Targeting PP2A and proteasome activity ameliorates features of allergic airway disease in mice

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Abstract

Background

Asthma is an allergic airway disease (AAD) caused by aberrant immune responses to allergens. Protein phosphatase-2A (PP2A) is an abundant serine/threonine phosphatase with anti-inflammatory activity. The ubiquitin proteasome system (UPS) controls many cellular processes, including the initiation of inflammatory responses by protein degradation. We assessed if enhancing PP2A activity with Fingolimod (FTY720) or 2-amino-4-(4-(heptyloxy) phenyl)-2-methylbutan-1-ol ($AAL_{(S)}$), or inhibiting proteasome activity with Bortezomib (BORT) could suppress experimental AAD.

Methods

Acute AAD was induced in C57BL/6 mice by intraperitoneal sensitisation with ovalbumin (OVA) in combination with intranasal (i.n) exposure to OVA. Chronic AAD was induced in mice with prolonged i.n exposure to crude house dust mite (HDM) extract. Mice were treated with vehicle, FTY720, $AAL_{(S)}$, BORT or $AAL_{(S)}$+BORT and hallmark features of AAD assessed.

Results

$AAL_{(S)}$ reduced the severity of acute AAD by suppressing tissue eosinophils and inflammation, mucus secreting cell (MSC) numbers, type-2 associated cytokines (Interleukin (IL)-33, thymic stromal lymphopoietin, IL-5 and IL-13), serum immunoglobulin (Ig)E, and airway hyper-responsiveness (AHR). FTY720 only suppressed tissue inflammation and IgE. BORT reduced bronchoalveolar lavage fluid (BALF) and tissue eosinophils and inflammation, IL-5, IL-13, and AHR. Combined treatment with $AAL_{(S)}$+BORT had complementary effects and suppressed BALF and tissue eosinophils and inflammation, MSC numbers, reduced the production of type-2 cytokines and AHR. $AAL_{(S)}$, BORT and $AAL_{(S)}$+BORT also reduced airway remodelling in chronic AAD.

Conclusion

These findings highlight the potential of combination therapies that enhance PP2A and inhibit proteasome activity as novel therapeutic strategies for asthma.

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Keywords: Allergic airway disease, asthma, inflammation, protein phosphatase 2A, ubiquitin proteasome system

Introduction

Asthma is a common chronic inflammatory allergic airway disease (AAD), typically caused by aberrant inflammatory responses to innocuous allergens. Exposure of the airway epithelium to allergens induces the release of the type-2 cytokines interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) (1). This promotes the development and activation of type-2 T-helper lymphocytes (Th2 cells) that release their characteristic cytokines IL-4, IL-5 and IL-13, promoting airway eosinophil accumulation, systemic immunoglobulin (Ig)E production, and airway mucus secreting cell (MSC) metaplasia and hyperresponsiveness (AHR) (2, 3). Together these events drive the development and progression of allergic asthma (2). The chronic release of cytokines and remodelling factors, such as transforming growth factor (TGF)-β by eosinophils and Th2 cells damages the epithelial lining, and leads to airway remodelling (4). Current mainstay asthma therapies include corticosteroids and long-acting β-agonists. These reduce symptoms but do not treat the underlying causes of the disease, and their use has numerous issues (2). There is an urgent need for effective alternative treatments.

PP2A is the most abundant serine/threonine phosphatase, is expressed ubiquitously and exists as a heterotrimeric enzyme consisting of structural (A), regulatory (multiple) (B) and catalytic (C) subunits (5, 6). Reduced PP2A activity occurs in animal models of AAD and patients with severe asthma (7-9), and enhancing activity with Fingolimod (FTY720) or 2-amino-4-(4-(heptyloxy) phenyl)-2-methylbutan-1-ol ($AAL_{(S)}$) abrogated the development of inflammation and AHR in AAD as well as inhibited IL-6 secretion in corticosteroid insensitive A549 lung epithelial cells (7, 10, 11). While these studies suggest that these agents may be potential asthma therapies, it is unknown which is more effective, as a side by side comparison has not been performed.

The ubiquitin proteasome system (UPS) is critical in regulating tissue homeostasis through the degradation of key proteins involved in cellular functions (12, 13). Ubiquitinated target proteins are selectively degraded by ubiquitin ligases. In the lungs, the ubiquitin E3 ligase, Itch, promotes immune tolerance by degrading the Th2-specific transcription factor, phosphorylated JunB (14). In murine AAD, Itch deficiency resulted in allergic inflammation in response to high-dose antigen (15). Another E3 ligase, Midline-1 (MID1), is upregulated in human primary bronchial epithelial cells (pBECs) upon house dust mite (HDM) and rhinovirus exposure and in HDM-induced AAD (7). The proteasome inhibitor, bortezomib (BORT) is approved for the treatment of multiple malignancies (16). Its protective effects are due in part to the suppression of nuclear factor kappa-light-chain-enhancer of activated B

cells (NF-κB) signalling, which leads to the down regulation of anti-apoptotic target genes (17). Thus, BORT may suppress airway inflammation driven by NF-κB, which indicates the potential for targeting the UPS by proteasome inhibition as a therapy for asthma.

Comparing and combining drugs that increase PP2A activity and suppress proteasome activity and the determination of any synergistic effects have not been assessed in asthma. Here we demonstrate that increasing PP2A activity with $AAL_{(S)}$ and inhibiting proteasome activity with BORT suppressed some of the same and also different features of acute AAD. Combined treatment with $AAL_{(S)}$+BORT had complementary effects and suppressed all the major hallmark features of acute AAD, with the inhibition of type-2 responses and AHR. Both drugs alone and combined also supressed airway remodelling in chronic HDM-induced AAD. Our study indicates that therapeutically targeting PP2A and proteasome activity, particularly in combination, may be effective asthma treatments.

Methods

Ethics statement

This study was performed in strict accordance with the recommendations in the Australian code of practise for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All protocols were approved by the Animal Ethics Committee of The University of Newcastle.

AAD

Acute AAD was induced in female, 6-8 week-old, C57BL/6 mice by intraperitoneal (i.p) sensitisation to ovalbumin (OVA, 100µg, Sigma-Aldrich, St. Louis, Missouri, USA) with Alhydrogel (1mg, InvivoGen, San Diego, California, USA) in sterile saline (200µl, 0.9%) on day 0 and 7. Mice were then challenged intranasally (i.n) with OVA (10µg, 50µl in sterile saline) on days 12-15. AAD was assessed on day 16. Chronic AAD was induced by i.n challenge with crude HDM extract (*Dermatophagoides pteronyssinus*, Greer Labs, Lenoir, North Carolina, USA) five times a week for five weeks. AAD was assessed on day 35.

Drug treatments

FTY720 (0.8mg/kg, 200µl PBS, Cayman Chemical, Michigan, USA, determined in optimisation studies), $AAL_{(S)}$ (0.8mg/kg, 200µl PBS, synthesised in-house as previously described (18)), BORT (0.2mg/kg, 200µl PBS, LC laboratories, Woburn, USA) or combined treatments ($AAL_{(S)}$+BORT) were administered i.p on days 12-15 (30 minutes before each challenge) in acute AAD, and everyday throughout the chronic model.

Airway inflammation, histopathology, mRNA expression, protein isolation, ELISA, serum antibodies, lung function and airway remodelling

Bronchoalveolar lavage (BAL) was performed and differential leukocyte counts determined. Histopathology, mRNA expression, protein isolation, ELISA, serum antibodies, lung function and airway remodelling were assessed as previously described and/or in the online supplementary material (19-27).

Results

$AAL_{(S)}$ but not FTY720 suppressed tissue inflammation and MSCs in acute AAD

To induce acute AAD, mice were sensitised (i.p day 0 and 7) and challenged (i.n days 12-15) with OVA and outcomes assessed (day 16, Fig. 1A). Vehicle, or the PP2A activators FTY720 or $AAL_{(S)}$ were administered i.p 30 minutes before each challenge. AAD was associated with increases in total leukocytes, eosinophils, neutrophils, macrophages and lymphocytes in BAL fluid (BALF, OVA+vehicle) compared to non-AAD (Saline+vehicle) controls (Figs. 1B and C, Figs. S1A-C). Treatment of mice during AAD with FTY720 (OVA+FTY720) or $AAL_{(S)}$ (OVA+$AAL_{(S)}$) had no significant effects on BALF leukocytes compared to vehicle-treated AAD controls. There were increases in airway eosinophils and total cellular inflammation in lung tissues, MSC numbers around the airways and mucin 5AC (Muc5AC) mRNA expression in lung homogenates in mice with AAD compared to non-AAD controls (Figs. 1D-G). FTY720 treatment reduced cellular inflammation but not tissue eosinophils, MSC numbers or Muc5AC mRNA expression compared to vehicle-treated controls. $AAL_{(S)}$ treatment reduced tissue eosinophils, cellular inflammation and MSC numbers, which was associated with reduced Muc5AC mRNA expression. $AAL_{(S)}$ treatment also significantly reduced airway eosinophils and cellular inflammation in lung tissues as well as Muc5AC mRNA expression compared to FTY720 treatment (Figs 1D, E and G).

