Articles in PresS. Am J Physiol Lung Cell Mol Physiol (August 21, 2015). doi:10.1152/ajplung.00194.2015

Title

Rebamipide suppresses mite-induced asthmatic responses in NC/Nga mice

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Running Head:

Rebamipide suppresses mite-induced asthmatic responses

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Abstract:

Allergic asthma caused by continuous allergen exposure evokes allergen-specific Th2 responses and is characterized by chronic airway inflammation and hyperresponsiveness. A previous report showed that rebamipide improved asthmatic symptoms in an ovalbumin/trypsin mice model. However, it is still unclear how rebamipide exerts its effects in asthma. In this study, rebamipide improved the asthmatic responses induced by mite exposure in NC/Nga mice, revealing the mechanism of this therapeutic effect. Rebamipide suppressed the infiltration of eosinophils into the airways and lung as well as attenuating the production of reactive oxygen species in tissues. In addition to these anti-inflammatory effects, rebamipide inhibited the production of IL-33, a member of the IL-1 family that drives the subsequent production of Th2-associated cytokines. These observations identify the point where rebamipide exerts its suppressive action on asthma and suggest that rebamipide has therapeutic potential in preventing mite-induced asthma.

Keywords:

rebamipide, asthma, eosinophil, IL-33, reactive oxygen species

Introduction

Asthma is a chronic respiratory disease that affects about 300 million people worldwide (32). The major characteristics of asthma are airway hyperresponsiveness (AHR), moist cough and variable airflow obstruction on the basis of airway inflammation (36). Such asthmatic symptoms are triggered by factors such as indoor allergens (house dust mites and pet dander), outdoor allergens (pollens, particulate matter and molds), tobacco smoke, exercise and viral respiratory infections (36). These factors evoke airway inflammation by means of the inflammatory mediators and cytokines derived from eosinophils, lymphocytes and airway epithelial cells (3). In addition, when exposed to allergens or non-allergenic substances these cells can produce reactive oxygen species (ROS), which causes tissue damage via lipid peroxidation of cell membranes and nucleotide damage within DNA (8, 18).

Rebamipide (2-{4-cholorobenzoylamino-3-[2(1H)-quinolinon-4-yl]} propionic acid) is widely used for mucosal protection, healing of gastric ulcers and treatment of gastritis. It has various biological effects that include up-regulation of growth factors such as epidermal growth factor and hepatocyte growth factor (31, 34); increased production of mucus and prostaglandins (34, 37); inhibition of oxygen radical production by neutrophils stimulated with N-formyl-methionyl-leucyl-phenylalanine

(fMLP), opsonized zymosan or Helicobacter pylori (20, 27, 39, 41); scavenging of hydroxyl radicals (40) and inhibition of the production of inflammatory cytokines and chemokines (4, 7). These effects of rebamipide suggest the possibility that rebamipide may be useful for the treatment and prevention for asthma. A previous report showed that oral administration of rebamipide improved asthmatic symptoms in an ovalbumin (OVA)/trypsin mice model; however, how rebamipide exerts these effects remains unclear (5).

The aim of this study was to examine whether rebamipide improved the asthmatic responses in a model where NC/Nga mice were exposed to mites, and to clarify the molecular mechanisms involved.

Materials and Methods

Chemicals and reagents.

Rebamipide was manufactured by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Carboxymethyl cellulose sodium (CMC) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetylcholine chloride was purchased from Daiichi Sankyo Pharmaceutical Co., Ltd. (Tokyo, Japan). Gallamine triethiodide was purchased from Sigma-Aldrich Co. (St. Louis, MO). Isoflurane was purchased from Intervet K. K. (Tokyo, Japan). Sodium pentobarbital was purchased from Kyoritsu Seiyaku Co., Ltd. (Tokyo, Japan).

Animals.

Five-week-old male NC/Nga mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan). The mice were maintained under specific pathogen-free conditions in 12/12-hour light/dark cycle and had free access to standard laboratory food and water. They were acclimatized for at least one week before the experiments. The care and handling of animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals at Shikata Campus of Okayama University. This study was approved by the Okayama University Institutional Animal Care and Use

87 Committee.

88

89 **Intranasal and intratracheal administrations.**

90 NC/Nga mice were sensitized to crude mite extract (*Dermatophagoides farinae*: Df,
91 Cosmo Bio, Tokyo, Japan) based on a previously described protocol (29) as shown in
92 Fig. 1. Briefly, mice were anesthetized with 3% isoflurane and treated with intratracheal
93 2% rebamipide (500 μg in 25 μl 0.1% CMC) for 14 consecutive days (days 0–13). One
94 hour after each treatment, the anesthetized mice received intranasal instillation of Df
95 crude extract (50 μg in 25 μl saline) for six days (days 0–4 and 11). Saline and 0.1%
96 CMC were used as controls, for comparison with the groups given Df or rebamipide.

97

98 **Measurement of AHR.**

99 On day 14, the degree of bronchoconstriction was measured according to the overflow
100 method (26). Briefly, mice were anesthetized with 50 mg/kg pentbalbital (i.p.) and
101 connected to an artificial ventilator following surgical incision of the trachea. A
102 pulmotor system was constructed with a rodent ventilator (model 55-7058; Harvard
103 Apparatus Canada, QC, Canada), a bronchospasm transducer (model 7020; Ugo Basile,
104 Comerio-Barese, Italy), and a data recorder (model WR300; Graphtec corporation,

105 Kanagawa, Japan). Gallamine triethiodide (350 μg/mouse) was immediately
106 administered intravenously to eliminate spontaneous respiration and followed by
107 repeated administration of acetylcholine with stepwise increases in the concentration
108 from 62.5 to 2,000 μg/kg. Dose-response curves were obtained for acetylcholine in
109 anesthetized, mechanically ventilated mice. Bronchoconstriction was expressed as the
110 respiratory overflow volume provoked by acetylcholine as a percentage of the maximal
111 overflow volume (100%) obtained by totally occluding the tracheal cannula (16).
112
113 **Bronchoalveolar lavage fluid.**
114 Immediately after the assessment of acetylcholine-induced AHR, the lungs were
115 lavaged with 1 ml aliquots of cold Hanks' balanced salt solution (HBSS) without
116 calcium and magnesium, containing 50 μM EDTA. The collected bronchoalveolar
117 lavage fluid (BALF) was then centrifuged at 200×*g* for 10 min at 4°C. The supernatant
118 was collected and stored at −80°C for further analysis, and the cell pellet was
119 resuspended in cold HBSS. The number of leukocytes in the BALF was determined
120 using a hematology analyzer (model XT-200i; Sysmex, Hyogo, Japan). Another aliquot
121 was used for cytospin preparations at 500 rpm for 5 min (Cytospin2; Thermo Fisher
122 Scientific, Kanagawa, Japan) and was stained with Diff-Quik for cell count (Sysmex).

Lung specimens.

The lung tissue was utilized for extraction of total RNA for RT-PCR and fixed in 10% buffered formalin for morphological examination. The remaining lung tissue was homogenized in a homogenizing buffer (T-PER tissue extraction reagent containing a protease inhibitor cocktail) with a Multi-Beads Shocker (model MB901OT; Yasui Kikai, Osaka, Japan) set to 1,900 rpm and 30 sec, with repetition for two cycles.

Quantitative PCR analysis.

