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ORIGINAL ARTICLE

Effects of the Histone Deacetylase Inhibitor, Trichostatin A, in a Chronic Allergic Airways Disease Model in Mice

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Abstract There is a need for new asthma therapies that can concurrently address airway remodeling, airway hyperresponsiveness and progressive irreversible loss of lung function, in addition to inhibiting inflammation. Histone deacetylase inhibitors (HDACi) alter gene expression by interfering with the removal of acetyl groups from histones. The HDACi trichostatin A (TSA) has pleiotropic effects targeting key pathological processes in asthma including inflammation, proliferation, angiogenesis and fibrosis. The aim was to evaluate the effects of TSA treatment in a mouse model of chronic allergic airways disease (AAD). Wild-type BALB/c mice with AAD were treated intraperitoneally with 5 mg/kg TSA or vehicle control. Airway inflammation was assessed by bronchoalveolar lavage fluid (BALF) cell counts and histological examination of lung tissue sections. Remodeling was assessed by morphometric analysis and airway hyperresponsiveness was assessed by invasive plethysmography. TSA-treated mice had a reduced number of total inflammatory cells and eosinophils within the BALF as compared to vehicle-treated mice (both $p < 0.05$). Furthermore, airway remodeling changes were significantly reduced with TSA compared to vehicle-treated mice, with fewer goblet cells ($p < 0.05$), less subepithelial collagen deposition ($p < 0.05$) and attenuated airway hyperresponsiveness at the highest methacholine dose. These findings demonstrate that treatment with an HDACi can concurrently reduce structural airway remodeling changes and airway hyperresponsiveness, in addition to attenuating airway inflammation in a chronic AAD model. This has important implications for the development of novel treatments for severe asthma.

Keywords Airway hyperresponsiveness · Airway remodeling · Asthma · Trichostatin A · Therapy

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Introduction

Asthma is characterized by airway inflammation, remodeling and hyperresponsiveness (Tang et al. 2006). Airway remodeling describes structural changes within the airway, including epithelial metaplasia, subepithelial fibrosis (deposition of extracellular matrix) components beneath the basement membrane (Belleguic et al. 2002), myofibroblast and goblet cell metaplasia, and angiogenesis (Davies and Holgate 2002). These events cause thickening of the airway wall which in turn contributes to airway hyperresponsiveness (AHR) and results in progressive irreversible loss of lung function (Tang et al. 2006).

Current asthma therapy relies primarily upon the use of anti-inflammatory agents, in particular the glucocorticosteroids. However, for a proportion of asthma patients, glucocorticosteroids are ineffective (steroid resistance) or only effective in high doses that may result in systemic side effects (Leung and Szefler 1998).

Moreover, glucocorticosteroids have limited actions against airway remodeling and AHR, which contribute to ongoing symptoms in these patients (Covar et al. 2004; Ward and Walters 2005). The identification of novel treatments that can prevent and/or reverse airway remodeling changes and inhibit AHR in addition to abrogating airway inflammation would be advantageous.

Histone deacetylase inhibitors (HDACi) modulate transcriptional activity by interfering with the removal of acetyl groups from histones and thereby alter gene expression. It is thought that they increase access to DNA transcription factors in chromatin (Sambucetti et al. 1999; Wolffe and Kurumizaka 1998). In general, HDACi have been shown to have anti-proliferation, anti-angiogenesis, anti-oxidative, anti-inflammatory and anti-fibrotic effects in cell culture experiments and in animal models of disease (Dani et al. 2008; Grabiec et al. 2010; Shankar and Srivastava 2008; Wang et al. 2009). Trichostatin A (TSA), a hydroxamic acid, is an organic antifungal antibiotic with broad-spectrum HDACi activity (Vanhaecke et al. 2004). This compound is the prototypical HDACi and has been widely investigated. It has broad-spectrum HDACi activity and it is established that it induces histone hyperacetylation and altered gene transcription in cell cultures and in vivo. Recent animal studies have revealed potent anti-inflammatory effects for TSA in mouse models of allergic airways disease (AAD). TSA treatment inhibited Th2 responses, with reduced interleukin 4 (IL-4), IL-5 and immunoglobulin E (IgE) (Choi et al. 2005) and attenuated airway inflammation, likely due to inhibition of T cell recruitment and Th2 cytokine production, in short-term models of AAD (Choi et al. 2005; Dombrowsky et al. 2009). Consistent with these anti-Th2 effects, a microarray study showed that TSA upregulated 310 genes and downregulated 313 genes, with genes for STAT5A (necessary for Th2 differentiation) and mucins (important in goblet cell metaplasia) downregulated by 80.4 and 77.3 %, respectively (Hong et al. 2009). TSA has also been shown to block transforming growth factor β (TGF-β) induced fibroblast differentiation into myofibroblasts, impair epithelial mesenchymal transition (Wang et al. 2009), and to have anti-angiogenic and anti-fibrotic effects, which together suggest likely beneficial effects against airway remodeling in asthma (Grabiec et al. 2010; Michaelis et al. 2004). TSA treatment has been shown to inhibit expression of fibrosis markers in patients with scleroderma (Hemmatazad et al. 2009), and to reduce extracellular matrix deposition (fibrosis) in a mouse bleomycin skin fibrosis model (Huber et al. 2007). The aim of this study was to evaluate the effects of the histone-modifying compound TSA in a chronic murine AAD model. In particular, we focused on characterizing the impact of TSA on various aspects of airway remodeling.

Materials and Methods

Animals

Six-week-old female BALB/c mice were housed under specific pathogen-free conditions and maintained on a fixed 12 h light, 12 h dark lighting schedule. This mouse strain demonstrates strong Th2 responses in ovalbumin (OVA)-induced AAD models (Corteling and Trifilieff 2004; Hayashi et al. 2003; Melgert et al. 2005). All experimental procedures were approved by the Murdoch Children's Research Institute Animal Ethics Committee and followed the Australian Guidelines for the Care and Use of Laboratory Animals for Scientific Purposes.