$AAL_{(S)}$ but not FTY720 suppressed OVA-induced type-2 associated airway and tissue cytokine levels and AHR

Next, the effects of FTY720 and $AAL_{(S)}$ treatment on type-2 associated cytokines, IgE and AHR were assessed. There were increased levels of IL-33 and TSLP in lung homogenates, IL-5 and IL-13 in BAL supernatants, IgE in serum and AHR, characterised by exaggerated transpulmonary resistance in response to increasing doses of methacholine in mice with AAD compared to non-AAD controls (Figs. 2A-F). Treatment of mice during AAD with $AAL_{(S)}$, but not FTY720 reduced the levels of all cytokines back to vehicle-treated AAD control levels. There were non-significant trends to reduced cytokine levels with FTY720 treatment. Both FTY720 and $AAL_{(S)}$ reduced IgE levels and AHR. $AAL_{(S)}$ treatment significantly reduced levels of IL-33 in the lung and AHR compared to FTY720 treatment (Figs 2A and F).

Treatment with BORT reduced OVA-induced airway and tissue inflammation and Muc5AC mRNA expression in the lung

Next, the effects of the proteasome inhibitor, BORT, on acute AAD were assessed (Fig. 3A). Treatment of mice during AAD with BORT (OVA+BORT) reduced total leukocytes, eosinophils, neutrophils, macrophages and lymphocytes in BALF compared to vehicle-treated AAD controls (Figs. 3b and c, Figs. S2A-C). There were also reduced eosinophils in lung tissue (Fig. 3D). Treatment did not affect tissue inflammation or MSC numbers, but reduced Muc5AC mRNA expression in lung homogenates (Figs. 3E-G).

Treatment with BORT reduced OVA-induced airway IL-5 and IL-13 levels and suppressed AHR

Treatment of mice during AAD with BORT did not significantly affect the levels of IL-33 and TSLP (trend to a decrease) in lung homogenates compared to vehicle-treated AAD controls (Figs. 4A and B). However, treatment did reduce the levels of IL-5 and IL-13 in BAL supernatants (Figs. 4C and D). Treatment had no effect on serum IgE but did decrease AHR (Figs. 4E and F).

Combined treatment with $AAL_{(S)}$+BORT had complementary effects and reduced OVA-induced eosinophilic pulmonary inflammation, MSC numbers and Muc5AC expression

Our data show that $AAL_{(S)}$ treatment had beneficial effects on several features of AAD including tissue eosinophils and inflammation, MSC numbers, Muc5AC expression, levels of type-2 associated cytokines, IgE production and AHR. FTY720 had lesser effects. BORT had complementary effects and suppressed the influx of inflammatory cells into the airways, tissue eosinophils, Muc5AC expression, IL-5 and IL-13 production and AHR (Table 1). This suggests that combined treatment with the PP2A activator $AAL_{(S)}$ and proteasome inhibitor BORT have complementary effects. Thus, we next assessed the effects of combining $AAL_{(S)}$+BORT into one treatment for acute AAD. Treatment of mice during AAD (OVA+$AAL_{(S)}$+BORT) had no effects on total leukocytes, neutrophils, macrophages, or lymphocytes in BALF compared to vehicle-treated AAD controls (Fig. 5B, Figs. S3A-C)**.** However, and critically, the combined treatment reduced eosinophils in both BALF and lung tissue, tissue inflammation, MSC numbers and Muc5AC mRNA expression (Figs. 5C-G).

Combined treatment with $AAL_{(S)}$+BORT had complementary effects and inhibited OVA-induced type-2 associated cytokines and AHR

Consistent with the reduction in allergic inflammation and mucus responses (Figs. 5C-F), treatment of mice during AAD with $AAL_{(S)}$+BORT significantly reduced levels of IL-33 and TSLP in lung homogenates and IL-5 and IL-13 in BAL supernatants compared to vehicle-treated AAD controls (Figs. 6A-D). Cytokine production was completely inhibited, with levels suppressed to those in vehicle-treated non-AAD controls. Combined treatment did not significantly reduce serum IgE, but inhibited AHR back to non-AAD levels (Figs. 6E and F).

Treatment with $AAL_{(S)}$, BORT and $AAL_{(S)}$+BORT reduced airway remodelling in chronic AAD

Acute OVA-induced AAD does not involve chronic features of asthma such as airway remodelling. Thus, the effects of $AAL_{(S)}$ and BORT alone or combined on airway remodelling were assessed by chronically exposing mice to HDM (Fig. 7A). Chronic exposure (HDM+vehicle) increased pulmonary inflammation, MSC numbers and AHR compared to vehicle-treated non-AAD controls (Saline+vehicle) (Figs. S4A-G). Treatment with $AAL_{(S)}$, BORT or $AAL_{(S)}$+BORT throughout HDM exposure again variously suppressed these features. Chronic HDM exposure also induced airway remodelling with increased collagen deposition around the airways (Fig. 7B). Treatment significantly reduced collagen deposition. This was associated with reductions in TGF-β mRNA expression (Fig. 7C).

Discussion

We assessed the effects of enhancing PP2A activity and inhibiting proteasome activity, either alone or in combination, as potential therapies for AAD. Enhancing PP2A activity with $AAL_{(S)}$ suppressed tissue eosinophils and inflammation, MSC numbers and Muc5AC expression, type-2 cytokines in the lungs, IgE levels in serum and AHR, in acute OVA-induced AAD. FTY720 only significantly suppressed tissue inflammation and IgE levels. Inhibiting proteasome activity with BORT reduced eosinophils in the airways and lung tissue, airway type-2 (IL-5, IL-13) levels and AHR. Importantly, we show for the first time that combined treatment with $AAL_{(S)}$ and BORT had complementary effects and was superior to either treatment alone, reducing eosinophil levels in the airways and lung tissue, tissue inflammation, MSC numbers and Muc5AC expression, type-2 cytokines and AHR. Notably, increases in cytokine levels and AHR were completely inhibited. Furthermore, $AAL_{(S)}$ and BORT alone or combined suppressed airway remodelling in chronic HDM-induced AAD. This combination, therefore, has potential as an effective asthma therapy.

PP2A is the most abundant serine/threonine phosphatase in mammals (6), and numerous studies show its activity is reduced in asthma (9, 28-30). PP2A activity was impaired in airway smooth muscle cells of asthmatics compared to non-asthmatics (28), in peripheral blood mononuclear cells from severe asthma patients (9), and in animal models of steroid-resistant AHR (29). These studies highlight the therapeutic potential of enhancing PP2A activity in asthma. Whilst some studies have reported the use of the two common PP2A activators, FTY720 and $AAL_{(S)}$, in murine AAD (7, 10), which one is more effective was unknown.

Our study, for the first time, compared the effects of $AAL_{(S)}$ and FTY720 on AAD. $AAL_{(S)}$ was more effective than FTY720. In acute OVA-induced AAD, $AAL_{(S)}$ treatment inhibited eosinophil influx into the lung, but not the airways, and tissue inflammation and to a greater extent than FTY720. Its effects were associated with reduced levels of innate (IL-33, TSLP) and adaptive (IL-5, IL-13) type-2 cytokines. Treatment with $AAL_{(S)}$, but not FTY720, reduced MSC numbers, which was associated with reduced levels of IL-13 and Muc5AC mRNA expression in the lungs. Both treatments reduced serum IgE levels. AHR was significantly reduced with $AAL_{(S)}$, but not FTY720, which was associated with reduced IL-13 levels. Others also showed that $AAL_{(S)}$ treatment reduced tissue inflammation, type-2

cytokines (IL-33, IL-5, IL-13) and AHR in acute HDM-induced AAD (7), and reduced eosinophilic airway inflammation and AHR in rhinovirus-induced exacerbation of acute AAD (8).

In contrast to our findings, others showed that oral treatment with FTY720 decreased airway inflammation, MSC numbers and AHR in T-cell transfer- and OVA-induced AAD (31). This was postulated to be due to the sequestration of T cells in lymphoid tissues. Another study showed that intratracheal administration of FTY720 during OVA-induced AAD reduced airway inflammation and type-2 cytokines (IL-5, IL-13) by altering the function of lung dendritic cells (10). The differences in our compared to other studies could be partly due to the off-target effects of FTY720, different routes of administration or mouse strains used. Since FTY720 is phosphorylated *in vivo* by sphingosine kinases to become FTY720-P and also binds to sphingosine 1-phosphate receptors (SIPR1) to cause lymphocyte trafficking, it does not specifically activate PP2A (32). In contrast, $AAL_{(S)}$ does not bind to SIPR1, and more specifically increases PP2A activity. In our study, FTY720 was administered systemically (i.p) compared to oral or intratracheal administration used by others, which may have resulted in greater metabolism to FTY720-P. Furthermore, others used BALB/c mice that are more susceptible to developing Th2-driven AAD, while we used C57BL/6 mice that can be considered to have more balanced immunity. By using C57BL/6 mice that are less susceptible to developing AAD, our data adds additional impact and demonstrates that the protective effects of targeting these pathways is not restricted only to susceptible strains. It also provides a platform for further mechanistic studies using factor deficient or transgenic mice that are typically generated on a C57BL/6 background (e. g. in mice with altered TTP activity) (33).

