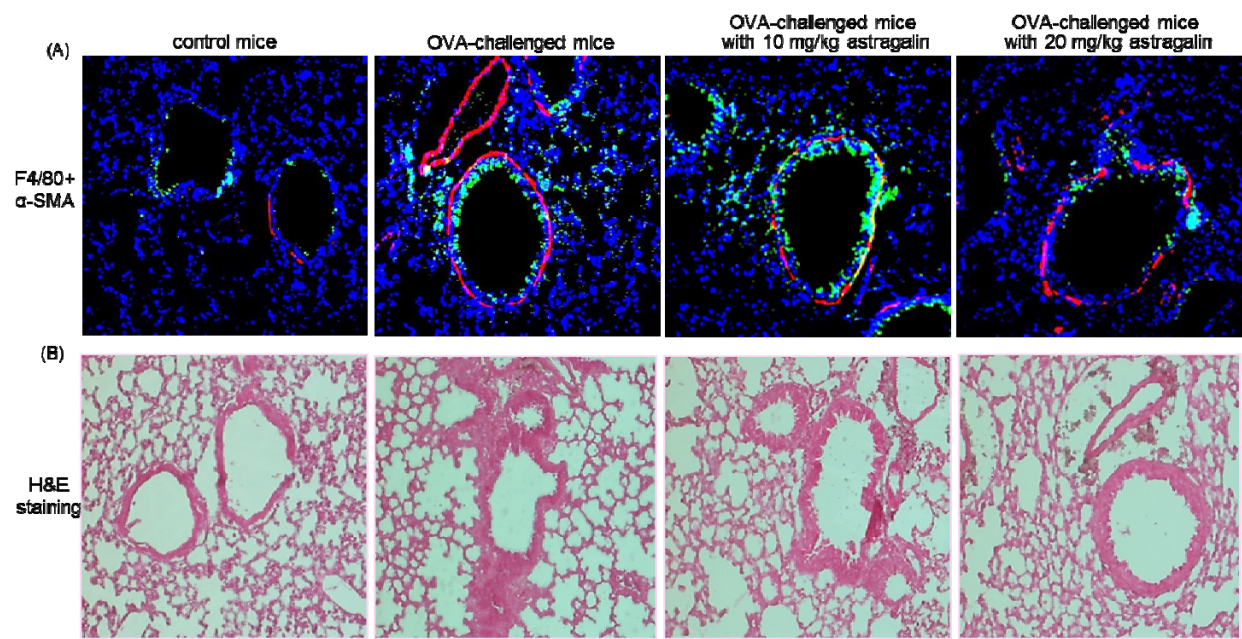
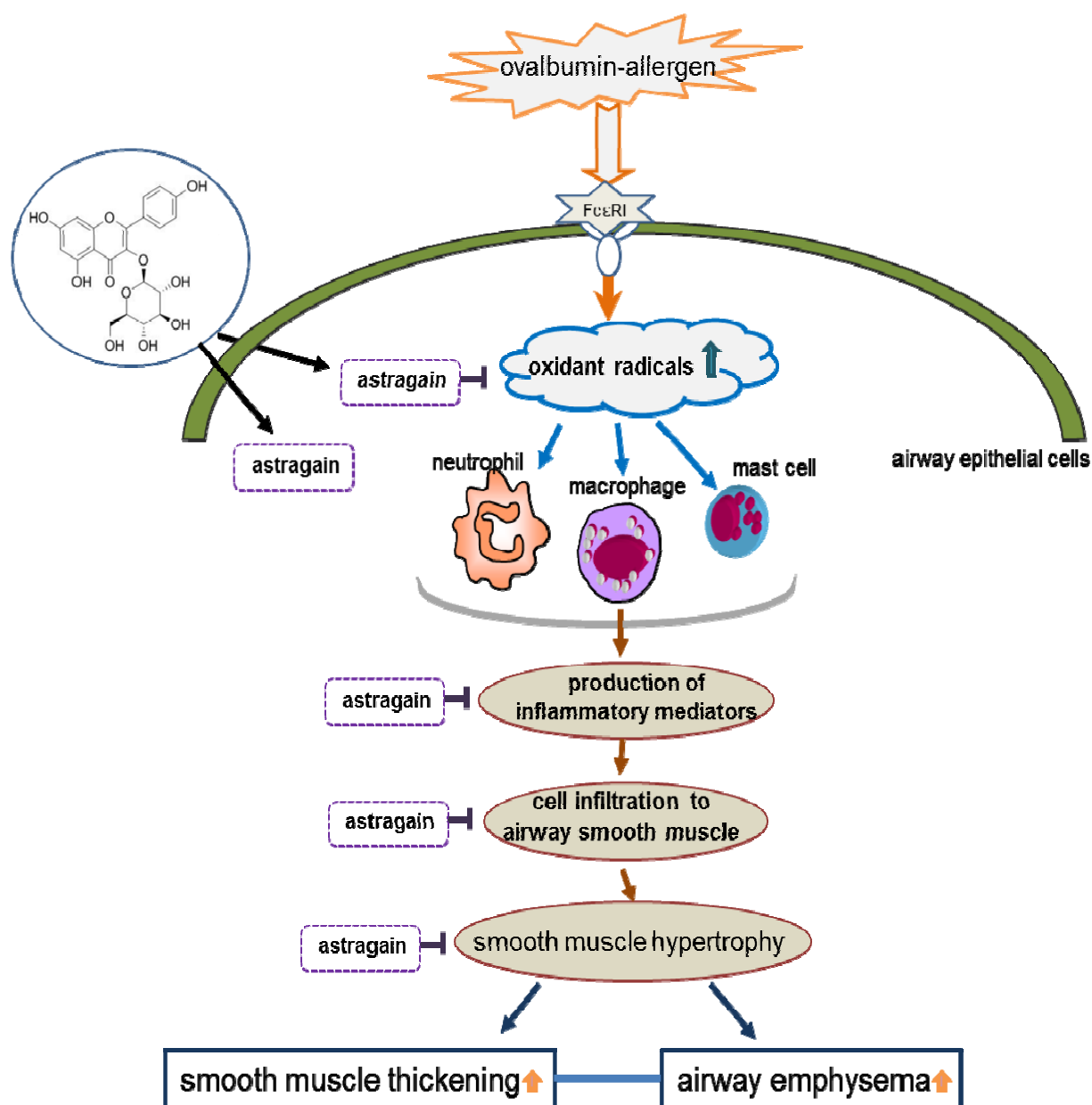