Mouse Model of Chronic AAD

An established model of OVA-induced chronic AAD was used as previously described (Mookerjee et al. 2006; Temelkovski et al. 1998). This model was chosen as it includes many of the pathological features of human asthma including increased allergic responses indicated by increased IgE against OVA (OVA-specific IgE), AHR, and remodeling changes such as epithelial remodeling, goblet cell metaplasia and peribronchial fibrosis. However, it does not display smooth muscle thickening (Mookerjee et al. 2006; Temelkovski et al. 1998). Briefly, mice were sensitized with 10 μg of grade V OVA (Sigma Chemical, St Louis, Missouri, USA) and 1 mg of aluminium potassium sulfate adjuvant (alum) in 500 μl saline intraperitoneally (i.p.) on day 0 and 14 and then challenged with nebulized 2.5 % (w/v) OVA in saline 3 days/week for 6 weeks to establish AAD.

OVA-exposed mice were treated with TSA (OVA-TSA, $n = 15$) or vehicle 1 % dimethyl sulfoxide (DMSO) in normal saline control (OVA-VEH, $n = 15$) i.p. following each OVA nebulisation (3 days/week for 6 weeks). A third group of mice, sensitized with saline/alum on days 0 and 14 and nebulized with saline 3 days/week for 6 weeks ($n = 15$), served as additional controls.

The TSA (Sigma Chemical, USA) was used at a low concentration of 5 mg/kg (dissolved in 1 % DMSO in normal saline). It is well established this dose results in histone hyperacetylation and is known to be well-tolerated in mice (Langley et al. 2009).

Methacholine-Induced AHR

Forty-eight hours after the last OVA nebulization/treatment with TSA/vehicle, AHR was measured by invasive plethysmography using a mouse plethysmograph (Buxco Electronics, Troy, New York, USA) as described previously (Mookerjee et al. 2006). Briefly, mice were anesthetized by i.p. injection of ketamine (200 μg/g) and xylazine (10 μg/g),

and tracheostomized. After baseline airway resistance had been recorded, increasing methacholine doses (0.25, 0.5, 1, 2, 4 and 8 mg/ml) were delivered by nebulizer (Buxco Electronics, Troy, New York, USA) and airway resistance measured (Finepointe, Buxco Electronics).

Quantitation of Serum OVA-Specific IgE Levels

Serum was obtained by lethal cardiac puncture of anesthetized mice after AHR measurement and stored at −70 °C for measurement of OVA-specific IgE by ELISA (Keramidaris et al. 2001; Locke et al. 2007; Mookerjee et al. 2006). OVA-specific IgE levels were expressed as arbitrary units (AU) where 1 AU = OD of 1:50 dilution of positive control serum.

Bronchoalveolar Lavage

After measurement of airway reactivity, bronchoalveolar lavage (BAL) was performed by three washes of 0.5 ml saline containing 5 % (v/v) fetal calf serum and total and differential cell counts of inflammatory cells determined (Fiscus et al. 2001; Mookerjee et al. 2006). Total viable cell counts were determined using a haemocytometer with trypan blue exclusion. Differential counts of eosinophils, neutrophils, lymphocytes, and monocytes/macrophages were determined on cytospin smears of BAL samples (4×10^5 cells) from individual mice stained with Diff-Quick (Life Technologies, Auckland, New Zealand) and identified by standard morphological criteria after counting 300 cells.

Tissue Collection

Lung tissues were immediately dissected and weighed (total lung weight) and then separated into individual lobes for histological analysis (Locke et al. 2007; Mookerjee et al. 2006; Samuel et al. 2003).

Lung Histopathology

The right lung was fixed in formalin, routinely processed and embedded in paraffin (Locke et al. 2007; Mookerjee et al. 2006; Samuel et al. 2003). Three micrometer lung tissue sections were stained with Masson trichrome for assessment of epithelial and subepithelial collagen thickness; and Alcian blue–periodic acid Schiff (AB–PAS) for assessment of goblet cell numbers (Locke et al. 2007; Mookerjee et al. 2006; Samuel et al. 2003).

Airway Tissue Inflammation Cell Score

The degree of airway inflammatory cell infiltration was scored by two independent blinded investigators. The degree of airway inflammatory cell infiltration around the bronchi was scored as 0 for no inflammatory cell infiltration: 1 for 3 layers of inflammatory cells; 2 for 6 layers; 3 for 10 layers and congestion; or 4 for more than 10 layers of inflammatory cells and severe congestion. For each mouse, 5 airways were observed and the average scores were taken.

Morphometric Analysis of Structural Changes

Morphometric analysis of lung tissue sections was performed as described previously (Locke et al. 2007; Mookerjee et al. 2006). Images of lung tissue sections were captured using a Digital camera (Q Imaging, Burnaby, British Columbia, Canada). A minimum of five bronchi measuring 150–350 μm luminal diameter were analyzed per mouse using Image Pro-Discovery software (Media Cybernetics, Silver Spring, MD), which was calibrated with a reference micrometer slide. The thickness of the bronchial epithelial layer was measured by tracing around the basement membrane and the luminal surface of epithelial cells using a digitizer (Aiptek, Irvine, CA) and calculating the mean distance between these lines by Image Pro-Discovery software (Media Cybernetics). Subepithelial collagen thickness was similarly measured by tracing around the outer extent of the total collagen layer in the submucosal region and around the basement membrane, and the mean distance between these lines calculated. The basement membrane length was measured by tracing in Image Pro-Discovery software (Media Cybernetics) and goblet cells were counted in AB–PAS-stained sections and expressed as percentage of cells.