The effects of $AAL_{(S)}$ on airway remodelling were also determined, and treatment suppressed chronic HDM-induced collagen deposition around the airways. This was associated with reduced TGF-β mRNA expression in the lungs. Others also showed that $AAL_{(S)}$ treatment reduced collagen deposition around the airways of mice chronically exposed to OVA (34). Our study used HDM, which is a clinically relevant allergen, and involves sensitisation solely *via* the airways instead of systemic sensitisation in the presence of an adjuvant used in OVA models (35). This is consistent with allergen exposure in humans and results in local, instead of systemic immune responses.

The UPS has been studied extensively, however, few have focussed on asthma (7, 36). In the lungs, the E3 ubiquitin ligase, Itch, is involved in maintaining tolerance by inducing anergy in Th2 cells (15). Other E3 ubiquitin ligases such as gene related to anergy in lymphocytes (GRAIL) are implicated

256 in inducing T cell tolerance by targeting Th2 transcription factors for degradation (36). Recently, MID1
257 was shown to be upregulated in pBECs from human asthma patients and in HDM-exposed mice (7).
258 Proteasome inhibition is emerging as a potential therapy in many diseases particularly cancer,
259 whereas studies in asthma are only commencing. Treatment with the inhibitor PS-519 in OVA-
260 induced pulmonary eosinophilia in rats significantly reduced eosinophil influx into the lungs (37). Only
261 one other study used BORT, which was tested in a chronic OVA mouse model. Long-term treatment
262 with high doses reduced OVA-specific IgE, but not airway inflammation or AHR (38). However, the
263 effects of treatment in acute models, with HDM or on features of airway remodelling have not been
264 assessed previously.

265 We assessed the effects of BORT on both acute and chronic models of AAD. Treatment
266 suppressed some features of acute AAD, including eosinophil infiltration into the airways and lung
267 tissue, which was associated with reduced levels of IL-5 and IL-13 in BAL supernatants. It also
268 attenuated AHR, which was consistent with reduced levels of IL-13. However, treatment did not alter
269 tissue inflammation, MSC numbers, levels of innate type-2 cytokines (IL-33 and TSLP) or IgE.
270 Importantly, it did prevent airway remodelling in chronic AAD, which was associated with reduced
271 TGF-β mRNA expression in the lung. Discrepancies between our and previous findings may be
272 attributed to the doses of BORT used (38). We used a moderate dose (0.2mg/kg) while a higher dose
273 of BORT (0.75mg/kg) was used previously. Others demonstrated that the attenuation of experimental
274 colitis in mice by BORT treatment was dose dependent (39). A low dose (0.1mg/kg) reduced
275 inflammation but did not affect cytokine or chemokine production, intermediate doses (0.2 and
276 0.35mg/kg) attenuated colitis while a higher dose (0.5mg/kg) caused mortality. Notably, BORT is a
277 non-selective proteasome inhibitor, hence it is possible that the general reduction of proteasome
278 activity is not optimal in suppressing all features of AAD, and specific inhibition may have greater
279 effects.

280 Several studies highlight associations between PP2A and the UPS. Inhibition of PP2A
281 augmented the proteolytic function of murine cardiac proteasomes (40). Others identified the
282 mediation of PP2A/C ubiqutination and degradation by the E3 ligase Cullin-3 (41), and in HDM-
283 induced AAD, MID1 protein decreases PP2A activity (7). Given that both PP2A and the UPS are
284 implicated in asthma pathogenesis, and that $AAL_{(S)}$ and BORT inhibit different features of AAD, the
285 complementary effects of enhancing PP2A activity and inhibiting proteasome activity concurrently

were determined. This has not been assessed previously. Treatment with $AAL_{(S)}$+BORT had complementary effects and suppressed the major hallmark features of AAD including eosinophil infiltration into the airways and lungs, tissue inflammation, MSC numbers, type-2 associated cytokines, AHR and collagen deposition. Notably type-2 cytokine production and AHR were completely inhibited. Interestingly, combined treatment reduced the levels of eosinophils in the airways, which was not observed with $AAL_{(S)}$ treatment alone. This is important since anti-IL-5 treatment in humans is only effective when there is major suppression of eosinophil levels (42, 43). The effects of combined treatment were stronger in the acute compared to the chronic model. In the chronic model, there were no additional beneficial effects of combined compared to individual treatments. Nevertheless, our data consistently show that combination treatment suppresses both acute and chronic features of asthma across different models. Ours is the first study to show the complementary effects of enhancing PP2A activity and inhibiting proteasome activity at the same time on both acute and chronic features of AAD.

The exact mechanisms of how these immunomodulatory drugs suppress AAD remains to be fully elucidated. A recent study showed that PP2A activators may promote increases in anti-inflammatory tristetraprolin (TTP) activity (30). The TTP protein exists in two forms, the phosphorylated form, which is inactive, and the unphosphorylated form, which is active and induces mRNA decay. The major targets of TTP are the mRNA transcripts of cytokines. Thus, when TTP is phosphorylated cytokine expression occurs but when TTP is unphosphorylated the production of target cytokines is inhibited. Unphosphorylated TTP is less stable and is degraded by the UPS (44, 45). PP2A is able to mediate the dephosphorylation of TTP protein, leading to an increase in the active unphosphorylated form and the mRNA decay of cytokines (46). Inhibition of PP2A with okadaic acid or siRNA leads to increased phosphorylation of TTP, thereby increasing the stability of cytokines, such as tumour necrosis alpha (TNFα) mRNA in mouse alveolar macrophage cell lines (46). Recently, it was demonstrated that $AAL_{(S)}$ suppressed the levels of TNFα-induced interleukin IL-8 and IL-6 in A549 lung epithelial cells (47). This was postulated to be due to the ability of $AAL_{(S)}$ to shift the equilibrium towards active TTP. As the active form of TTP is unstable and prone to degradation by the UPS (45), preventing the degradation of unphosphorylated TTP by BORT would favour the degradation of pro-inflammatory cytokine mRNA. Treatment of RAW 264.7 with MG-132, an inhibitor of the 20S/26S proteasome increased TTP protein levels also by preventing its degradation (44).

These findings suggest that enhancing PP2A activity and inhibiting proteasome activity, as we have done in this study, could potentially increase the active and stable form of TTP, hence leading to reduced levels of pro-inflammatory cytokines and the suppression of AAD.

Another possible mechanism is through the inhibition of NF-κB activity, which regulates the expression of many cytokines (48). PP2A is a crucial regulator of NF-κB (49). Its inhibition increases the activity of inhibitor of κB (IκB) kinaseβ (IKKβ), which subsequently leads to the proteasomal degradation of IκBα, allowing NF-κB to translocate into the nucleus to activate responsive genes (49). The UPS also controls NF-κB activity through IκB degradation (50). The inhibition of tumour growth in human T-cell lymphoma cells by BORT may be due to nuclear translocation of IκB and the inactivation of NF-κB (51). The mechanisms of action of these drugs clearly need further study.

While we did not directly confirm the activity of the drugs ($AAL_{(S)}$ and BORT) against their targets (PP2A and UPS) at the doses provided, several studies have confirmed this. PP2A activity was shown to be increased in mice with AAD after treatment with $AAL_{(S)}$ (7). BORT is a known proteasome inhibitor that has been approved for use in multiple myeloma, and it inhibits proteasomal activity by up to 70% in whole blood samples (52). Similarly, proteasome activity was inhibited both *in vitro* and *in vivo* (53, 54). Notably, the doses used in these studies were lower than the dose used in our study, indicating that we would also observe the desired effects on the target pathways. Importantly, we did not observe any adverse effects on the mice at the doses used in our study.

In summary, we demonstrate that enhancing PP2A activity and inhibiting proteasome activity, either alone or in combination has beneficial effects in acute and chronic AAD. Enhancing PP2A activity with $AAL_{(S)}$ more effectively suppressed hallmark features of AAD than FTY720, while inhibiting proteasome activity with BORT had some beneficial effects. Combining $AAL_{(S)}$ and BORT had complementary effects and was more effective compared to any treatment alone. Our findings highlight the importance of PP2A and the UPS in AAD and suggest that their complementary targeting may have therapeutic potential in asthma.

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Author's contribution

PMN, MRS, ARC, AJA and PMH participated in the conception and design of the study. PMN performed all the experiments, analysis and wrote the draft of the manuscript. MRS, TJH, GL and JCH assisted with mouse experiments. JCM and NMV provided the $AAL_{(S)}$. All authors participated in the interpretation of data and editing of the manuscript for intellectual content. All authors read and approved the final manuscript.