Figure 7



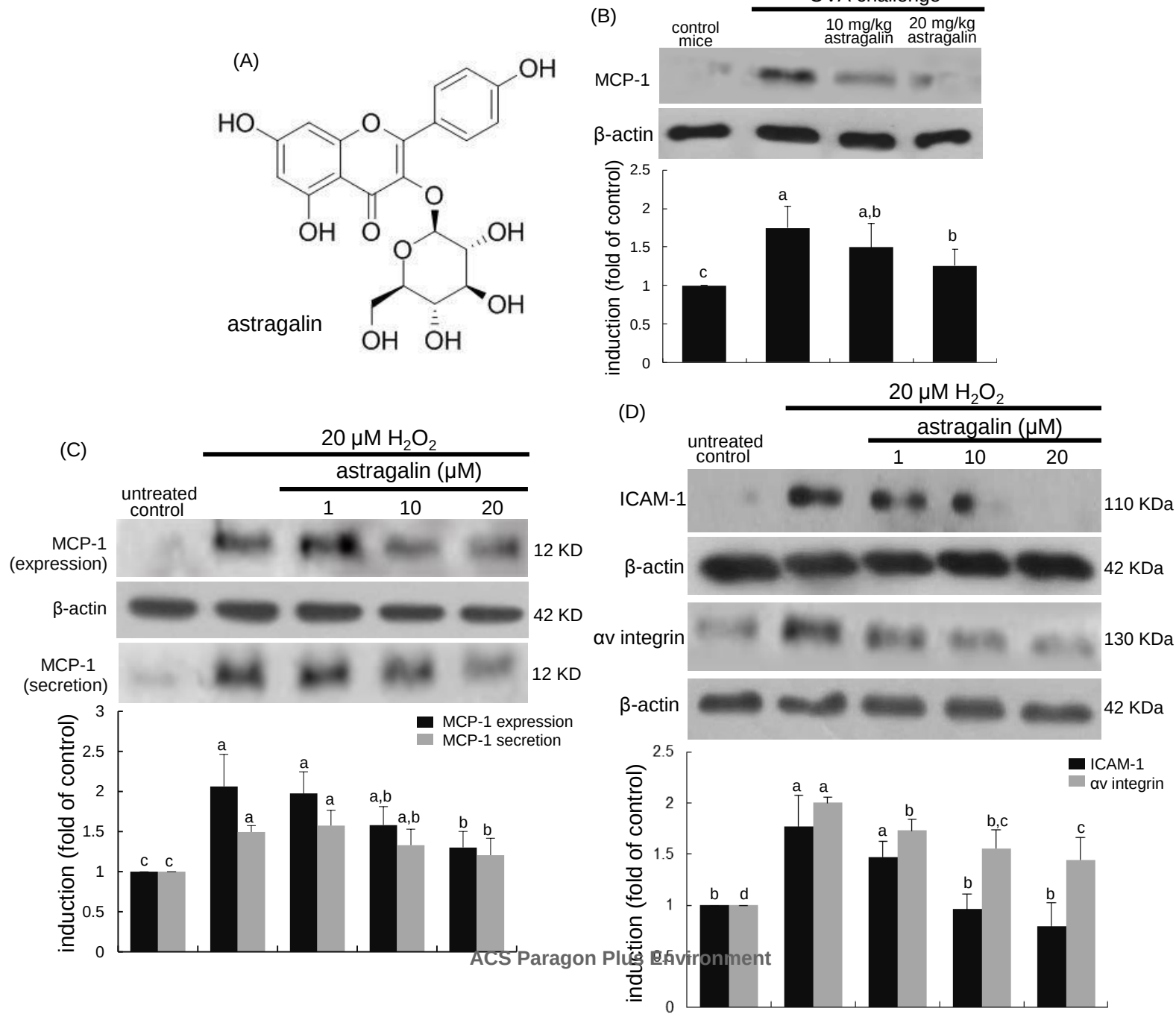