Total RNA was isolated using the RNeasy mini kit (Qiagen, Redwood City, CA), and reverse transcription was performed with the High Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Quantitative RT-PCR was carried out using the ABI 7500 Fast Real-Time PCR System and the Taqman Fast Universal PCR Master Mix (Life Technologies). The reaction mixture was prepared with TaqMan Gene Expression Primer and Probe (Life Technologies) according to the manufacturer's protocol. Primers specific for interleukin-4 (IL-4, Assay ID: Mm00445259_m1), IL-5 (Mm00439646_m1), IL-13 (Mm00434204_m1), interferon gamma (IFNγ, Mm99999071_m1), Eotaxin-1 (Mm00441238_m1), Eotaxin-2

(Mm00444701_m1), IL-33 (Mm00505403_m1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 4352932E), arginase-1 (Arg1, Mm00475988_m1), Fizz1 (Mm00445109_m1), Ym1 (Mm00657889_m1) and nitric oxide synthase 2 (NOS2, Mm00440502_m1) were used. The thermal cycling conditions were at 95°C for 20 sec, followed by 40 cycles of amplification at 95°C for 3 sec and 60°C for 30 sec. The expression of mRNA was standardized to GAPDH mRNA, and the relative expression of each gene was quantified by the ddCt method, expressed as a ratio, related to the mean value for vehicle-treated lungs (17).

EIA analysis.

The concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was determined by a DNA/RNA oxidative stress EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The detection limit of this assay was 10.3 pg/ml.

ELISA analysis.

Protein concentrations were determined by the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific Inc., Waltham, MA) using bovine serum albumin standards. The concentrations of eotaxin-1, eotaxin-2 and IL-33 were determined by ELISA kit or

159 DuoSet (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's
160 instructions and these were adjusted by total protein. The detection limits of the assays
161 for eotaxin-1, eotaxin-2 and IL-33 were 15.6 pg/ml each.
162
163 **Histopathological observations.**
164 The fixed lung tissues were dehydrated, embedded in paraffin, and sectioned.
165 Hematoxylin and eosin (H&E) staining was used to assess the degree of inflammation
166 (11). Periodic acid-Schiff (PAS) staining was also performed to observe interstitial
167 goblet cell hyperplasia (30). Images of lung tissue sections stained with H&E and PAS
168 were acquired with a BZ-9000 microscope (Keyence, Osaka, Japan) equipped with a
169 40× objective lens. The levels of inflammation in peribronchial areas of the lung were
170 determined according to an ordinal scale of grades 0 to 3, as described elsewhere (33),
171 in a blinded fashion with examination of at least five airway cross-sections per slide. A
172 value of 0 indicated that no inflammation was detectable; a value of 1, occasional
173 cuffing with inflammatory cells; a value of 2, most bronchi were surrounded by a thin
174 layer (1–5 cells thick) of inflammatory cells; and a value of 3, most bronchi were
175 surrounded by a thick layer (more than 5 cells thick) of inflammatory cells. Goblet cell
176 hyperplasia was semiquantitatively scored in a blinded fashion in at least five airway

177 cross-sections per slide on a scale of grades 0 to 4 (absence, < 25% of epithelial lining
178 cells, 25–50% of epithelial lining cells, 50–75% of epithelial lining cells, > 75% of
179 epithelial lining cells, respectively) as previously described (21).
180
181 **Statistical analysis.**
182 All data, unless otherwise noted, are expressed as mean ± SE. One-way analysis of
183 variance (ANOVA) followed by Dunnett's test was used to evaluate the significance of
184 differences between more than two groups. A mixed model for repeated measures
185 (MMRM) method was used to determine significant differences in the dose-response
186 studies. The threshold for statistical significance was set at $P < 0.05$. Statistical analyses
187 were performed using SAS software Release 9.3 (SAS Institute, Tokyo, Japan).
188

Results

Effect of rebamipide on AHR

Mite antigen is one of the major allergic substrates that induce asthma. Here we used *Dermatophagoides farinae* (Df) for the induction of asthma in NC/Nga mice. To confirm asthmatic features, airway hyperresponsiveness (AHR), which is a basic and reproducible indicator of asthma, was measured. First we examined whether Df treatment changes AHR in response to acetylcholine. In the vehicle group, AHR increased in a dose-dependent manner and such increments in AHR were further enhanced by Df administration ($P < 0.01$, **Fig. 2**). We then evaluated the effects of rebamipide on Df-treated mice and found that rebamipide reduced AHR significantly ($P < 0.01$, **Fig. 2**). Thus intratracheal administration of rebamipide improved AHR in response to acetylcholine in Df-treated NC/Nga mice.

Effect of rebamipide on histopathological findings

Histopathological changes in lungs of mice treated with Df were examined to estimate the degree of inflammation, using H&E staining. Df treatment increased the inflammation score to about twice that of the vehicle group (0.9 ± 0.0 vs 2.0 ± 0.2, $P < 0.01$, **Fig. 3A, B and G**) and such Df-induced inflammation was suppressed by

207 rebamipide (2.0 ± 0.2 vs 1.4 ± 0.1, $P < 0.01$, **Fig. 3C and G**). Since it is known that
208 airway inflammation causes hyperplasia of goblet cells, we attempted to estimate it
209 using PAS staining. Similar to the results of inflammatory scoring, the goblet cell
210 hyperplasia score in the Df-treated group was higher than that in the vehicle group (0.0
211 ± 0.0 vs 2.5 ± 0.4, $P < 0.01$, **Fig. 3D, E and H**), while the score in the rebamipide group
212 was lower than in the Df-treated group (2.5 ± 0.4 vs 1.6 ± 0.2, $P < 0.05$, **Fig. 3F and H**).
213 These results confirmed that rebamipide exhibits anti-inflammatory effects on
214 Df-elicited inflammation in lung.
215
216 *Effect of rebamipide on the accumulation of immune cells in BALF*
217 To explore the mechanisms of inflammation in the airway and lung, we collected
218 bronchoalveolar lavage fluid (BALF) and analyzed the number of accumulated cells and
219 types in BALF using a hematology analyzer. Df treatment increased total cell number
220 about nine times that in the vehicle group ($2.64 \pm 0.43 \times 10^4$ cells vs $23.58 \pm 4.07 \times 10^4$
221 cells) (**Fig. 4**). We further analyzed BALF to identify the cell types present and found
222 that the numbers of inflammatory cells including eosinophils, macrophages, neutrophils
223 and lymphocytes were notably increased by Df treatment (**Fig. 4**). In particular, the
224 number of eosinophils in the BALF increased about 4000-fold in comparison with

225 vehicle control (0.04 ± 0.04 × 10^3 cells vs 157.89 ± 29.82 × 10^3 cells), suggesting that
226 Df-induced an inflammatory response mainly mediated by excess numbers of
227 infiltrating eosinophils in the airways and lungs. This increased number of inflammatory
228 cells was reduced for every cell type when rebamipide was administered intratracheally
229 for 14 days, where the attenuation rate in eosinophil number was about 64%.
230
231 *Effect of rebamipide on expression of eotaxins*
232 Next we measured the expression of eotaxin-1 and eotaxin-2 in lung tissue to confirm
233 the involvement of eosinophils, as suggested in the results of BALF (**Fig. 4**). We found
234 that Df challenge increased eotaxin-1 mRNA expression about 20-fold and eotaxin-2
235 mRNA 80-fold compared with those in the vehicle group (**Fig. 5A**). Elevated protein
236 expression was also detected for both eotaxin-1 and eotaxin-2 (**Fig. 5B**. eotaxin-1: 36.1
237 ± 9.1 vs 219.0 ± 32.8 pg/mg protein, $P < 0.01$, eotaxin-2: 59.2 ± 6.0 vs 679.5 ± 185.6
238 pg/mg protein, $P < 0.01$). When rebamipide was administered, augmented mRNA
239 expression induced by Df was reduced about one half and one quarter for eotaxin-1 and
240 eotaxin-2, respectively. In addition, increased protein expression of both eotaxin-1 and
241 eotaxin-2 were also significantly decreased by rebamipide (eotaxin-1: 90.8 ± 12.7
242 pg/mg protein, $P < 0.01$; eotaxin-2: 174.7 ± 31.5 pg/mg protein, $P < 0.05$).