Conflict of interest

PMH reports grants from National Health and Medical Research Council of Australia, during the conduct of the study; funding/consultancies from Pharmaxis, AstraZeneca, Sanofi, Pharmakea, Ausbio, and Allakos outside the submitted work; NMV reports grants from Cancer Institute NSW during the conduct of the study. All other authors declared no conflict of interest.

Figure 1 $AAL_{(S)}$ but not FTY720 suppressed tissue inflammation and mucus secreting cell (MSC) numbers in ovalbumin (OVA)-induced acute allergic airway disease (AAD). (A) AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. FTY720 or $AAL_{(S)}$ were administered i.p 30 minutes before each OVA challenge. Controls were vehicle-treated. Features of AAD were assessed 24 hours after the final OVA challenge. (B) Total leukocytes and (C) eosinophils in bronchoalveolar lavage fluid (BALF). (D) Numbers of airway tissue eosinophils per 100µm basement membrane (BM) in Lendrum's Carbolchromotrope stained lung sections. (E) Histopathology score in haematoxylin and eosin stained lung sections. Scale bars= 200µm. (F) MSC numbers per 100µm BM in periodic acid-Schiff stained lung sections. Scale bars= 50µm. (G) Mucin 5AC (Muc5AC) mRNA expression in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-AAD vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to AAD vehicle-treated (OVA+vehicle) controls, $^{+}$ represents $P \leq 0.05$ compared to AAD FTY720-treated (OVA+FTY720) mice.

Figure 2 $AAL_{(S)}$ but not FTY720 suppressed type-2 associated airway and tissue cytokines levels and airway hyper-responsiveness (AHR) in OVA-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. FTY720 or $AAL_{(S)}$ were administered i.p 30 minutes before each OVA challenge. Controls were vehicle-treated. Features of AAD were assessed 24 hours after the final OVA challenge. (A) Interleukin (IL)-33 and (B) thymic stromal lymphopoietin (TSLP) in lung homogenates. (C) IL-5 and (D) IL-13 in bronchoalveolar lavage supernatants. (E) Total immunoglobulin (Ig)E in serum. (F) AHR in terms of transpulmonary resistance in response to increasing doses of methacholine (left) and at the maximal dose of methacholine (50mg/ml; right). Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-AAD vehicle-treated (Saline+vehicle) controls, $^{\#}$ represents $P \leq 0.05$ compared to AAD vehicle-treated (OVA+vehicle) controls, $^{+}$ represents $P \leq 0.05$ compared to AAD FTY720-treated (OVA+FTY720) mice.

Figure 3 Treatment with BORT suppressed eosinophil influx into the airways and lung tissues as well as mucin 5AC (Muc5AC) expression in ovalbumin (OVA)-induced acute allergic airway disease (AAD). (A) AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA

challenges. Controls were sham-sensitised and challenged with OVA. BORT was administered i.p 30 minutes before each OVA challenge. Controls were vehicle-treated. Features of AAD were assessed 24 hours after the final OVA challenge. (B) Total leukocytes and (C) eosinophils in bronchoalveolar lavage fluid (BALF). (D) Numbers of airway tissue eosinophils per 100μm basement membrane (BM) in Lendrum's Carbolchromotrope stained lung sections. (E) Histopathology score in haematoxylin and eosin stained lung sections. Scale bars= 200μm. (F) Mucus secreting cell numbers per 100μm BM in periodic acid-Schiff stained lung sections. Scale bars= 50μm. (G) Muc5AC mRNA expression in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-AAD vehicle-treated (Saline+vehicle) controls, # represents $P \leq 0.05$ compared to AAD vehicle-treated (OVA+vehicle) controls.

Figure 4 Treatment with BORT reduced airway interleukin (IL)-5 and IL-13 levels in the lungs and airway hyper-responsiveness (AHR) in ovalbumin (OVA)-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. Vehicle or BORT were administered i.p 30 minutes before each OVA challenge. Features of AAD were assessed 24 hours after the final OVA challenge. (A) IL-33 and (B) thymic stromal lymphopoietin (TSLP) in lung homogenates. (C) IL-5 and (D) IL-13 in bronchoalveolar lavage supernatants. (E) Total immunoglobulin (Ig)E in serum. (F) AHR in terms of transpulmonary resistance in response to increasing doses of methacholine (left) and at the maximal dose of methacholine (50mg/ml; right). Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-allergic vehicle-treated (Saline+vehicle) controls, # represents $P \leq 0.05$ compared to allergic vehicle-treated (OVA+vehicle) controls.

Figure 5 Combined treatment with $AAL_{(S)}$+BORT had complementary effects and reduced eosinophilic pulmonary inflammation, mucus secreting cell (MSC) numbers and mucin 5AC (Muc5AC) expression in ovalbumin (OVA)-induced acute allergic airway disease (AAD). (A) AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. $AAL_{(S)}$+BORT were administered i.p 30 minutes before each OVA challenge. Controls were vehicle-treated. Features of AAD were assessed 24 hours after the final OVA challenge. (B) Total leukocytes and (C) eosinophils in bronchoalveolar lavage fluid (BALF). (D)

Numbers of airway tissue eosinophils per 100µm basement membrane (BM) in Lendrum's Carbolchromotrope stained lung sections. (E) Histopathology score in haematoxylin and eosin stained lung sections. Scale bars= 200µm. (F) MSC numbers per 100µm BM in periodic acid-Schiff stained lung sections. Scale bars= 50µm. (G) Muc5AC mRNA expression in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. Data from controls (Saline+vehicle and OVA+vehicle) are recapitulated from Figure 3 To facilitate comparisons of data to single treatments, data from OVA+$AAL_{(S)}$ groups are recapitulated from figure 1, and data from OVA+BORT groups are recapitulated from figure 3. * represents $P \leq 0.05$ compared to non-AAD vehicle-treated (Saline+vehicle) controls, # represents $P \leq 0.05$ compared to AAD vehicle-treated (OVA+vehicle) controls.

Figure 6 Combined treatment with $AAL_{(S)}$+BORT had complementary effects and inhibited type-2 associated cytokines and airway hyper-responsiveness (AHR) in ovalbumin (OVA)-induced acute allergic airway disease (AAD). AAD was induced by intraperitoneal (i.p) OVA sensitisation and intranasal OVA challenges. Controls were sham-sensitised and challenged with OVA. $AAL_{(S)}$+BORT were administered i.p 30 minutes before each OVA challenge. Controls were vehicle-treated. Features of AAD were assessed 24 hours after the final OVA challenge. (A) Interleukin (IL)-33 and (B) thymic stromal lymphopoietin (TSLP) in lung homogenates. (C) IL-5 and (D) IL-13 in bronchoalveolar lavage supernatants. (E) Total immunoglobulin (Ig)E in serum. (F) AHR in terms of transpulmonary resistance in response to increasing doses of methacholine (left) and at the maximal dose of methacholine (50mg/ml; right). Data (n=6-8) are presented as means ± s.e.m. Control mice (Saline+vehicle and OVA+vehicle) is recapitulated from Figure 4. To facilitate comparisons of data to single treatments, data from OVA+$AAL_{(S)}$ groups are recapitulated from figure 2, and data from OVA+BORT groups is recapitulated from figure 4. *represents $P \leq 0.05$ compared to non-AAD vehicle-treated (Saline+vehicle) controls, # represents $P \leq 0.05$ compared to AAD vehicle-treated (OVA+vehicle) controls.

Figure 7 Treatment with $AAL_{(S)}$, BORT and $AAL_{(S)}$+BORT reduced airway remodelling in house dust mite (HDM)-induced chronic allergic airway disease (AAD). (A) Chronic AAD was induced by administration of HDM intranasally five times a week for five weeks. $AAL_{(S)}$, BORT or $AAL_{(S)}$+BORT

were administered intraperitoneally daily. Controls were vehicle-treated. Airway remodelling in terms of collagen deposition around the airways were assessed after 5 weeks. (B) Area of collagen deposition (μm^2) per basement membrane perimeter in Masson's Trichrome stained lung sections. Scale bars= 50μm. (C) Transforming growth factor (TGF)-β mRNA expression in lung homogenates. Data (n=6-8) are presented as means ± s.e.m. * represents $P \leq 0.05$ compared to non-AAD vehicle-treated (Saline+vehicle) controls, # represents $P \leq 0.05$ compared to AAD vehicle-treated (HDM+vehicle) controls.