Immunohistochemistry for TGF-β1 and MMP-9

Sections of lung tissue were stained immunohistochemically to detect and localize TGF-β1, and MMP-9 protein expression. TGF-β and MMP-9 were identified using rabbit polyclonal antibodies, sc-146 specific for TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA) and ab16306 specific for latent and active forms of MMP-9 (Abcam, Cambridge, UK), respectively. Bound primary antibody was detected using anti-rabbit EnVision (Dako, Glostrup, Denmark). The chromagen 3,3-diaminobenzidine was used and sections counterstained with haematoxylin.

Statistical Analysis

Results were expressed as mean ± SEM with 95 % confidence interval and analyzed using the Mann–Whitney test. Airway hyperresponsiveness measurements were compared using a two-way ANOVA and Bonferroni post-test.

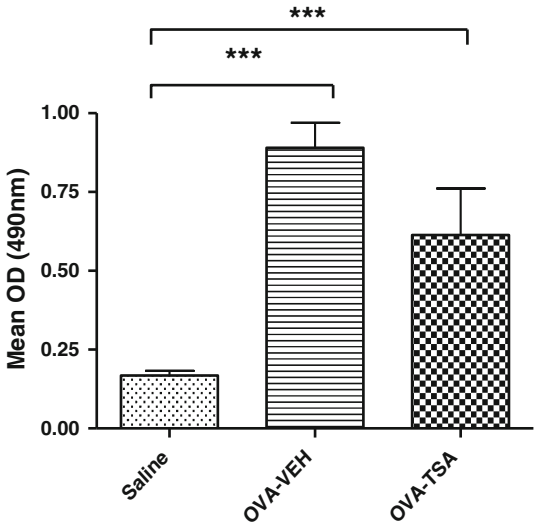

Fig. 1 Serum levels of OVA-specific IgE in the chronic AAD model. Enzyme-linked immunosorbent assay was performed on mice blood serum samples. OD values were read at 490 nm, with a reference wavelength of 650 nm. There was a significant increase in the OVA-VEH and the OVA-TSA sensitized groups with respect to saline group (***$p < 0.001$). In each group $n = 15$

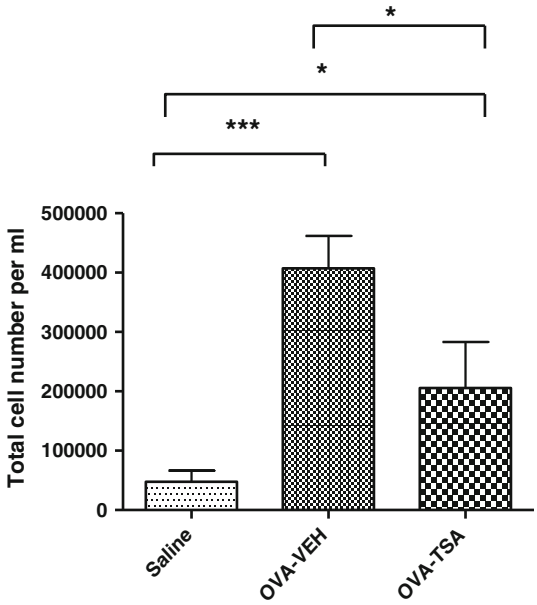

Fig. 2 Total inflammatory cell count in BALF. Following the killing, BAL was performed and total inflammatory cell number per 1 ml of BALF determined. The OVA-VEH and OVA-TSA groups showed a significant increase in total inflammatory cell numbers compared to the saline mice. The OVA-TSA group had reduced cell number compared to OVA-VEH. (*$p < 0.05$; ***$p < 0.001$). In each group $n = 15$

Results

Validation of the Chronic AAD Model

It was first necessary to validate that OVA sensitisation/challenge had been successfully established in the OVA mice treated with vehicle (OVA-VEH). Allergen-specific IgE (OVA-specific IgE), and BAL inflammatory cells were assessed.

Adequate OVA sensitisation was verified by measurement of OVA-specific IgE in serum samples at the end of the 8-week OVA sensitisation/challenge protocol. As expected, OVA-VEH mice demonstrated increased levels of OVA-specific serum IgE compared to saline control mice ($p < 0.001$; Fig. 1).

Total and differential BAL cell counts were significantly increased in OVA-VEH mice as compared with the saline-sensitized/challenged control mice (all $p < 0.001$; Fig. 2).

These results confirmed that the AAD model was successfully established with development of airway inflammation, airway remodeling and AHR as previously reported (Locke et al. 2007; Mookerjee et al. 2006; Royce and Tang 2009; Temelkovski et al. 1998).

TSA Treatment Reduces Key Aspects of Airway Inflammation in Chronic AAD

OVA-specific IgE levels in OVA sensitized/challenged mice treated with TSA (OVA-TSA) were similar to levels in OVA sensitized/challenged mice treated with vehicle (OVA-VEH), and levels in both the OVA-TSA and OVA-VEH groups were significantly higher than levels in saline-sensitized/challenged control mice confirming successful OVA sensitisation in both groups ($p < 0.001$; Fig. 1).

Despite adequate OVA sensitisation in the OVA-TSA mice, total and differential BAL cell counts were significantly reduced when compared to the vehicle-treated mice (OVA-VEH; all $p < 0.05$; Figs. 2, 3). However, total and differential BAL cell counts in the OVA-TSA group remained significantly higher than in the saline-sensitized/challenged control mice (total BAL cell count $p < 0.05$; eosinophil count $p < 0.001$; neutrophil count $p < 0.01$; lymphocytes $p < 0.01$; Figs. 2, 3).

The degree of airway inflammatory cell infiltration around the bronchi was higher in OVA-VEH and OVA-TSA groups as compared to saline control mice ($p < 0.001$). Treatment with TSA led to decreased inflammatory cell infiltration with OVA-TSA mice having a lower score compared to OVA-VEH mice ($p < 0.05$; Fig. 4). These results indicate that systemic (i.p.) administration of TSA can inhibit the development of airway inflammation following sensitisation/challenge with OVA.