243 These results demonstrate that main cell infiltrating the lung was the eosinophil, which
244 was also the most abundant cell type in BALF after Df treatment; rebamipide
245 suppressed this infiltration.
246
247 *Effect of rebamipide on 8-OHdG in BALF*
248 Reactive oxygen species (ROS) are detected extensively in patients with asthma, where
249 they are mainly produced by granulocytes and epithelial cells. Because rebamipide is a
250 direct scavenger of ROS (40) and also inhibits ROS production by activated neutrophils
251 through the competitive inhibition of the formyl peptide receptor (20), we measured
252 8-OHdG in BALF as an indicator of ROS production to examine the suppressive effect
253 of rebamipide in our model. The level of 8-OHdG in BALF increased after Df treatment
254 (78.0 ± 12.6 vs 159.6 ± 24.6 pg/ml BALF, $P < 0.05$) and this increase in 8-OHdG was
255 significantly reduced to the same level seen with the vehicle control when rebamipide
256 was administered (74.3 ± 9.4, $P < 0.01$) (**Fig. 6**).
257
258 *Effect of rebamipide on expression of IL-33 and cytokines*
259 It has been revealed that the epithelium-derived cytokine IL-33 plays important roles in
260 asthma in Th2-dependent and independent ways (23), where IL-33 activates effector

261 cells such as eosinophils, mast cells and basophils directly and also induces activation
262 of these cells via IL-4, IL-5 and IL-13 produced by Th2 cells (13, 19). We thus
263 examined changes in the expression of IL-33 and Th2 cytokines in lung, before and
264 after Df treatment.

265 We first examined IL-33 expression by measuring mRNA and protein levels.
266 Df treatment increased IL-33 expression significantly compared with the vehicle group
267 (690.1 ± 73.7 vs 3306.5 ± 493.8 pg/mg protein, $P < 0.01$) (**Fig. 7A and B**). We then
268 tested the effect of rebamipide on the expression of IL-33. Rebamipide reduced IL-33
269 expression by about half, with respect to both mRNA and protein (1742.1 ± 178.0
270 pg/mg protein, $P < 0.01$). We next measured the expression of IL-4, IL-5 and IL-13 as a
271 representative Th2 cytokines and IFNγ as a Th1 cytokine, respectively. For all cytokines
272 examined except IFNγ, mRNA expression was elevated, suggesting that Df treatment
273 selectively evokes a Th2 response. These augmented Th2 responses were dramatically
274 suppressed when rebamipide was administered (**Fig. 8**).

275

276 *Effect of Rebamipide on macrophage polarization*

277 Based on the result of enhanced Th2 cytokine productions shown in Figure 8, we further
278 analyzed macrophage polarization to confirm Th2 response by testing the expression of

279 series of polarization marker. Here we utilized arginase-1 (Arg1), found in inflammatory
280 zone-1 (Fizz1) and chitinase-like 3 (Ym1) as a marker for M2 macrophage and nitric
281 oxide synthase 2 (NOS2) was selected for M1 macrophage marker (25, 38). All mRNA
282 levels in M2 macrophage markers increased after Df treatment. On the other hand, M1
283 macrophage maker did not show any changes **(Fig. 9**). These enhanced expression of
284 M2 macrophage makers were remarkably decreased after rebamipide treatment
285 suggesting the possibility that rebamipide suppress M2 macrophage polarization
286 through the inhibition of production of Th2 cytokines.
287

Discussion

Previous studies reported that rebamipide attenuated the allergic response and respiratory symptoms by improving AHR, reducing leukocyte numbers in BALF and suppressing goblet cell hyperplasia regardless of the different animals, allergens and routes of administration of rebamipide. For example, Lee et al. reported that oral administration of rebamipide reduced the number of infiltrating leukocytes and the amount of TNFα in BALF, and downregulated MUC5AC mucin synthesis in the airway epithelium in a rat model of cigarette smoke-induced mucin production (12). Another group used a different approach, examining the effects of rebamipide on an OVA/trypsin-induced asthmatic mouse model, and found that oral administration of rebamipide decreased the eosinophil number in BALF and improved the respiratory rate, air-flow rate and tidal volume (5). In this study, we used a mite-induced asthmatic model in NC/Nga mice, which resembles human asthma, to examine the therapeutic effects of rebamipide and to clarify the molecular mechanisms involved (26, 29, 30).

Our results suggest that rebamipide improves features of asthma in NC/Nga mice through three pathways. First, rebamipide suppressed eosinophil invasion of the airways and lungs (**Fig. 4 and 5**). It is known that an excess number of infiltrating eosinophils in respiratory tissues is one of the characteristic features of asthma and it is

306 strongly associated with the development of AHR. Such chemotaxis of eosinophils
307 towards inflamed tissue may be inhibited by rebamipide directly or indirectly. As
308 previous reports have shown that rebamipide exerts an antagonistic effect on the formyl
309 peptide receptor (FPR) expressed in neutrophils and eosinophils, an essential receptor
310 for the expression of chemotaxis (20, 28), there is a possibility that rebamipide
311 suppresses chemotactic behavior of eosinophils through the inhibition of the FPR. In
312 addition to the direct effects of rebamipide on eosinophils, we found that rebamipide
313 reduced the expression of eotaxin-1 and eotaxin-2 in lung (**Fig. 5**). The eotaxin family
314 has been identified as chemoattractant for eosinophils, implying that rebamipide may
315 suppress chemotaxis of eosinophils by eliminating the chemoattractant source. Second,
316 rebamipide improved asthmatic symptoms by reducing ROS production by eosinophils
317 and epithelial cells. A marker of oxidative stress formed by ROS is 8-OHdG (10).
318 Several studies demonstrated that 8-OHdG expression was induced and enhanced in the
319 lungs as a result of several types of oxidative stress (1, 24, 35). Similar to previous
320 studies, the 8-OHdG level in BALF was elevated by Df treatment and was reduced
321 significantly by rebamipide administration, suggesting that rebamipide may work as a
322 scavenger of ROS (**Fig. 6**). Alternatively, rebamipide may reduce ROS produced by
323 activated eosinophils through inhibition of FPR agonist-induced eosinophil activation as