Table 1 Summary of the effects of different treatments on features of acute AAD

	FTY720	$AAL_{(S)}$	BORT	$AAL_{(S)}$+BORT
	Fig. 1		Fig. 3	Fig. 5
All BALF cells	-	-	↓	↓ (eosinophils)
Tissue eosinophils	-	↓	↓	↓
Tissue inflammation	↓	↓	-	↓
MSCs	-	↓	-	↓
Muc5AC mRNA	-	↓	↓	↓
	Fig. 2		Fig. 4	Fig. 6
IL-33, TSLP	-	↓↓	-	↓↓
IL-5, IL-13	-	↓	↓↓	↓↓
IgE	↓	↓	-	-
AHR	-	↓	↓	↓↓

- represents no effect, ↓ represents reduced, ↓↓ represents reduced to baseline, bronchoalveolar lavage fluid (BALF), mucus secreting cells (MSCs), mucin 5AC (Muc5AC), interleukin (IL), thymic stromal lymphopoietin (TSLP), immunoglobulin (Ig), airway hyper-responsiveness (AHR)

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Targeting PP2A and proteasome activity ameliorates features of allergic airway disease in mice

Main Figures

Figure 1

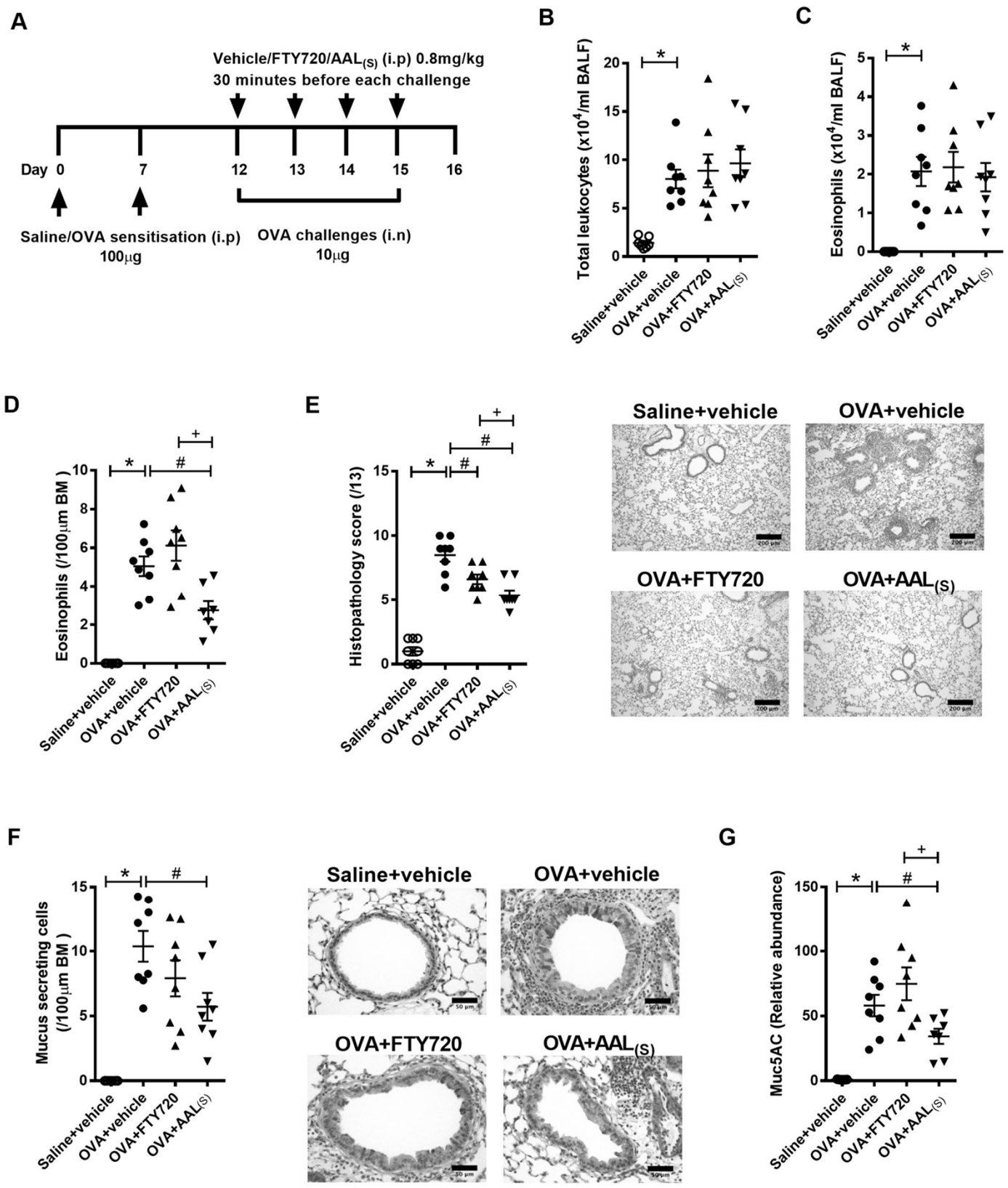


Figure 2

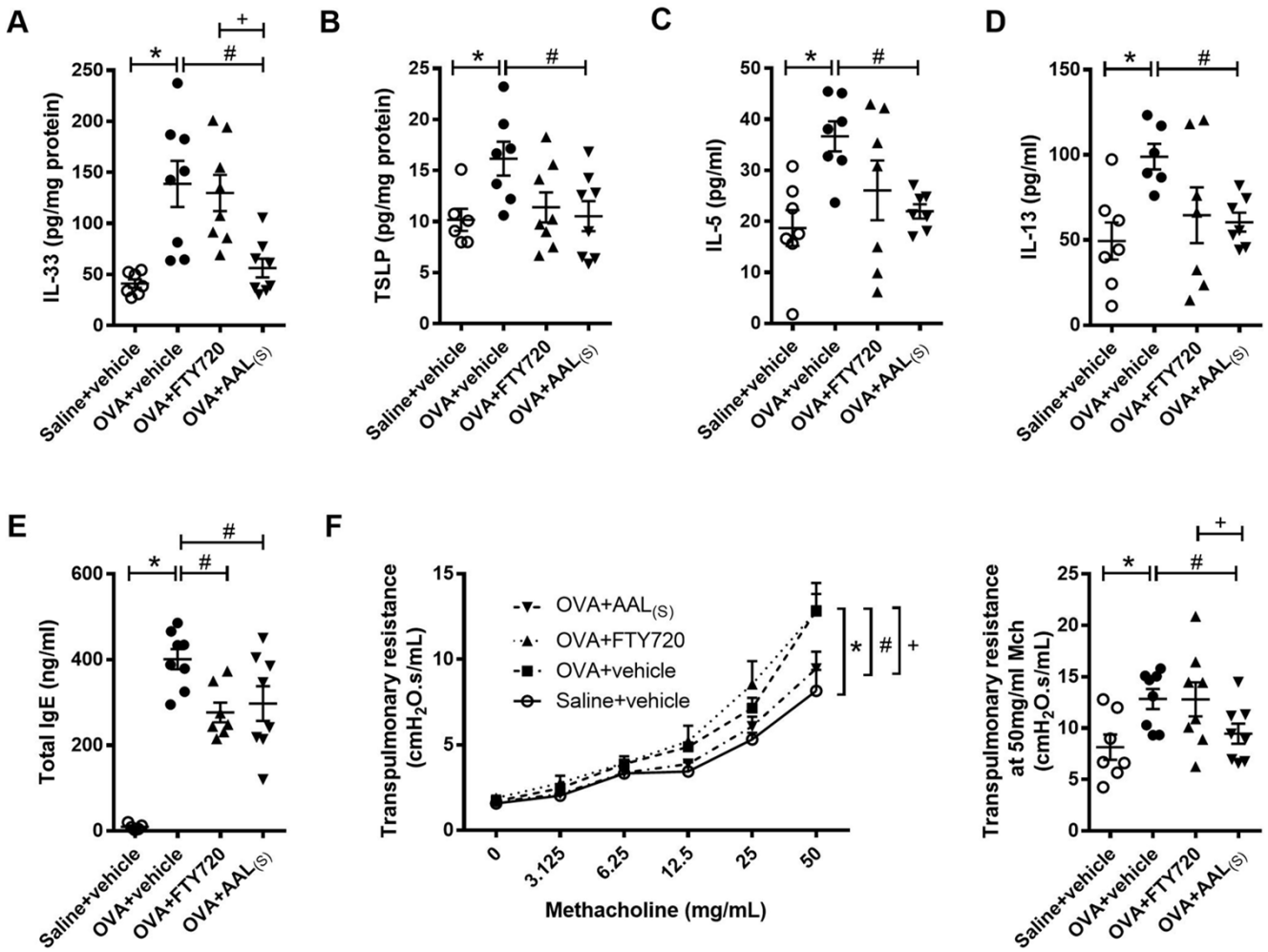

A
IL-33 (pg/mg protein)
B
TSLP (pg/mg protein)
C
IL-5 (pg/ml)
D
IL-13 (pg/ml)
E
Total IgE (ng/ml)
F
Transpulmonary resistance (cmH2O.s/mL)
OVA+AAL(S)
OVA+FTY720
OVA+vehicle
Saline+vehicle
Methacholine (mg/mL)
Transpulmonary resistance at 50mg/ml Mch (cmH2O.s/mL)
Saline+vehicle
OVA+vehicle
OVA+FTY720
OVA+AAL(S)