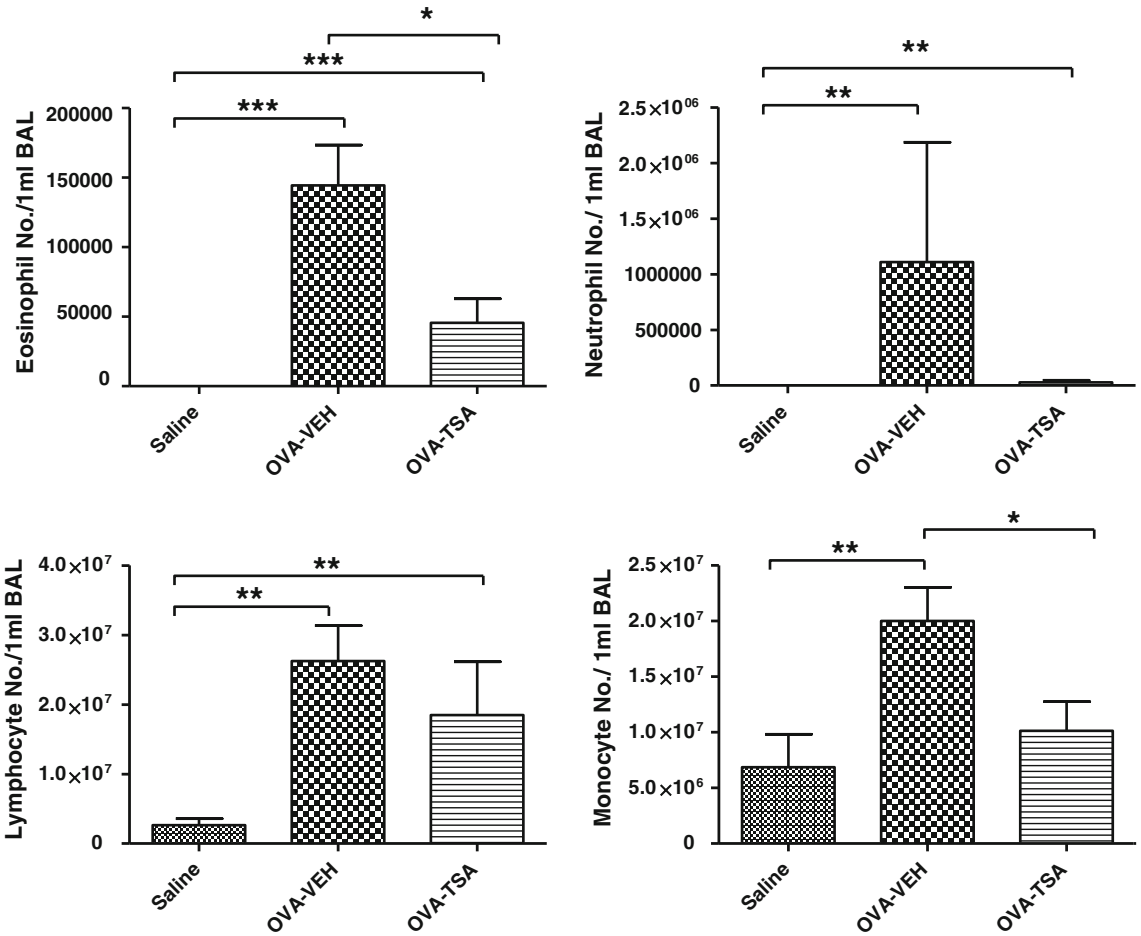

Fig. 3 BALF differential cell counts. Following Diff-Quick staining, numbers of differential cells were counted under the light microscope. The value expressed as total number in 1 ml sample. Eosinophils, neutrophils, lymphocytes and monocytes were significantly increased in OVA-VEH as compared to saline mice. Eosinophil and monocyte numbers were decreased in the OVA-TSA group as compared to the OVA-VEH group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). In each group $n = 10$

Treatment with TSA Suppresses Airway Remodeling Changes Including Goblet Cell Metaplasia and Subepithelial Collagen Deposition but Not Epithelial Thickening in Chronic AAD

Goblet cell numbers were determined in AB–PAS-stained lung tissue sections, while airway epithelial thickness and subepithelial extracellular matrix thickness were determined in Masson trichrome stained lung tissue sections.

Numbers of goblet cells in TSA-treated mice were significantly lower than in vehicle-treated mice ($p < 0.05$; Fig. 5), but remained significantly higher than in the saline-sensitized/challenged mice ($p < 0.001$). Similarly, subepithelial extracellular matrix thickness was significantly lower in TSA-treated mice as compared to vehicle-treated mice ($p < 0.05$), but remained higher than that observed in the saline-sensitized/challenged mice ($p < 0.05$; Fig. 6). In contrast, the mean epithelial thickness was not significantly different in OVA-TSA and OVA-VEH mice, and airway epithelial thickness in both groups was significantly increased as compared to saline-sensitized/challenged mice ($p < 0.001$; Fig. 6). These results suggest that TSA can regulate some aspects of airway remodeling (goblet cell metaplasia and subepithelial collagen deposition) in a mouse model of chronic AAD.

Treatment with TSA Attenuates Airway Hyperresponsiveness in Chronic AAD

Anesthetized tracheotomised mice were challenged with nebulized methacholine and the change in resistance from baseline was measured by invasive plethysmography. Methacholine-induced AHR was reduced in the OVA-TSA group as compared to the OVA-VEH group, with statistical significance reached at the highest dose of 8 mg/ml methacholine ($p < 0.001$; Fig. 7). These results show that TSA treatment can suppress AHR at this dose in the chronic AAD model.