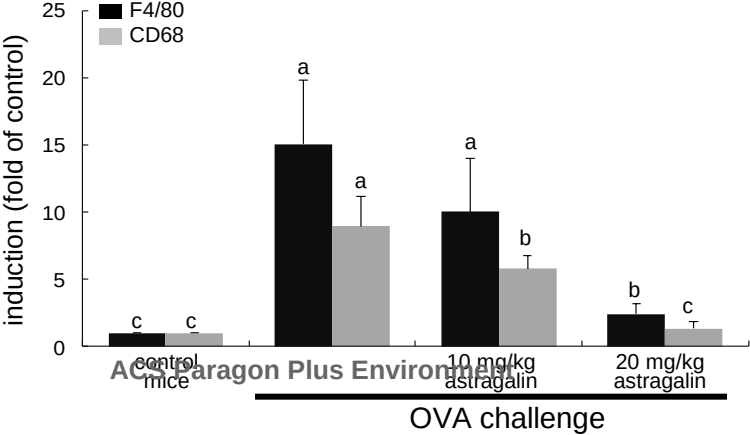
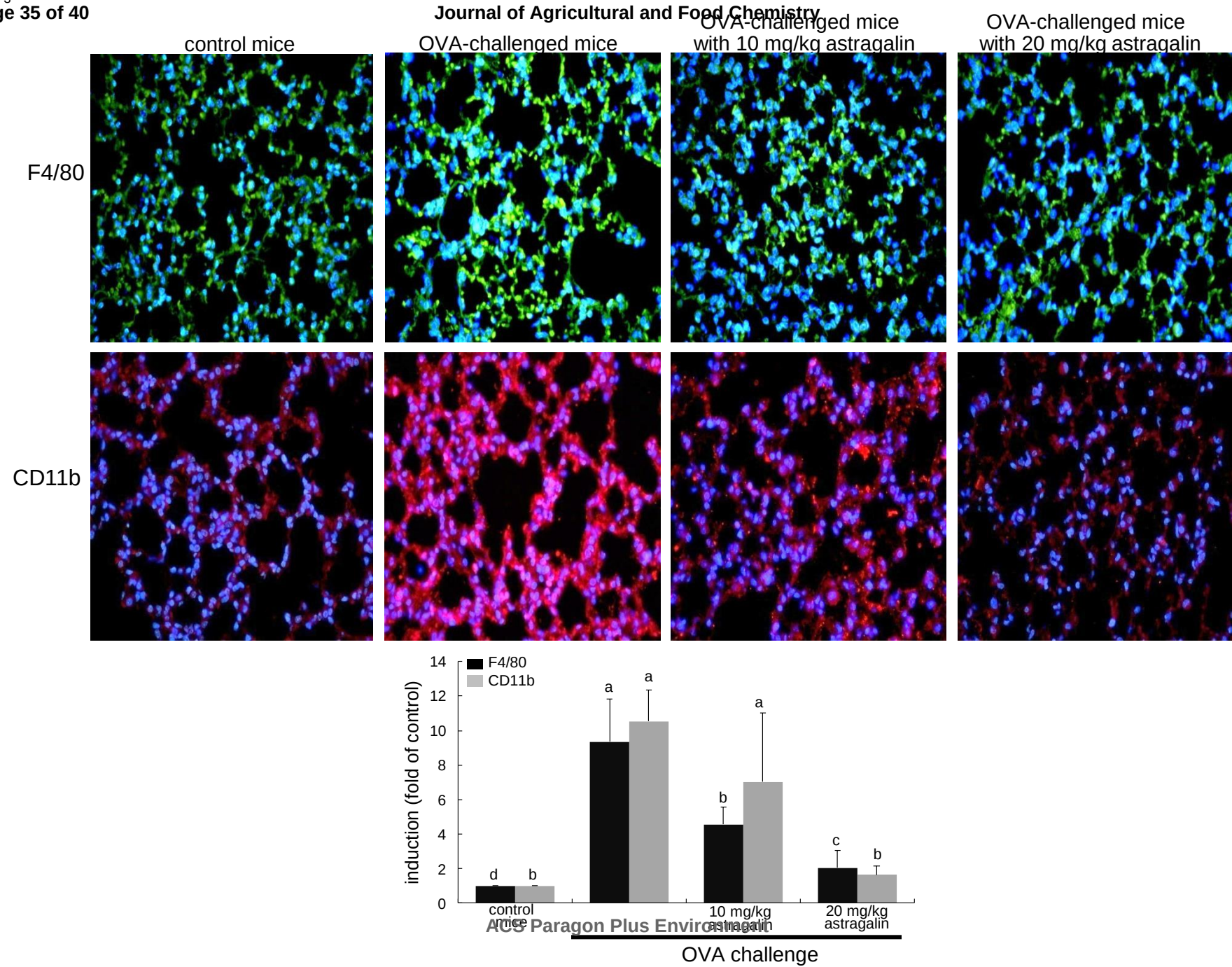


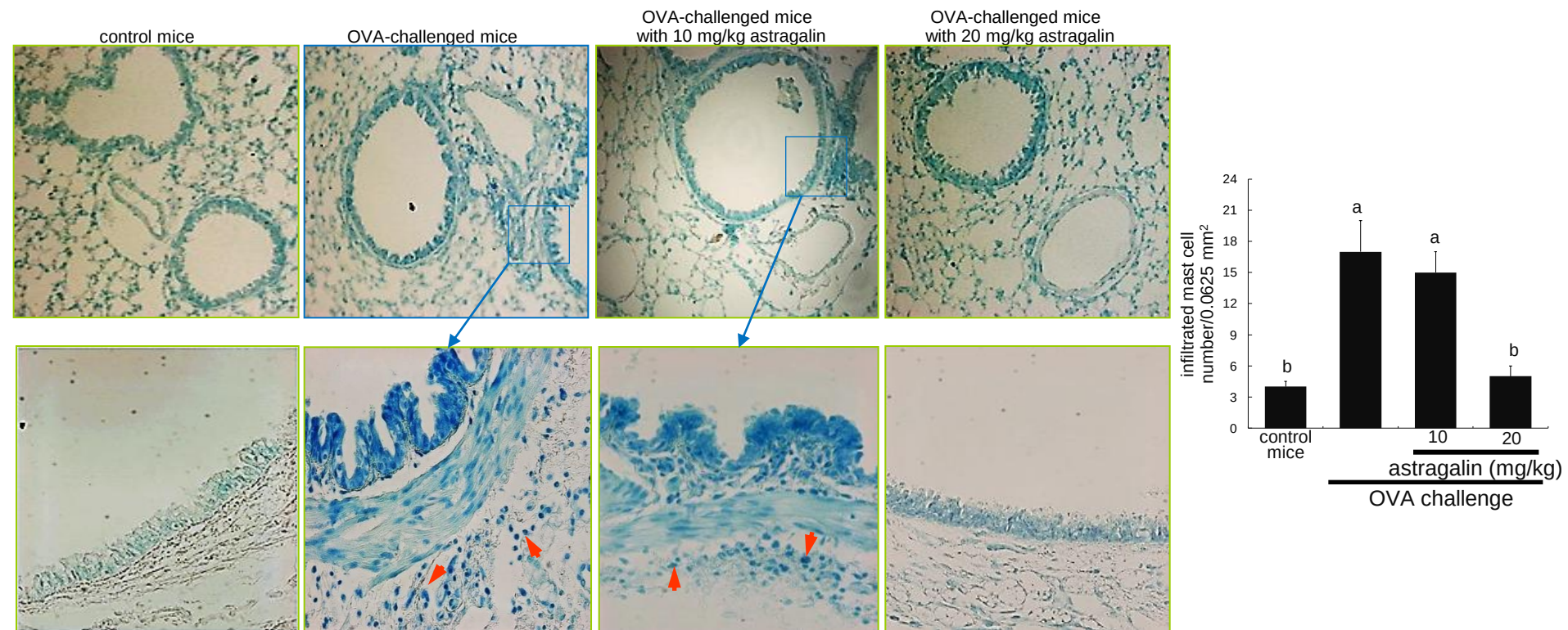