Figure 3

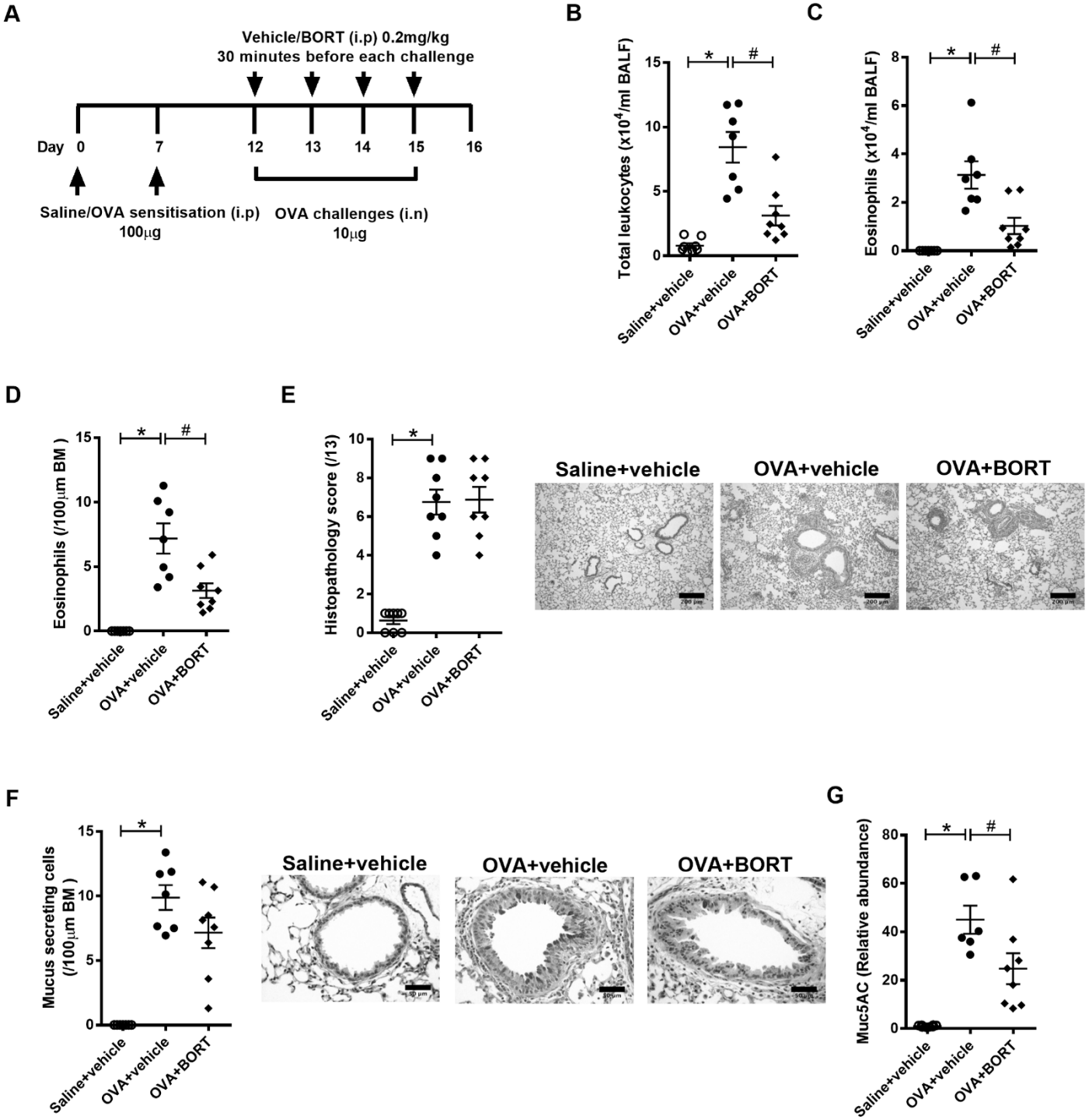

A
Vehicle/BORT (i.p) 0.2mg/kg
30 minutes before each challenge
Day 0 7 12 13 14 15 16
Saline/OVA sensitisation (i.p) 100μg
OVA challenges (i.n) 10μg
B
Total leukocytes (x10⁴/ml BALF)
C
Eosinophils (x10⁴/ml BALF)
Saline+vehicle
OVA+vehicle
OVA+BORT
D
Eosinophils (/100μm BM)
E
Histopathology score (/13)
Saline+vehicle
OVA+vehicle
OVA+BORT
F
Mucus secreting cells (/100μm BM)
Saline+vehicle
OVA+vehicle
OVA+BORT
G
Muc5AC (Relative abundance)
Saline+vehicle
OVA+vehicle
OVA+BORT

Figure 4

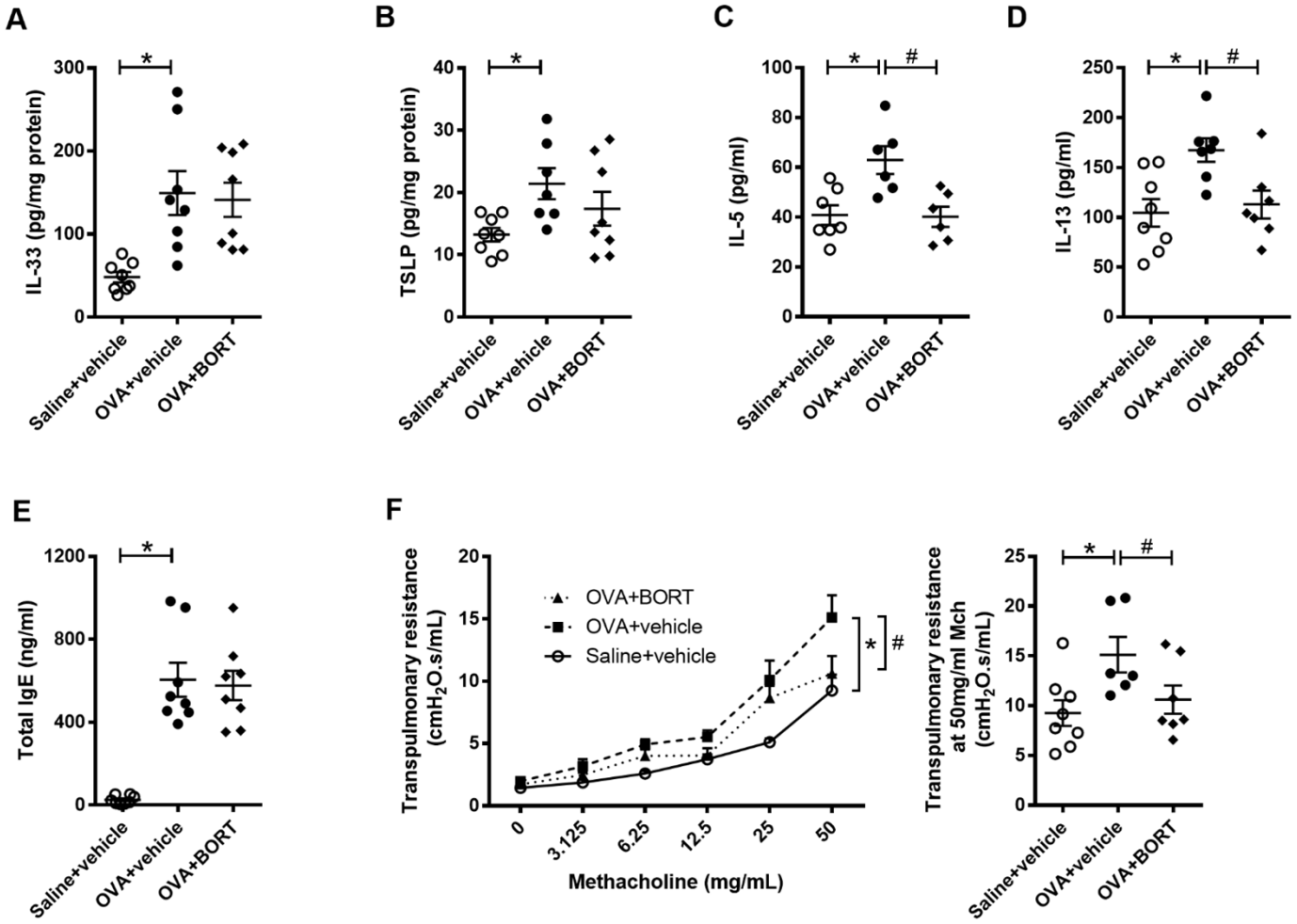

A
IL-33 (pg/mg protein)
300
200
100
0
*
Saline+vehicle
OVA+vehicle
OVA+BORT
B
TSLP (pg/mg protein)
40
30
20
10
0
*
Saline+vehicle
OVA+vehicle
OVA+BORT
C
IL-5 (pg/ml)
100
80
60
40
20
0
*
#
Saline+vehicle
OVA+vehicle
OVA+BORT
D
IL-13 (pg/ml)
250
200
150
100
50
0
*
#
Saline+vehicle
OVA+vehicle
OVA+BORT
E
Total IgE (ng/ml)
1200
800
400
0
*
Saline+vehicle
OVA+vehicle
OVA+BORT
F
Transpulmonary resistance (cmH2O.s/mL)
20
15
10
5
0
OVA+BORT
OVA+vehicle
Saline+vehicle
*
#
0
3.125
6.25
12.5
25
50
Methacholine (mg/mL)
Transpulmonary resistance at 50mg/ml Mch (cmH2O.s/mL)
25
20
15
10
5
0
*
#
Saline+vehicle
OVA+vehicle
OVA+BORT