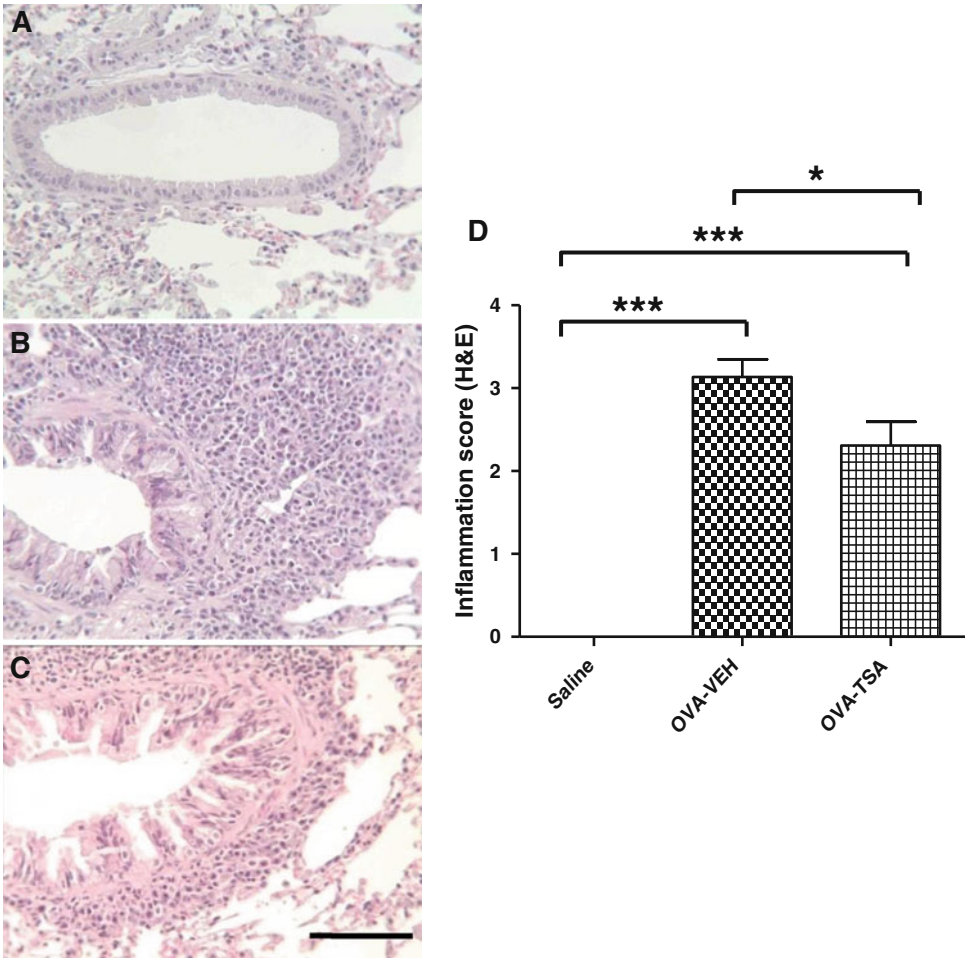

Fig. 4 Inflammatory score of hematoxylin and eosin-stained sections of mouse airways. Formalin-fixed paraffin-embedded sections of mouse lung tissue were stained with hematoxylin and eosin. Saline-treated mouse airway showing little or no peribronchial inflammatory infiltrate (**a**). OVA-VEH shows severe peribronchial inflammatory infiltrate (**b**). OVA-TSA shows moderate peribronchial inflammatory infiltrate (**c**). In each group $n = 15$. *Bar* 100 μm. Inflammatory cell infiltration was scored by two independent blinded investigators as 0 for no inflammation; 1 for 3 layers of inflammatory cells; 2 for 6 layers; 3 for 10 layers and congestion; or 4 for more than 10 layers of inflammatory cells and severe congestion. Both OVA-VEH and OVA-TSA had elevated inflammation scores as compared to saline mice (**d**). OVA-TSA was lower than OVA-VEH (*$< p < 0.05$; ***$p < 0.001$). In each group $n = 15$

Immunohistochemical Analysis of TGF-β1 and MMP-9 Expression is not Affected by TSA Treatment

TGF-β1 and MMP-9 were investigated by immunohistochemistry in formalin-fixed, paraffin-embedded lung tissue sections (Fig. 8). Little constitutive staining for TGF-β1 and MMP-9 was observed in the saline-sensitized and -challenged control mice. In the lungs of mice receiving chronic OVA sensitization and challenge, staining for TGFβ-1 and MMP-9 was increased. Cytoplasmic staining was present in bronchial epithelial cells, connective tissue cells in the lamina propria and adventitia, and smooth muscle cells. Staining was also seen in inflammatory cells. Treatment with TSA did not markedly reduce staining for TGF-β1 or for MMP-9 in the lung tissue.

Discussion

Human asthma is a chronic disease associated with structural changes to the airways including epithelial thickening and goblet cell metaplasia, subepithelial collagen deposition and smooth muscle hyperplasia. These pathological changes are believed to contribute to AHR and irreversible airway obstruction which is responsible for morbidity and mortality in severe asthma (Chetta et al. 1997; Hoshino et al. 1998). Remodeling can occur early in childhood and largely develops independent of airway inflammation (Chakir et al. 1996; Phunek et al. 1997; Warner et al. 2000). Unlike inflammation (which can be treated with inhaled corticosteroids), there is no effective therapy targeting remodeling. Airway remodeling cannot be investigated in short-term animal models of AAD and can only be investigated using longer term models which replicate a number of the key features of human asthma.