described above. Finally, rebamipide exhibited a suppressive action on the Th2 response by inhibition of IL-33 production. Previous reports have demonstrated the involvement of IL-33 in asthmatic symptoms (6, 9, 22). The IL-33 level is elevated in asthmatic patients when compared with healthy individuals, and a similar tendency was also observed in mice (6, 9, 22), where IL-33 is released from bronchial epithelial cells in response to allergen exposure and this secreted IL-33 then induces Th2 responses from macrophages, eosinophils and mast cells (19). Direct administration of IL-33 induces eosinophilic inflammation and AHR in mice and administration of neutralizing antibodies against IL-33 and IL-33 receptor ST2 attenuates eosinophilic inflammation and AHR in an OVA-induced airway inflammation model in mice (2, 9, 14, 15). In the present study, rebamipide decreased the expression of IL-33 in lung tissue (**Fig. 7**) and also attenuated the expression of Df-elicited Th2 cytokines, including IL-4, IL-5 and IL-13 (**Fig. 8**). Taken together, the inhibition of IL-33 production by rebamipide appears to suppress subsequent Th2 responses. Another approach used to check Th2 response involves examining the polarization of macrophages: macrophage have been classified as M1 (classically activated) or M2 (alternatively activated) macrophages. M1 macrophages express inducible nitric oxide synthase (NOS2) and proinflammatory cytokines, and these are essential for protection against infection. Conversely, M2

342 macrophages express arginase-1 (Arg1), chitinase-like 3 (Ym1), found in inflammatory
343 zone-1 (Fizz1), and chemokines such as Chemokine (C-C motif) ligand 17 (CCL17),
344 CCL24 (eotaxin-2) and these play important roles in responses to parasite infection,
345 tissue remodeling, angiogenesis and tumor progression (25, 38). Gene expression levels
346 of M2 macrophage markers such as Arg1, Fizz1 and Ym1 were significantly
347 upregulated by Df administration and downregulated by rebamipide treatment, while
348 M1 macrophage marker NOS2 showed no change (**Fig. 9**). Because both IL-33 and
349 IL-13 can polarize macrophages from M1 to M2 types, it is likely that rebamipide
350 impairs the polarization towards M2 macrophages due to the decreased expression of
351 IL-33 and IL-13.

352 In conclusion, our current study indicates the therapeutic potential of
353 rebamipide in the prevention of mite-induced asthma. Our data demonstrate that
354 rebamipide improves mite-induced asthmatic symptoms through the inhibition of
355 eosinophil infiltration, as well as attenuating oxidative stress and IL-33 production.

356

357 **Acknowledgements:**

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Disclosures:

IM is employed by Otsuka Pharmaceutical Co., Ltd.

Author contributions:

Conception and design of research: IM, KO; Performed experiments: IM, RZ, MK, KN; Analyzed data: IM, EE, KO; Interpreted results of experiments: IM, RZ, MK, KN, EE, KO; Prepared figures: IM; Drafted manuscript: IM; Edited and revised manuscript: MK, KN, EE, KO; Approved final version of manuscript: IM, RZ, MK, KN, EE, KO.

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505

506 **Figure Legends:**

507 **Fig. 1.** Schematic representation of the experiment. The vehicle group received 25 μl of
508 saline intranasally on days 0–4 and day 11 and 25 μl of 0.1% CMC intratracheally on
509 days 0–13. The Df group received 50 μg of *Dermatophagoides farinae* (Df) extract
510 dissolved in 25 μl of saline intranasally on days 0–4 and day 11 and 25 μl of 0.1% CMC
511 intratracheally on days 0–13. The Df plus rebamipide treatment group received 50 μg of
512 Df extract dissolved in 25 μl of saline intranasally on days 0–4 and day 11 and 500 μg
513 of rebamipide dissolved in 25 μl of 0.1% CMC intratracheally. Rebamipide or
514 0.1% CMC was administered one hour before Df or saline administration.

515

516 **Fig. 2.** Airway hyperresponsiveness (AHR) to acetylcholine. Effect of rebamipide on

517 Df-induced increase of AHR in NC/Nga mice. Data were obtained from 5–12
518 mice/group. The bronchoconstriction (%) is expressed as mean ± SE. ** $P < 0.01$ Df
519 group vs. Vehicle group. ## $P < 0.01$ Df plus rebamipide group vs. Df group. (MMRM
520 method).
521
522 **Fig. 3.** Histopathology of H&E-stained lungs or periodic acid-Schiff (PAS)-stained
523 lungs from Vehicle group (A, D), Df group (B, E), and Df plus rebamipide group (C, F).
524 For A, B, C, D, E and F, scale bars are 50 μm and pictures were taken at 40×
525 magnification. Inflammation score (G) and goblet-cell hyperplasia score (H) are
526 expressed as mean ± SE. Data were obtained from 5–12 mice/group. * $P < 0.05$, ** $P <$
527 0.01 vs. Df group.
528
529 **Fig. 4.** Effect of rebamipide on BALF cell numbers. Enlarged view display the results
530 of neutrophil and lymphocyte (Inlet). Data were obtained from 5–12 mice/group. The
531 total cell, eosinophil, macrophage, neutrophil and lymphocyte counts are expressed as
532 mean ± SE. ** $P < 0.01$ vs. Df group.
533
534 **Fig. 5.** Effect of rebamipide on Df-induced mRNA and protein expression of eotaxin-1

535 and eotaxin-2 in lung tissues (A, B). The expressions of mRNA for eotaxin-1 and
536 eotaxin-2 were standardized to GAPDH mRNA and the relative expression of each gene
537 was quantified by the ddCt method. The mean expression levels in lung tissues exposed
538 to vehicle were normalized to 1. Data were obtained from 5–12 mice/group.
539 Concentrations of eotaxin-1 and eotaxine-2 are expressed as mean ± SE. * $P < 0.05$, **
540 $P < 0.01$ vs. Df group.

541

542 **Fig. 6.** Effect of rebamipide on Df-induced 8-OHdG production in BALF. Data were
543 obtained from 5–12 mice/group. Concentration of 8-OHdG in BALF are expressed as
544 mean ± SE. * $P < 0.05$, ** $P < 0.01$ vs. Df group.

545

546 **Fig. 7.** Effect of rebamipide on Df-induced mRNA and protein expression of IL-33 in
547 lung tissues (A, B). The expression of mRNA for IL-33 was standardized to GAPDH
548 mRNA and the relative expression of IL-33 was quantified by the ddCt method. The
549 mean expression levels of vehicle lung tissues were normalized to 1. Data were obtained
550 from 5–12 mice/group. Data are expressed as mean ± SE. * $P < 0.05$, ** $P < 0.01$ vs. Df
551 group.

552

553 **Fig. 8.** Effect of rebamipide on Df-induced mRNA expression of cytokines in lung
554 tissues. The expression of mRNA for IL-4, IL-5, IL-13 (Th2) and IFNγ (Th1) were
555 standardized to GAPDH mRNA and the relative expression of IL-33 was quantified by
556 the ddCt method. The mean expression levels of lung tissue exposed to vehicle were
557 normalized to 1. Data were obtained from 5–12 mice/group. Data are expressed as mean
558 ± SE. ** $P < 0.01$ vs. Df group.

559

560 **Fig. 9.** Effect of rebamipide on Df-induced mRNA expression of M2 and M1
561 macrophage markers in lung tissue. The expression of mRNA for Arg1, Fizz1, Ym1 and
562 NOS2 was standardized to GAPDH mRNA and the relative expression of each gene
563 was quantified by the ddCt method. The mean expression levels of lung tissue exposed
564 to vehicle were normalized to 1. Data were obtained from 5–12 mice/group. Data are
565 expressed as mean ± SE. * $P < 0.05$, ** $P < 0.01$ vs. Df group.