Figure 2

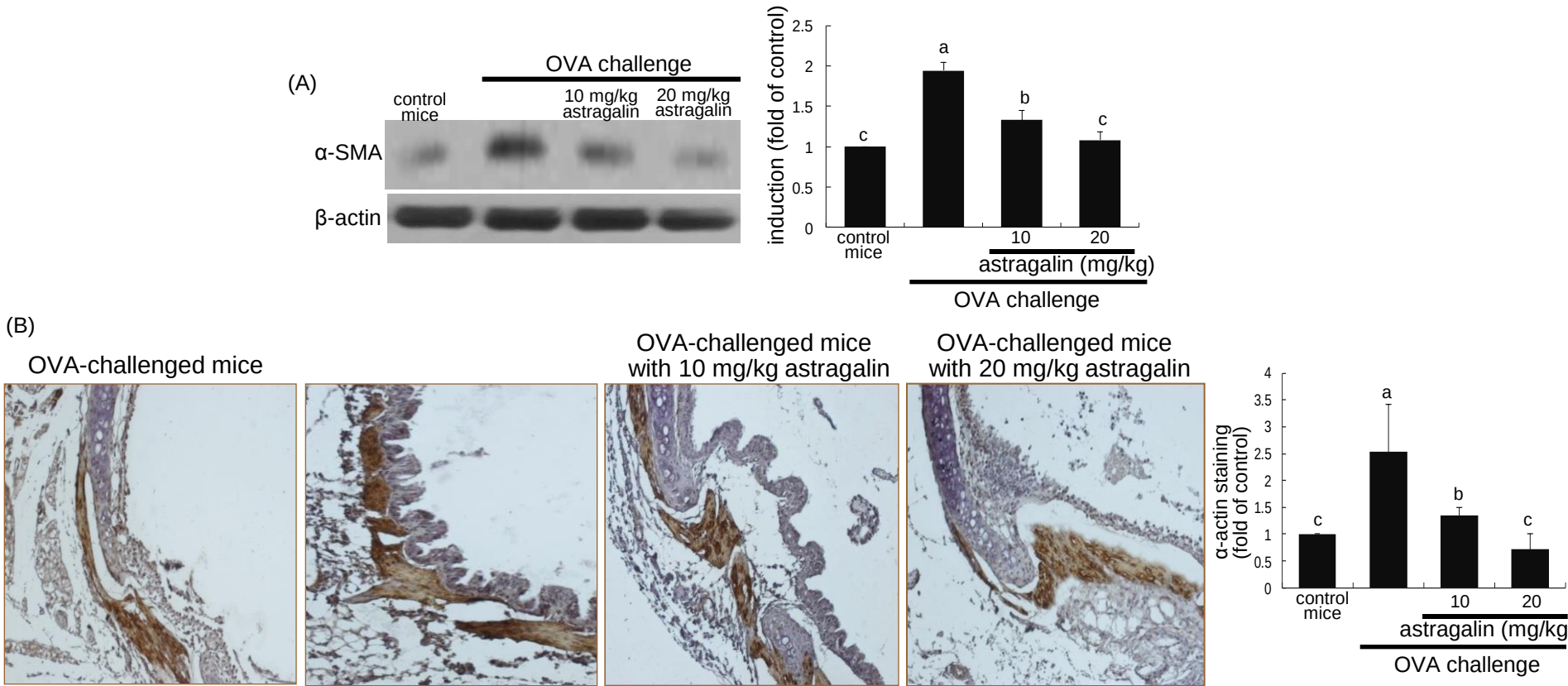