In the current study, we investigated the effects of TSA on airway inflammation, AHR and airway remodeling processes in a mouse model of chronic AAD that replicates many of the features of human asthma. Our findings indicate that at the highest dose of methacholine TSA reduced AHR, the hallmark feature of asthma that is most strongly associated with symptoms. Further TSA attenuated some aspects of airway remodeling (reduced goblet cell numbers and subepithelial fibrosis) in addition to reducing airway inflammation in this animal model of asthma. The mechanisms of these effects may include reduced eosinophilia

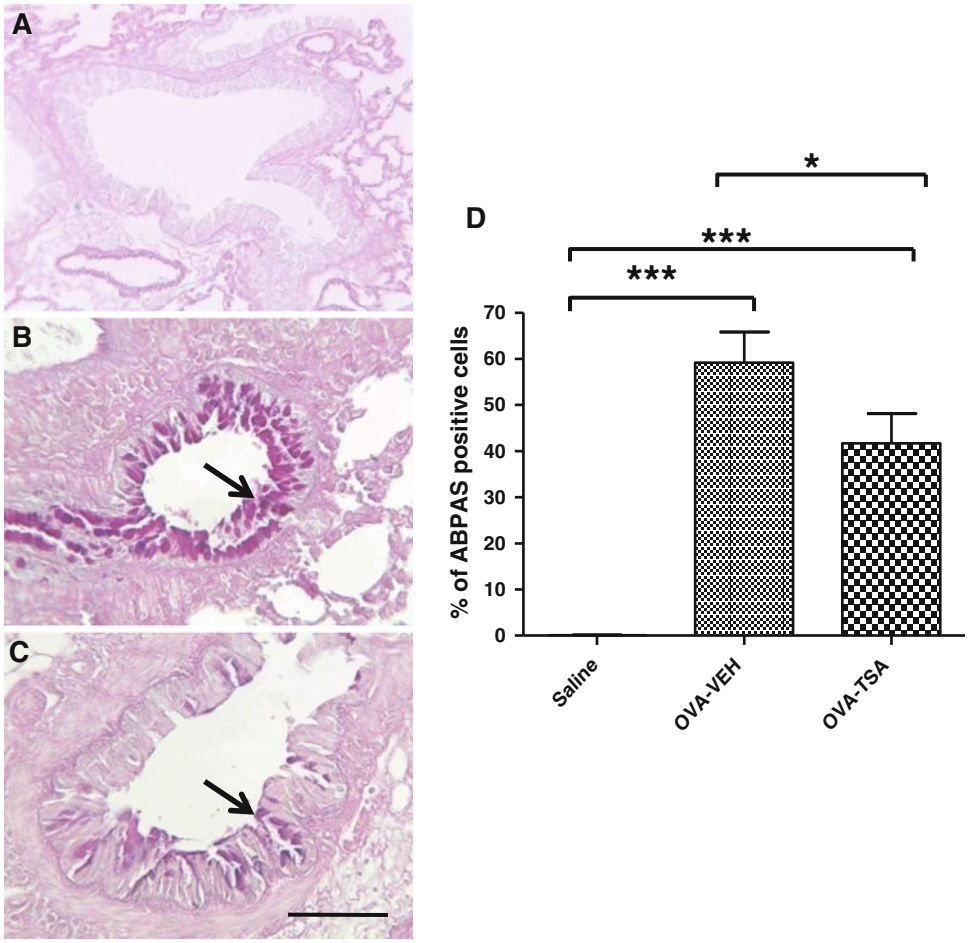

Fig. 5 Percentage of AB–PAS-positive goblets cells in mouse airways. Formalin-fixed paraffin-embedded sections of mouse lung tissue were stained with AB–PAS. Saline-treated mouse airway showing little or no goblet cell metaplasia (**a**). OVA-VEH shows severe goblet cell metaplasia (*arrows* **b**). OVA-TSA shows mild goblet cell metaplasia (*arrows*; **c**). In each group $n = 15$. *Bar* 100 μm. Both OVA-VEH and OVA-TSA had elevated goblet cells percentages as compared to saline mice (**d**). OVA-TSA was lower than OVA-VEH ($*p < 0.05$; $***p < 0.001$). In each group $n = 15$

and inflammatory cell infiltration to the airway which we were able to detect in our model, but also by direct action on effector cells in structural remodeling in the airway.

Broad-spectrum HDACi, such as TSA, are most well known for their potent anti-proliferative effects, which has already resulted in clinical application of the compounds suberoylanilide hydroxamic acid (SAHA, Vorinostat) and depsipeptide (Romidepsin, Istodax), for the treatment of cutaneous T cell lymphoma and numerous other analogues are currently in clinical trials as antineoplastic agents (Marks 2010). However, more recently there has been interest in the anti-inflammatory properties of TSA (Wang et al. 2009). TSA has been reported to significantly reduce airway inflammation, decrease expression of Th2 cytokines (IL-4 and IL-5) as well as total IgE in an acute (short term) model of AAD (Choi et al. 2005). Similar anti-inflammatory effects have been reported for a class III HDACi, sirtinol in an acute model of AAD (Kim et al. 2010a).

Eosinophils are potent inflammatory cells and are critical for the progression of asthma and exacerbations of chronic obstructive pulmonary disease. A large influx of eosinophils in BAL fluid was observed in the OVA-challenged group. Numerous studies have shown that HDACi can promote the production of IL-5, which is a life-supporting cytokine for eosinophils (Donnelly et al. 2004). It has been suggested that the mechanism of HDACi effect on eosinophilia is induction of granulocyte apoptosis (Kankaanranta et al. 2010). Recent experiments have shown that HDACi could induce apoptosis in human eosinophils and neutrophils in the absence and presence of survival-prolonging cytokines and glucocorticoids. The possible mechanism of TSA on granulocytes apoptosis is by targeting non-histone proteins, including c-jun-N-terminal kinase and caspases 3 and 6 (Zhang et al. 2008).