566

567 **Fig. 10.** Schematic representation of mechanisms by which rebamipide affects
568 Df-induced asthmatic symptoms.

Fig. 1

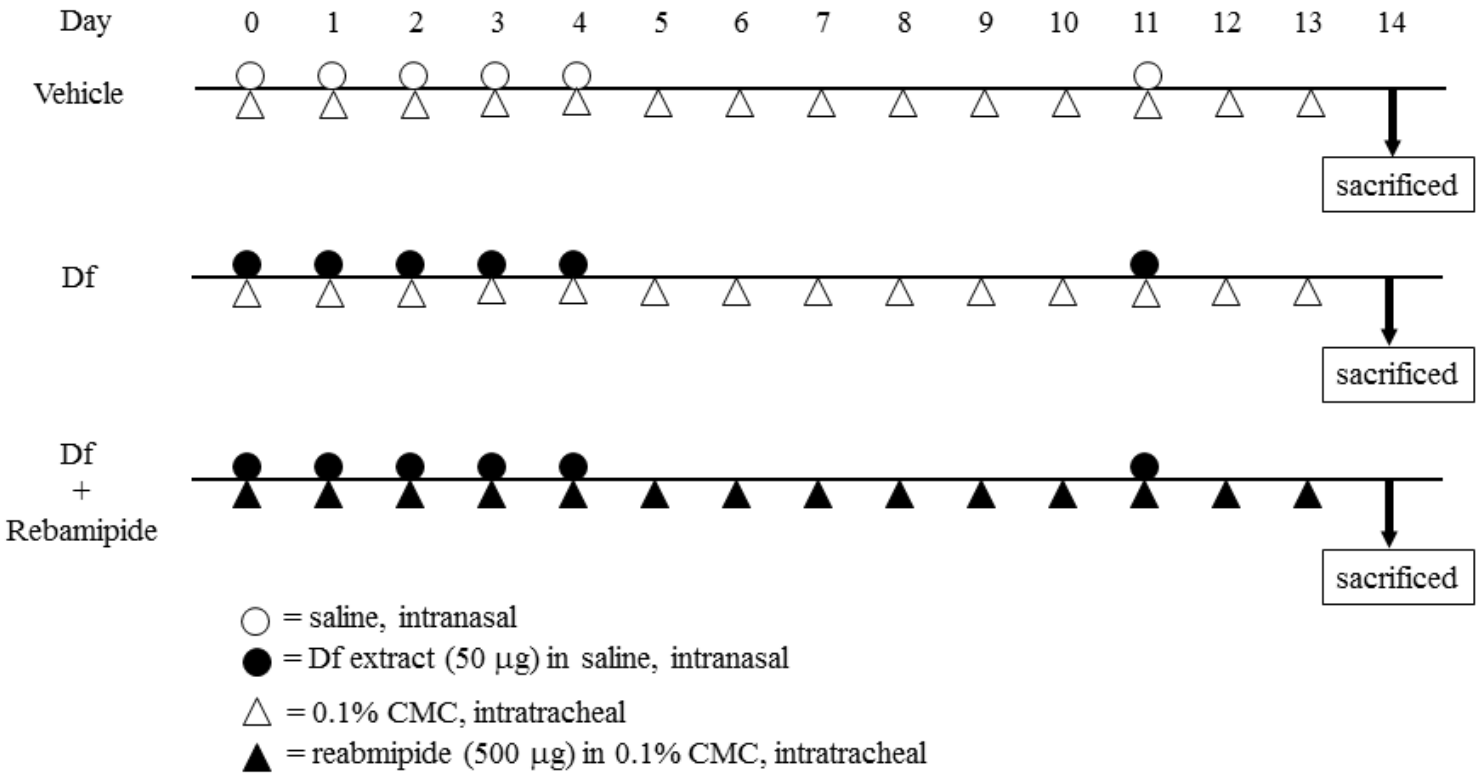

Day
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Vehicle
sacrificed
Df
sacrificed
Df
+
Rebamipide
sacrificed
○ = saline, intranasal
● = Df extract (50 μg) in saline, intranasal
△ = 0.1% CMC, intratracheal
▲ = reabmipide (500 μg) in 0.1% CMC, intratracheal

Fig. 2

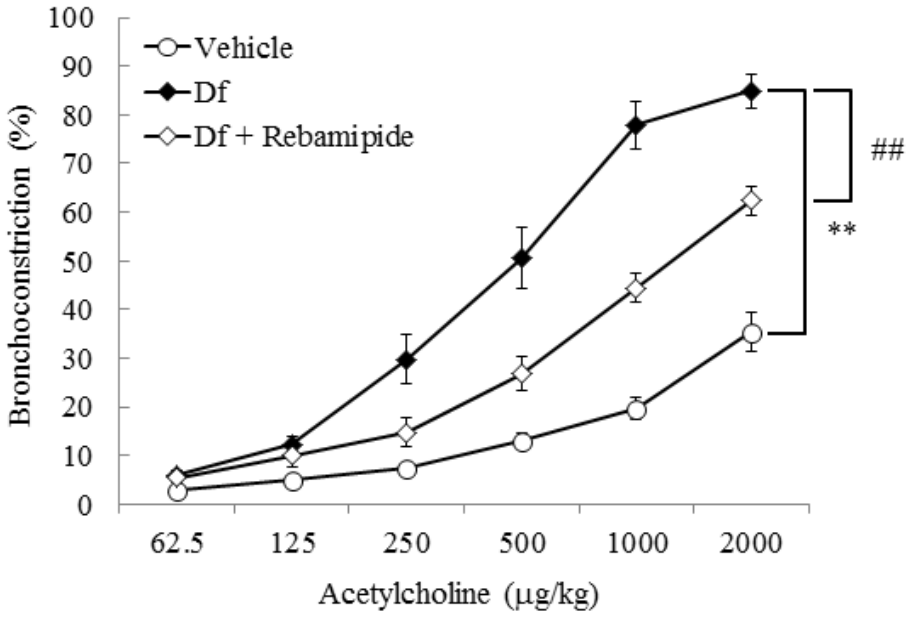
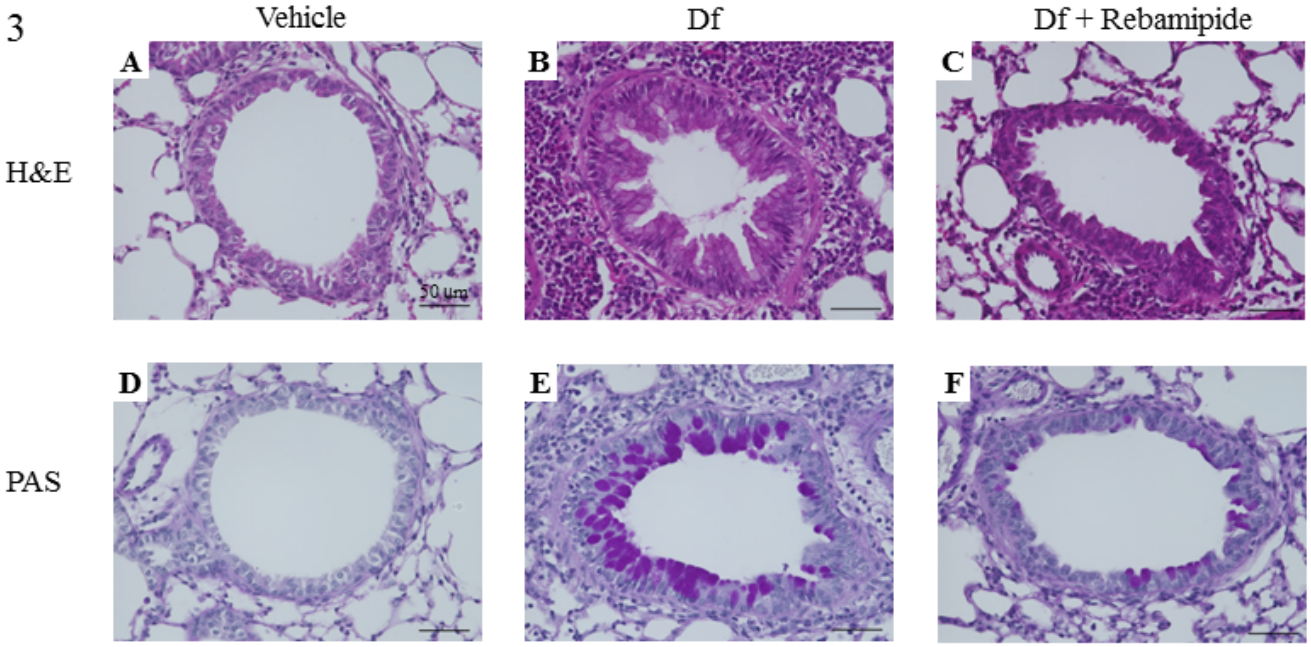
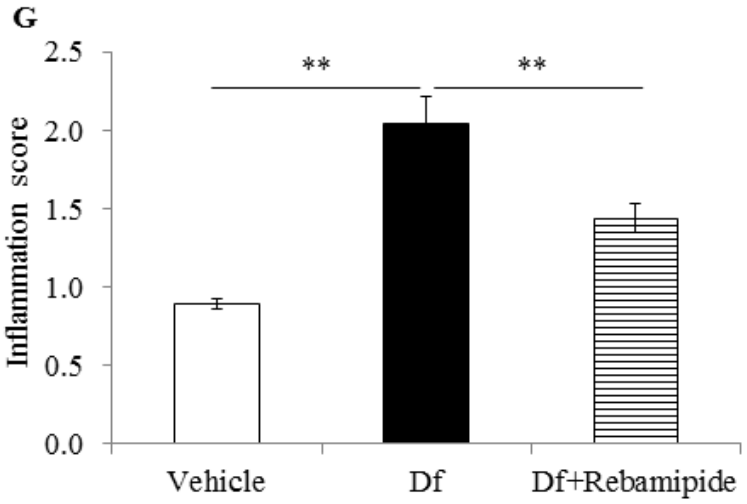