F4/80

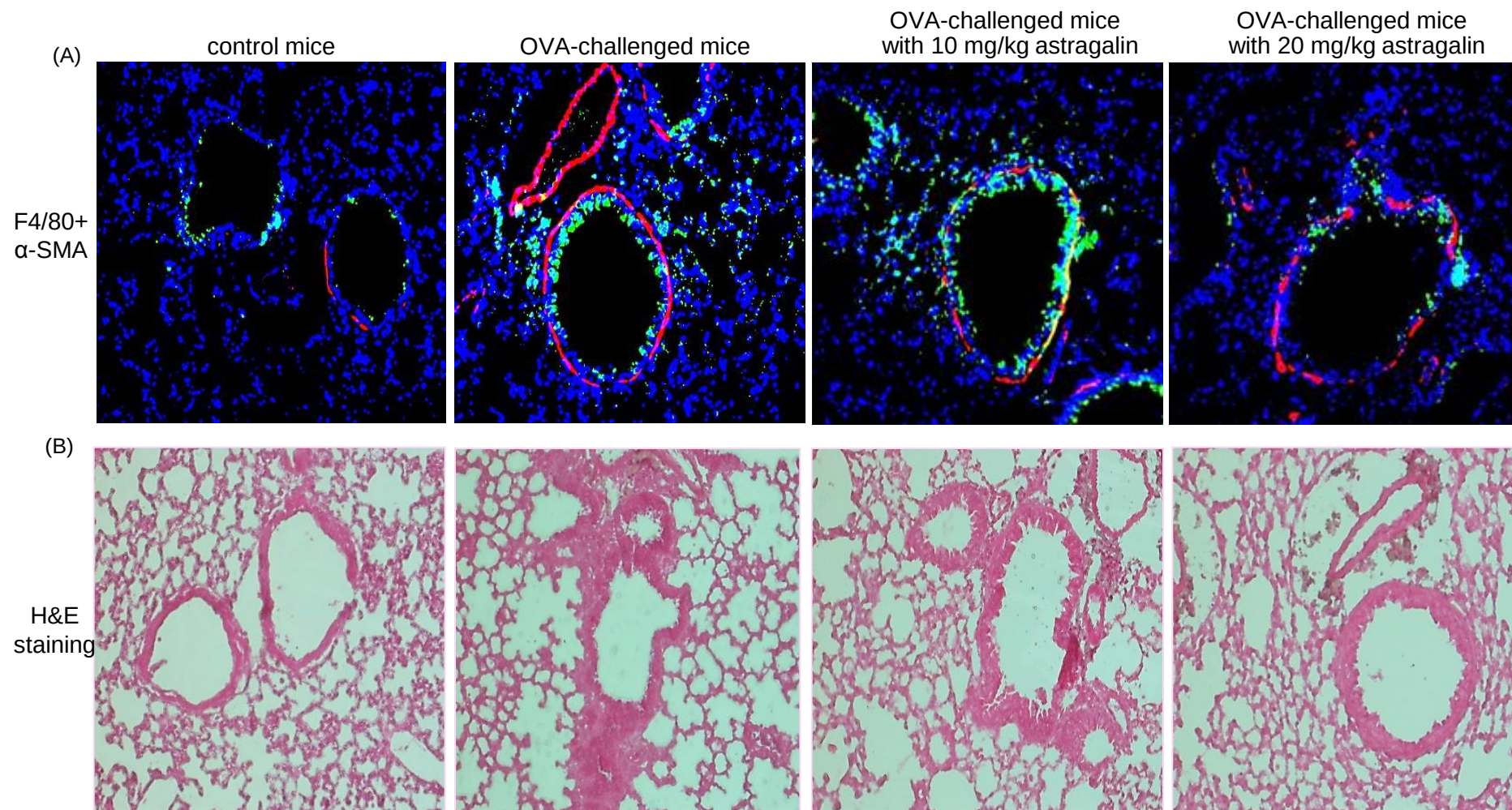
CD68