Figure 5

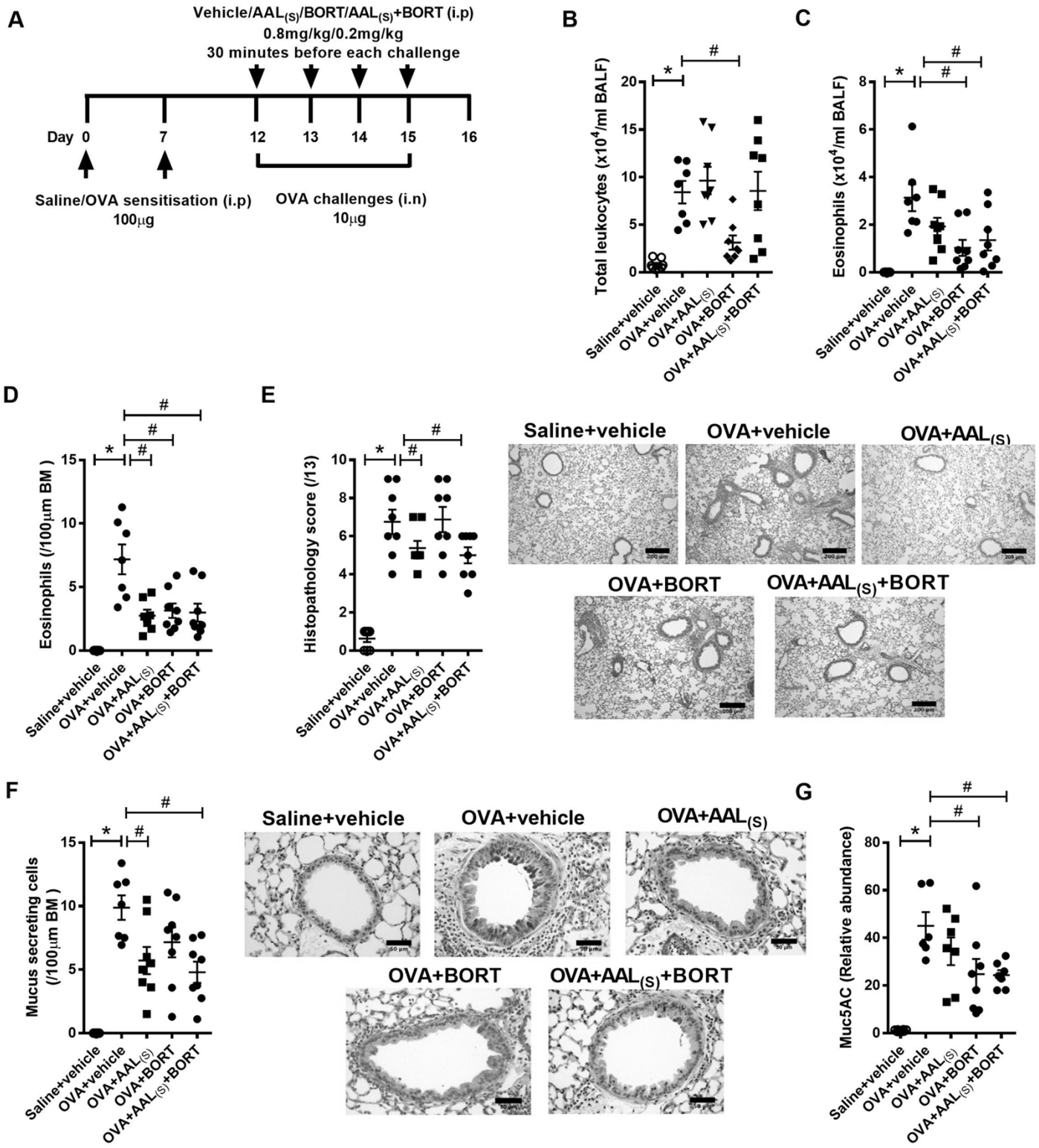

A
Vehicle/AAL(S)/BORT/AAL(S)+BORT (i.p)
0.8mg/kg/0.2mg/kg
30 minutes before each challenge
Day 0
7
12
13
14
15
16
Saline/OVA sensitisation (i.p)
100μg
OVA challenges (i.n)
10μg
B
Total leukocytes (x10^4/ml BALF)
C
Eosinophils (x10^4/ml BALF)
D
Eosinophils (/100μm BM)
E
Histopathology score (/13)
Saline+vehicle
OVA+vehicle
OVA+AAL(S)
OVA+BORT
OVA+AAL(S)+BORT
F
Mucus secreting cells (/100μm BM)
G
Muc5AC (Relative abundance)

Figure 6

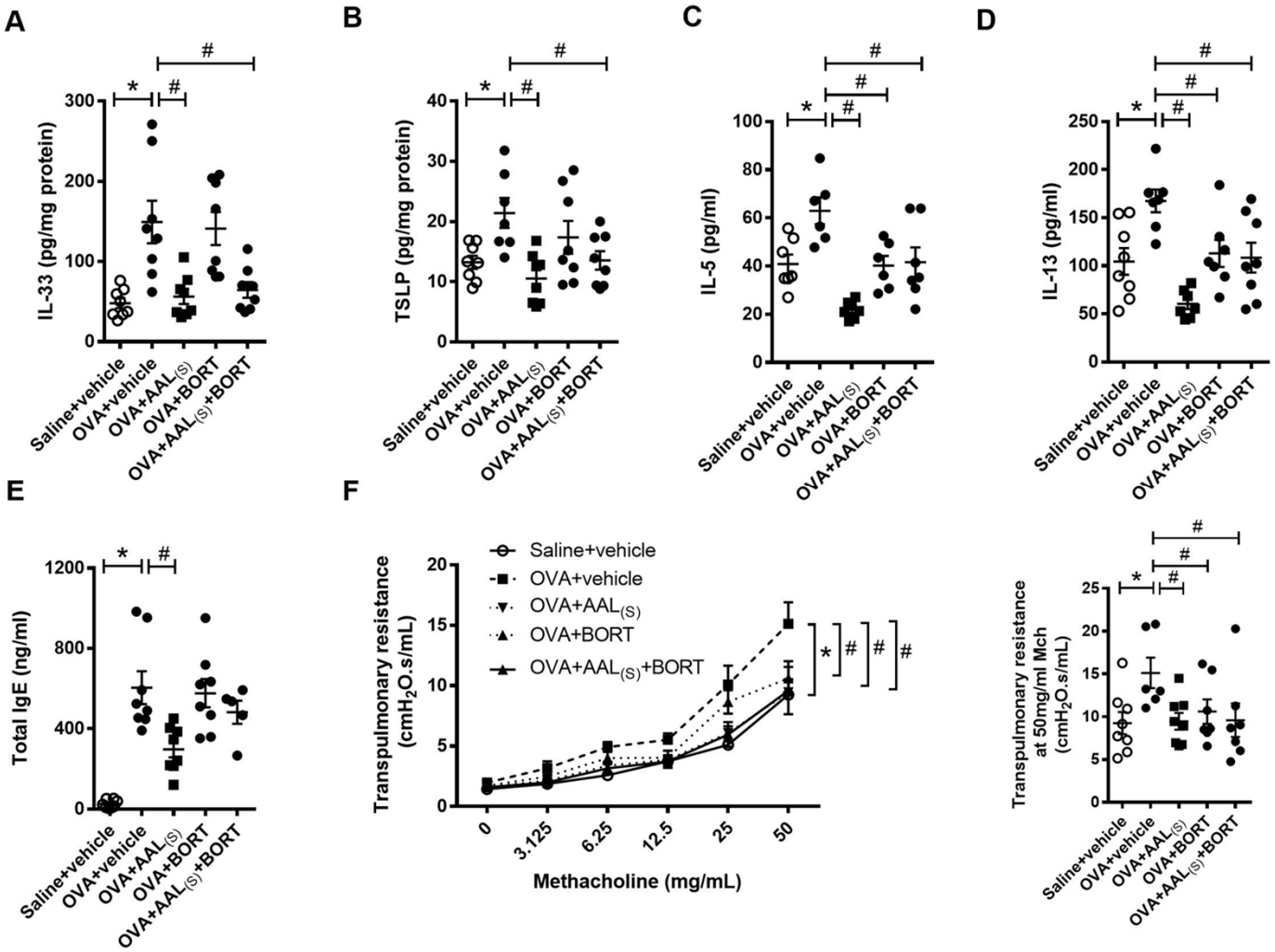
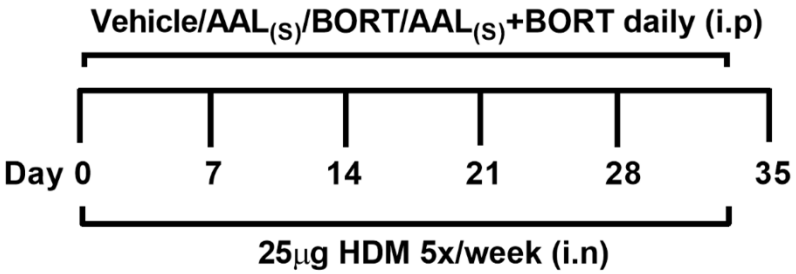
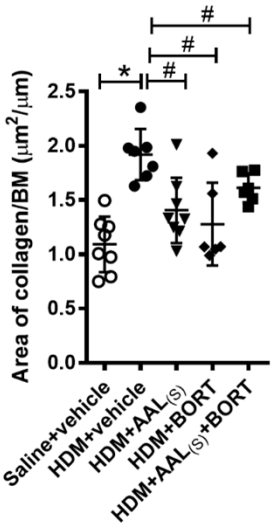