Vehicle
Df
Df + Rebamipide
Bronchoconstriction (%)
100
90
80
70
60
50
40
30
20
10
0
62.5
125
250
500
1000
2000
Acetylcholine (μg/kg)
##
**

Fig. 3


Vehicle
Df
Df + Rebamipide
A
B
C
H&E
50 um
D
E
F
PAS


G
Inflammation score
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Vehicle
Df
Df+Rebamipide

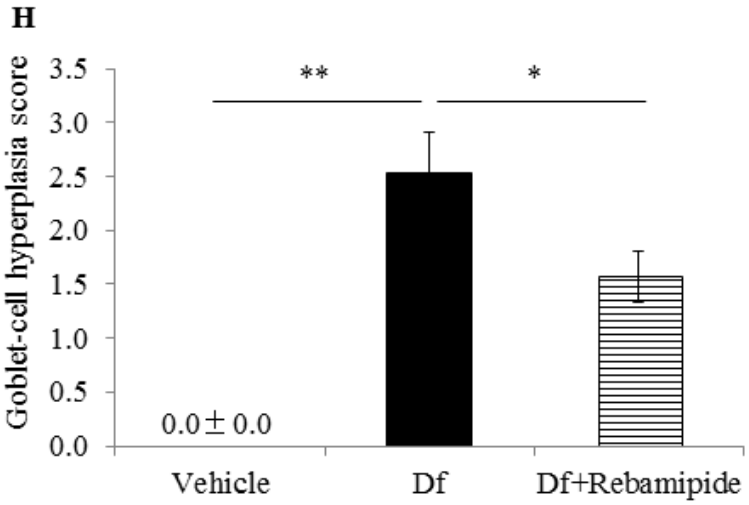

H
Goblet-cell hyperplasia score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
0.0 ± 0.0
Vehicle
Df
Df+Rebamipide

Fig. 4

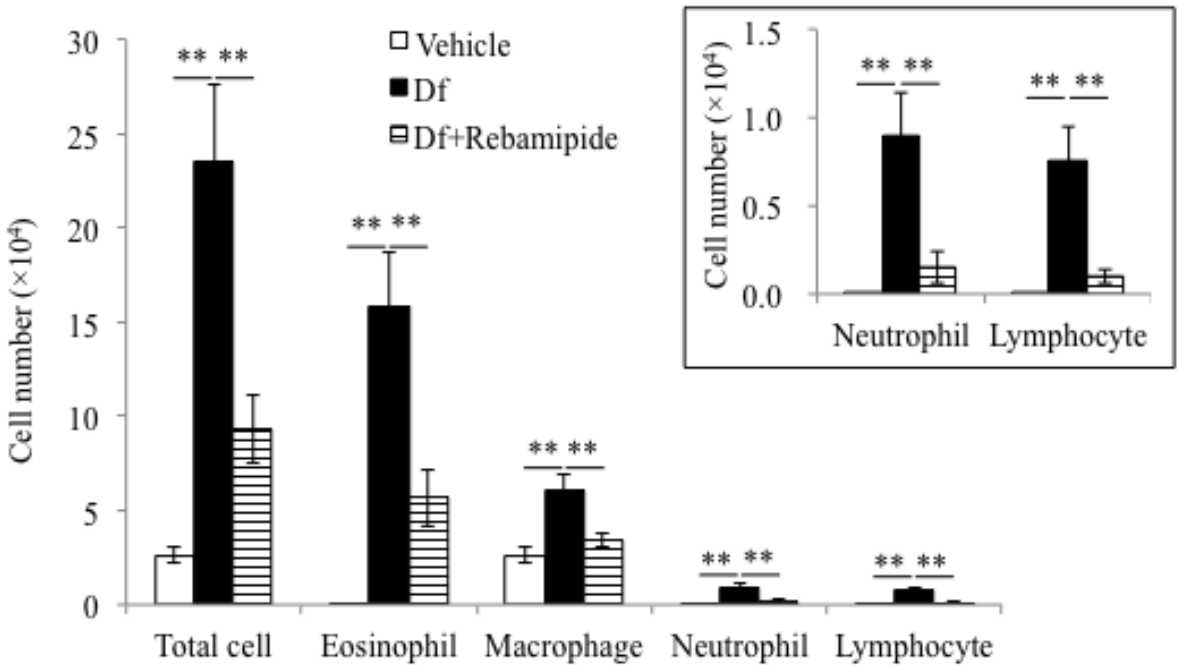

Vehicle
Df
Df+Rebamipide
Cell number (×10⁴)
30
25
20
15
10
5
0
** **
Total cell
Eosinophil
Macrophage
Neutrophil
Lymphocyte
1.5
1.0
0.5
0.0
Neutrophil
Lymphocyte