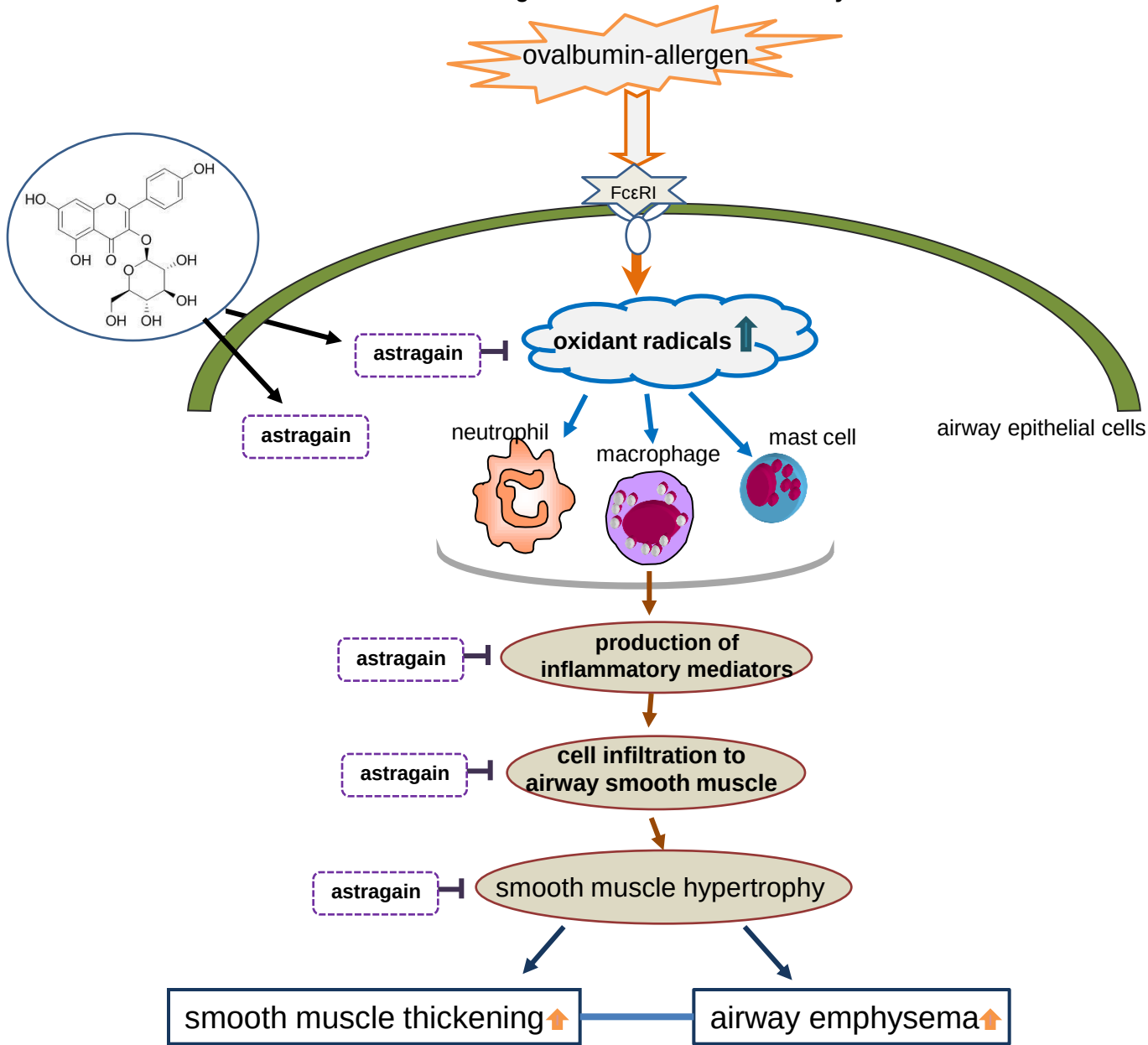


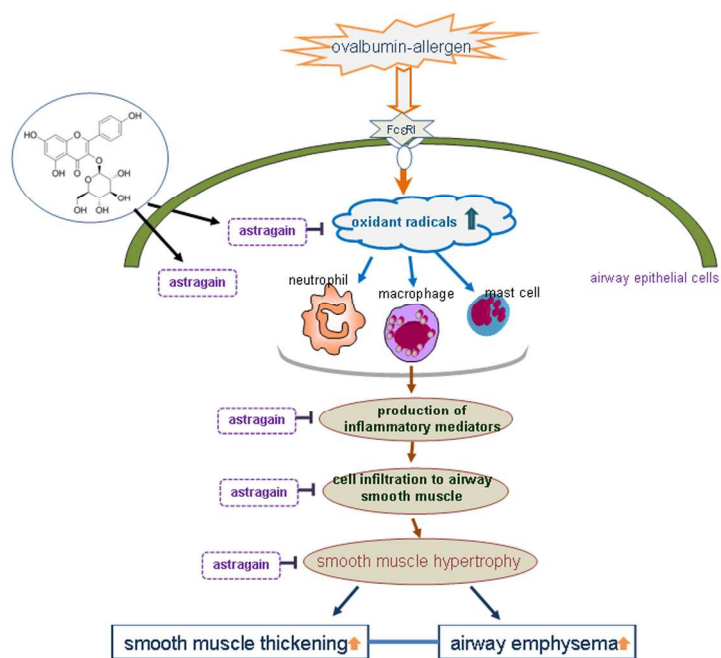












graphic abstract

254x190mm (300 x 300 DPI)