A
IL-33 (pg/mg protein)
B
TSLP (pg/mg protein)
C
IL-5 (pg/ml)
D
IL-13 (pg/ml)
E
Total IgE (ng/ml)
F
Transpulmonary resistance (cmH2O.s/mL)
Saline+vehicle
OVA+vehicle
OVA+AAL(S)
OVA+BORT
OVA+AAL(S)+BORT
Methacholine (mg/mL)
0
3.125
6.25
12.5
25
50
Transpulmonary resistance at 50mg/ml Mch (cmH2O.s/mL)

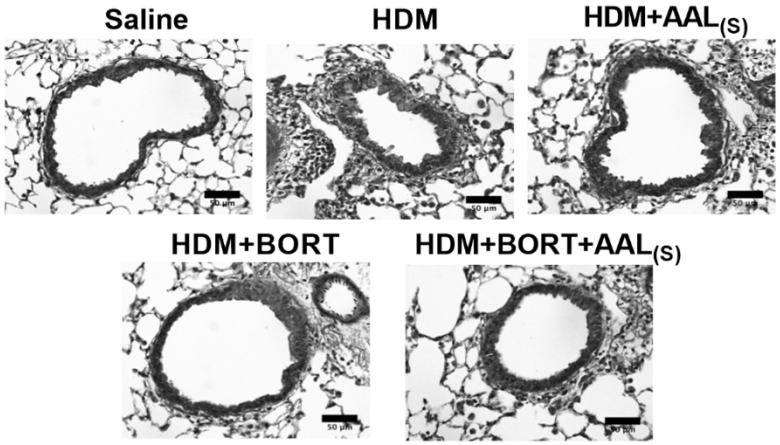
Figure 7

A

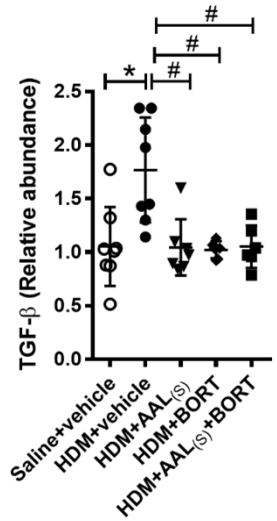

Vehicle/AAL(S)/BORT/AAL(S)+BORT daily (i.p)
Day 0
7
14
21
28
35
25μg HDM 5x/week (i.n)

B

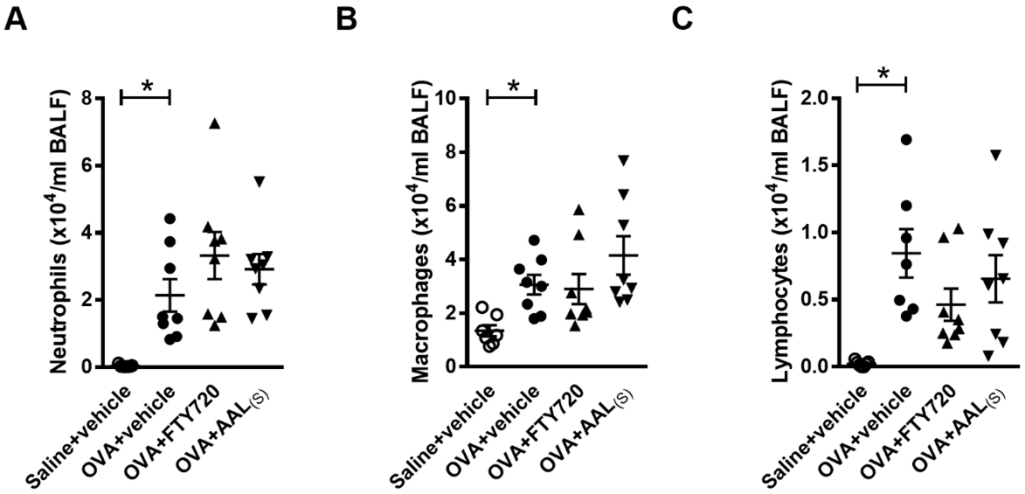

Area of collagen/BM (μm²/μm)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
#
#
Saline+vehicle
HDM+vehicle
HDM+AAL(S)
HDM+BORT
HDM+AAL(S)+BORT


Saline
HDM
HDM+AAL(S)
HDM+BORT
HDM+BORT+AAL(S)

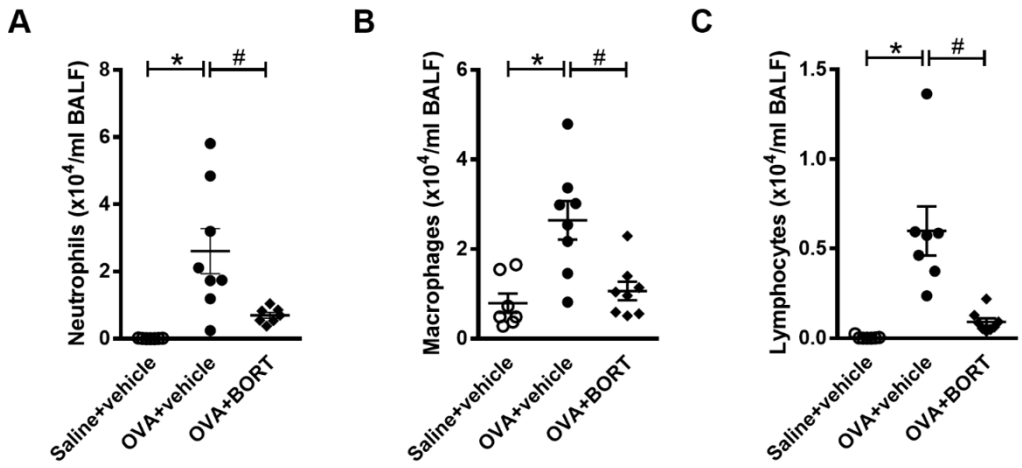
C


TGF-β (Relative abundance)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
#
#
Saline+vehicle
HDM+vehicle
HDM+AAL(S)
HDM+BORT
HDM+AAL(S)+BORT

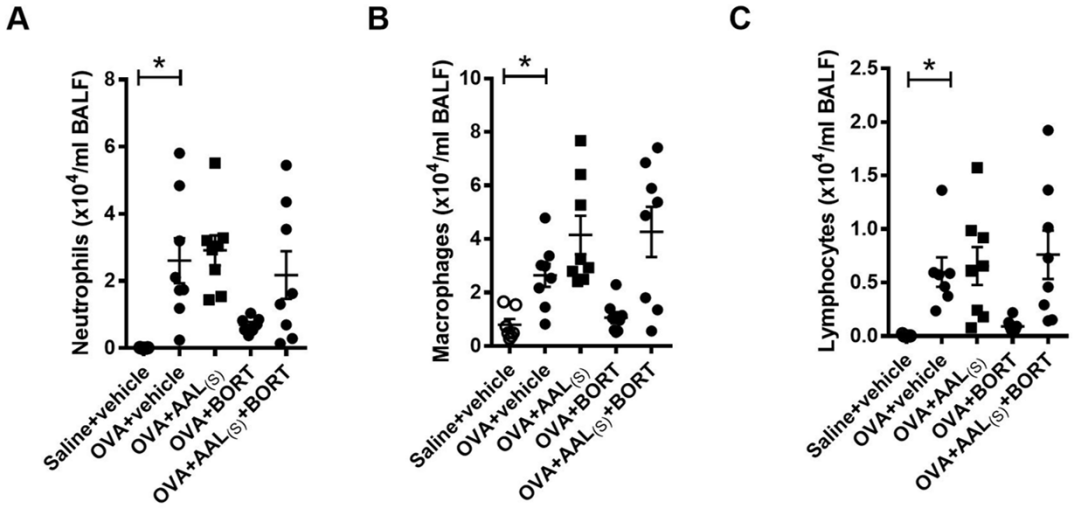
Supplementary figure 1



Supplementary figure 2



Supplementary figure 3



Supplementary figure 4