Fig. 5

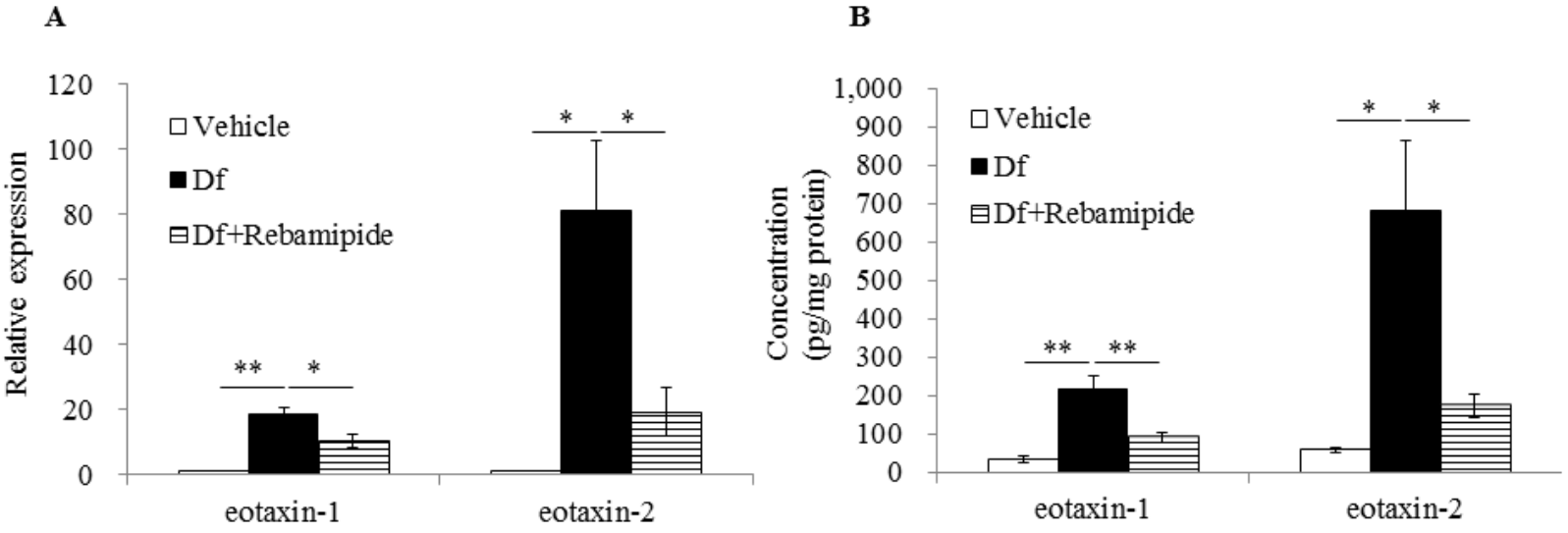

A
Relative expression
120
100
80
60
40
20
0
Vehicle
Df
Df+Rebamipide
**
*
*
*
eotaxin-1
eotaxin-2
B
Concentration (pg/mg protein)
1,000
900
800
700
600
500
400
300
200
100
0
Vehicle
Df
Df+Rebamipide
**
**
*
*
eotaxin-1
eotaxin-2

Fig. 6

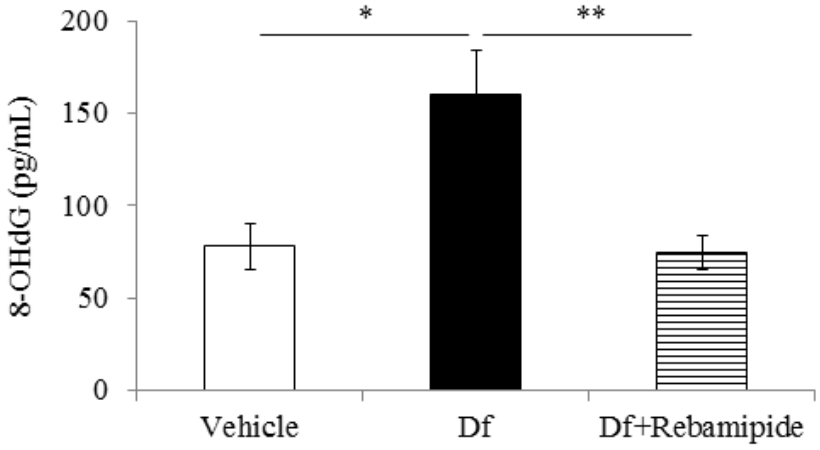


Fig. 7

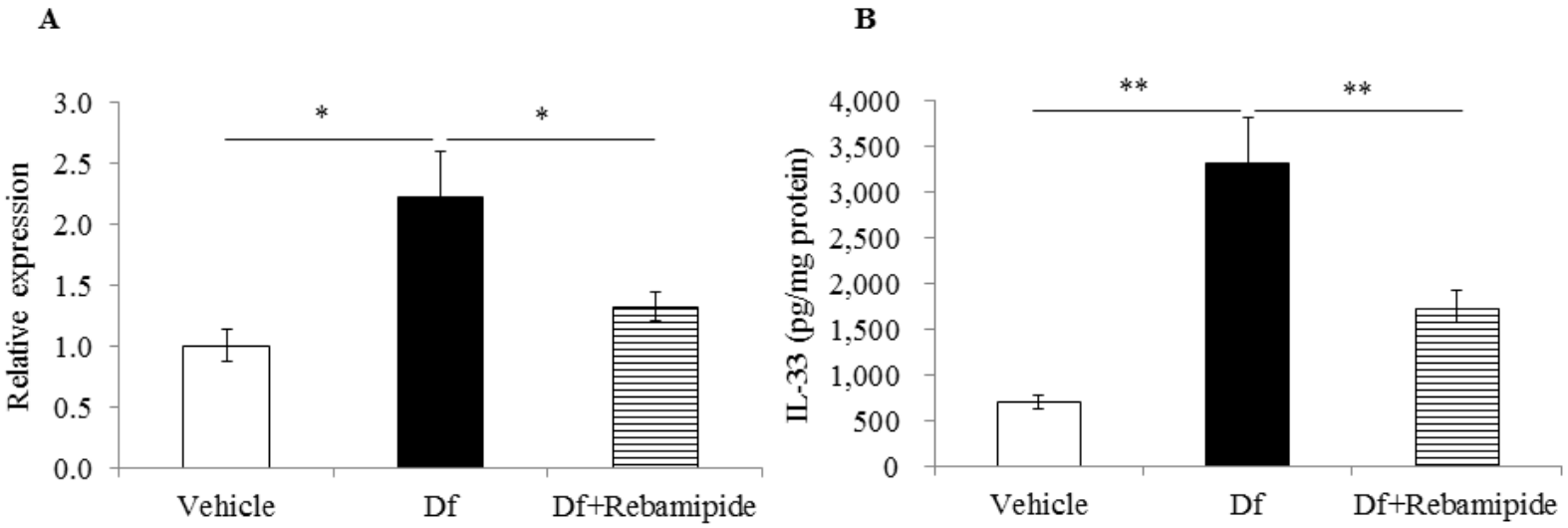

A
Relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Vehicle
Df
Df+Rebamipide
B
IL-33 (pg/mg protein)
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
**
**
Vehicle
Df
Df+Rebamipide