This is the first study to demonstrate the therapeutic effects of an HDACi on airway remodeling in a chronic in vivo model. Importantly, TSA was shown to suppress goblet cell metaplasia, a response to epithelial damage in asthma which can be highly deleterious. It is associated both with increased mucus secretion which can lead to occlusion of the airway (Ordonez et al. 2001) and also activation of airway epithelial cells which secrete cytokines, matrix metalloproteases and growth factors into the lamina propria which, have in turn been shown to act on

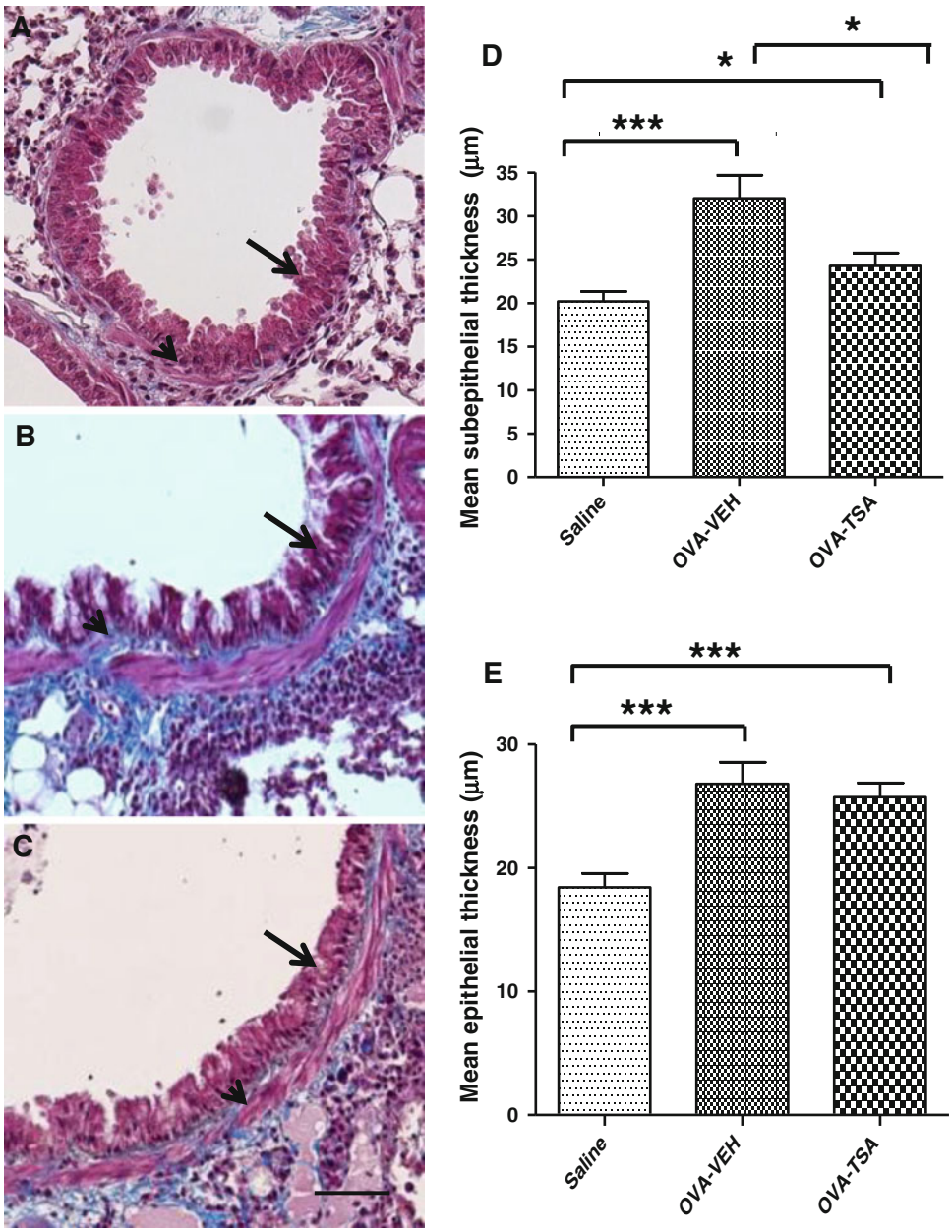

Fig. 6 Morphometric analysis of subepithelial collagen and epithelial thickness in the chronic AAD model. Formalin-fixed paraffin-embedded sections of mouse lung tissue were stained with Masson trichrome. Saline-treated mouse airway shows thin airway wall with thin epithelial layer (*arrow*) and little subepithelial collagen deposition (*arrowhead* **a**). OVA-VEH with thickened epithelial layer (*arrow*) and severe subepithelial collagen deposition (*arrowhead* **b**). OVA-TSA with thickened epithelial layer (*arrow*) but little subepithelial collagen deposition (*arrowhead* **c**). In each group $n = 15$. *Bar* 100 μm. **d** OVA-VEH mice had elevated subepithelial collagen thickness as compared to saline mice. OVA-TSA had significantly less collagen deposition compared to OVA-VEH and was not significantly different to saline (NS). In each group $n = 15$. **e** Both OVA-VEH and OVA-TSA had elevated epithelial thickness as compared to saline mice. OVA-TSA was not significantly different to OVA-VEH (NS; $*p < 0.05$; $***p < 0.001$). In each group $n = 15$

fibroblasts and airway smooth muscle cells to promote mesenchymal remodeling and airway thickening (Knight 2001). These changes all contribute to AHR (Tang et al. 2006). TSA has been shown to downregulate STAT5A, a gene important in goblet cell metaplasia (Michaelis et al. 2004; Grabiec et al. 2010). However, it is also possible that TSA regulates goblet cell metaplasia by its anti-proliferative activity. The suppression of airway fibrosis in the current study is a powerful effect of TSA and the level of collagen reduction was comparable to that of relaxin. Relaxin is an anti-fibrotic and we have demonstrated in a number of experiments using chronic AAD mice and relaxin-deficient mice has direct effects on fibrosis that contribute to AHR suppression (Lekgabe et al. 2006; Mookerjee et al. 2006; Royce et al. 2009; Samuel et al. 2007, 2009). Others have shown TSA and other HDACi to have anti-fibrotic effects in animal models of inflammatory diseases (Grabiec et al. 2010; Michaelis et al. 2004). A recent report by Sanders et al. (2011) provides the strongest evidence for direct anti-fibrotic effects of TSA. This group treated rat fibroblasts with TSA and found the drug epigenetically restored Thy-1 fibrosis suppressor expression. They also found normalized α-smooth muscle actin (αSMA; marker of myofibroblast differentiation) expression. This is directly relevant to airway remodeling as αSMA is also a marker of airway smooth muscle