Fig. 8

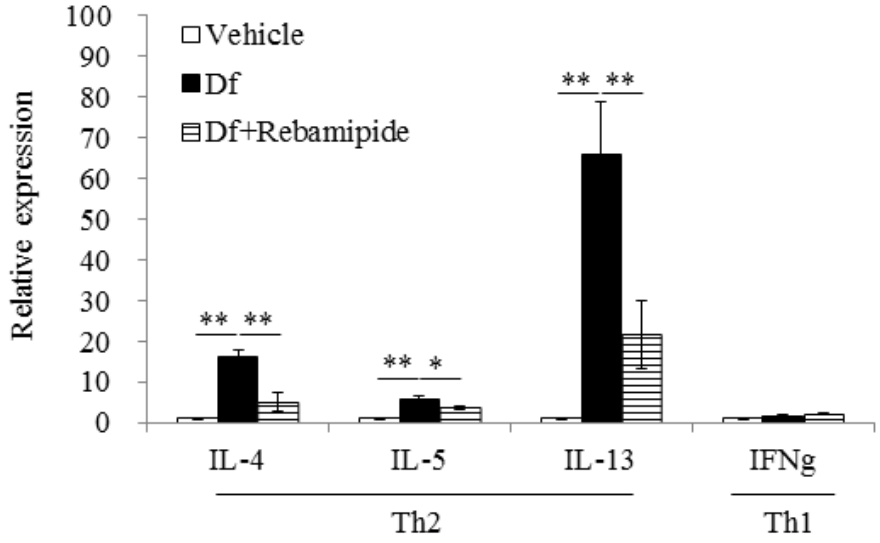

Relative expression
100
90
80
70
60
50
40
30
20
10
0
Vehicle
Df
Df+Rebamipide
** **
** *
** **
IL-4
IL-5
IL-13
IFNg
Th2
Th1

Fig. 9

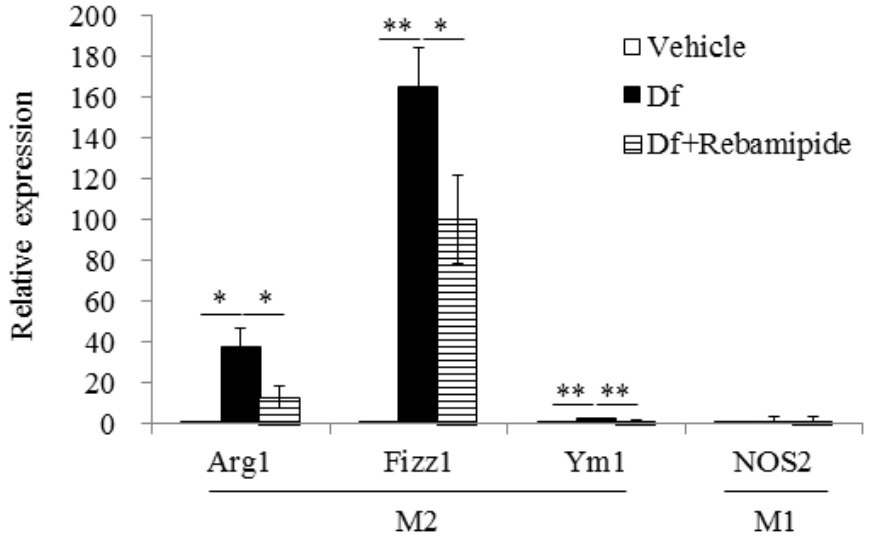
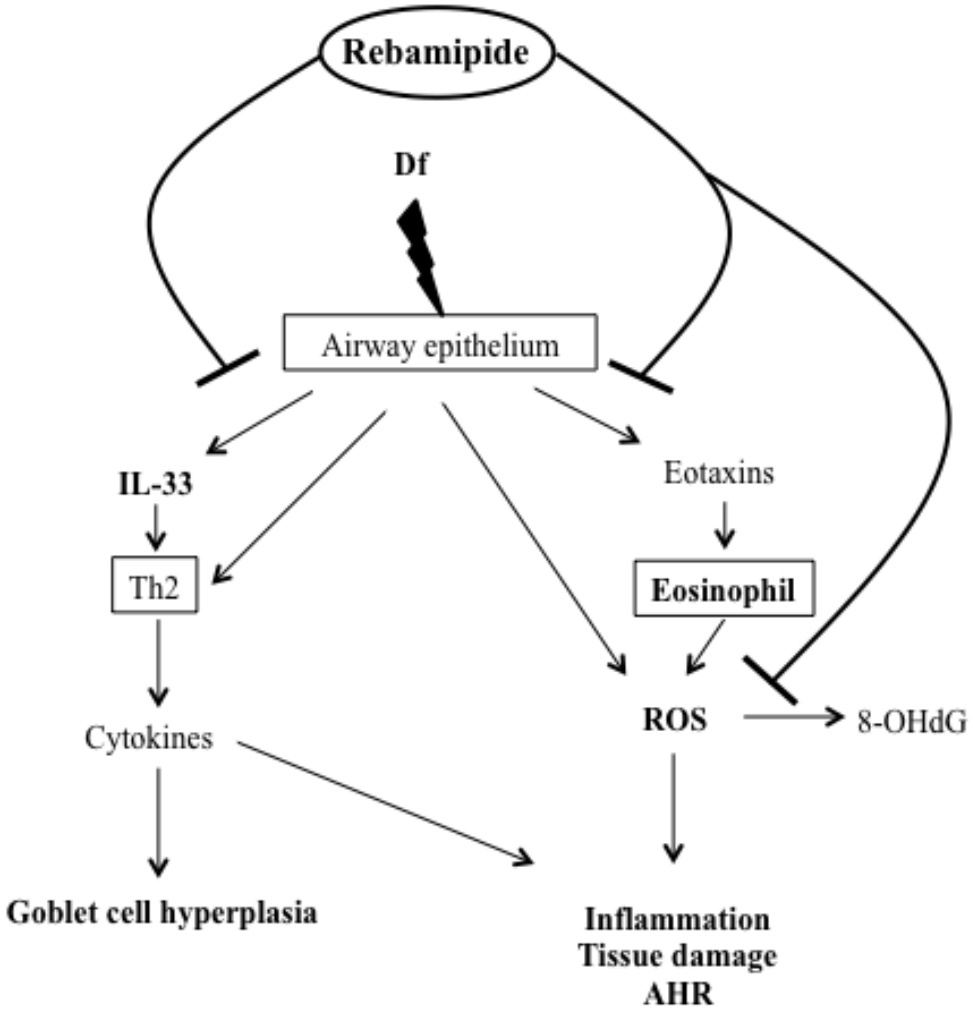

Relative expression
200
180
160
140
120
100
80
60
40
20
0
** *
* *
** **
Vehicle
Df
Df+Rebamipide
Arg1
Fizz1
Ym1
NOS2
M2
M1

Fig. 10


Rebamipide
Df
Airway epithelium
IL-33
Th2
Cytokines
Goblet cell hyperplasia
Eotaxins
Eosinophil
ROS
8-OHdG
Inflammation
Tissue damage
AHR