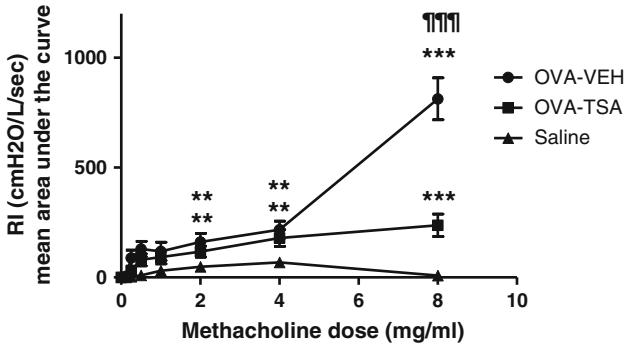

Fig. 7 Airway hyperresponsiveness to methacholine in the chronic AAD model. Plethysmography was performed 48 h following the final treatment and nebulization. Increasing doses of methacholine was administered and maximal resistance values [$cmH_2O/(L\ s)$] after 5 min was recorded. OVA-VEH mice had elevated airway resistance at the highest methacholine dose as compared to saline mice. OVA-TSA had significantly lower airway resistance at the highest methacholine dose compared to OVA-VEH. In each group $n = 10$. ($^{**}p < 0.01$; $^{***}p < 0.001$ as compared to saline group; $^{¶¶¶}p < 0.001$ as compared to OVA-TSA group)

(Westergren-Thorsson et al. 2010). Further experiments into the effect of TSA on airway smooth muscle expansion need to be performed and as mentioned above this aspect of asthma pathology is not present in our model (Locke et al. 2007). We were unable to observe differences in protein expression in the lungs of αSMA between mice treated with TSA compared to vehicle controls (data not shown). No differences were observed between staining for the pro-fibrotic growth factor TGF-β or MMP-9 between OVA-TSA and OVA-VEH groups. TSA has also been tested on human lung fibroblasts (Wi38 cell line) and was shown to inhibit the proliferation of these cells through cyclin-dependent kinase inhibitor p21waf and induced senescence-associated morphology (Place et al. 2005).

HDACi have possible direct or indirect effects on AHR given our findings and the findings of Kim et al. (2010a) using invasive plethysmography and methacholine challenge and the findings of Choi et al. (2005) using non-invasive techniques. Whilst the strong but indirect anti-inflammatory and anti-remodeling effects of TSA are likely to account for the attenuation of AHR, there is a possibility of direct effects on airway contraction. Preincubation of guinea pig tracheal rings with certain HDACi causes inhibition of antigen-induced contraction of sensitized rings (Assem el et al. 2008). The inhibition may be caused by an effect on mast cell activation and may be partly due to inhibition of mediators of the contractile response such as histamine (Assem el et al. 2008).

Despite the strong theoretical and experimental evidence of the potential utility of HDACi in treating airway remodeling, further studies are required. Previously, inhibitors of HDAC often led to a further induction of inflammatory gene expression in vitro. Some studies suggest TSA could shift the Th1–Th2 balance towards Th2, up-regulate secretion of IL-13 and IL-15 and alter tumor necrosis factor-α-induced gene expression via reducing interferon-γ. Also, HDACi may impair the glucocorticoid receptor (GR)-induced repression of inflammatory genes. The activated GR could interact with specific transcription factors such as AP-1 and NF-κB and thus prevent the transcription of targeted inflammation genes. HDACi-induced loss of HDAC2 disrupts the association between GR and NF-κB (Kankaanranta et al. 2010). In addition, in one study TSA was shown to enhance lipopolysaccharide-stimulated IL-8 and block attenuation of IL-8 transcription (Adcock et al. 2007). However, most animal studies suggest HDACi possess anti-inflammatory effects via different mechanisms such as the acetylation of non-histone proteins or the up-regulation of Tregs (Kim et al. 2010b). In general, the effect of HDACi on pro-inflammation or anti-inflammation is likely to be cell-type dependent. In vitro or in vivo studies have showed different effects of TSA either by pro or anti-inflammation with anti-inflammation strongly predominant in vivo studies (Xu et al. 2007).

One caveat of the current study is that TSA was given during the OVA challenge period in the chronic AAD model. Therefore, it is not known if similar changes can be achieved in a clinical setting where pathologies are already established. Further studies introducing TSA after remodeling changes are established would be warranted. In the current study we used systemic i.p. treatment in order to maximize delivery of TSA. In our long-term treatment study and the shorter systemic treatment regimen of Choi et al. (2005) there were no apparent side effects. Some HDACi are safe for use in humans and have been used in clinical trials for cancer. Oral Vorinostat (suberoylanilide hydroxamic acid) was the first HDACi to be approved by the Federal Drug Administration to treat cutaneous T cell lymphoma (Marks and Breslow 2007). Many other HDACi are in clinical trials currently, including lung cancer. The current study showed that TSA could be safely administered to mice with no side effects observed by histopathological analysis and animal welfare checks. There was also no significant weight loss in OVA-TSA mice as compared to controls (data not shown). Exploration of delivery methods such as intranasal administration may increase efficacy of TSA treatment in the lung, whilst minimizing side effects.

Airway remodeling involved in asthma is associated with AHR. By influencing goblet cell metaplasia, proliferation and fibrosis through the use of HDACi, we may in turn influence AHR. We have found an agent that targets all three fundamental pathologies in asthma which is

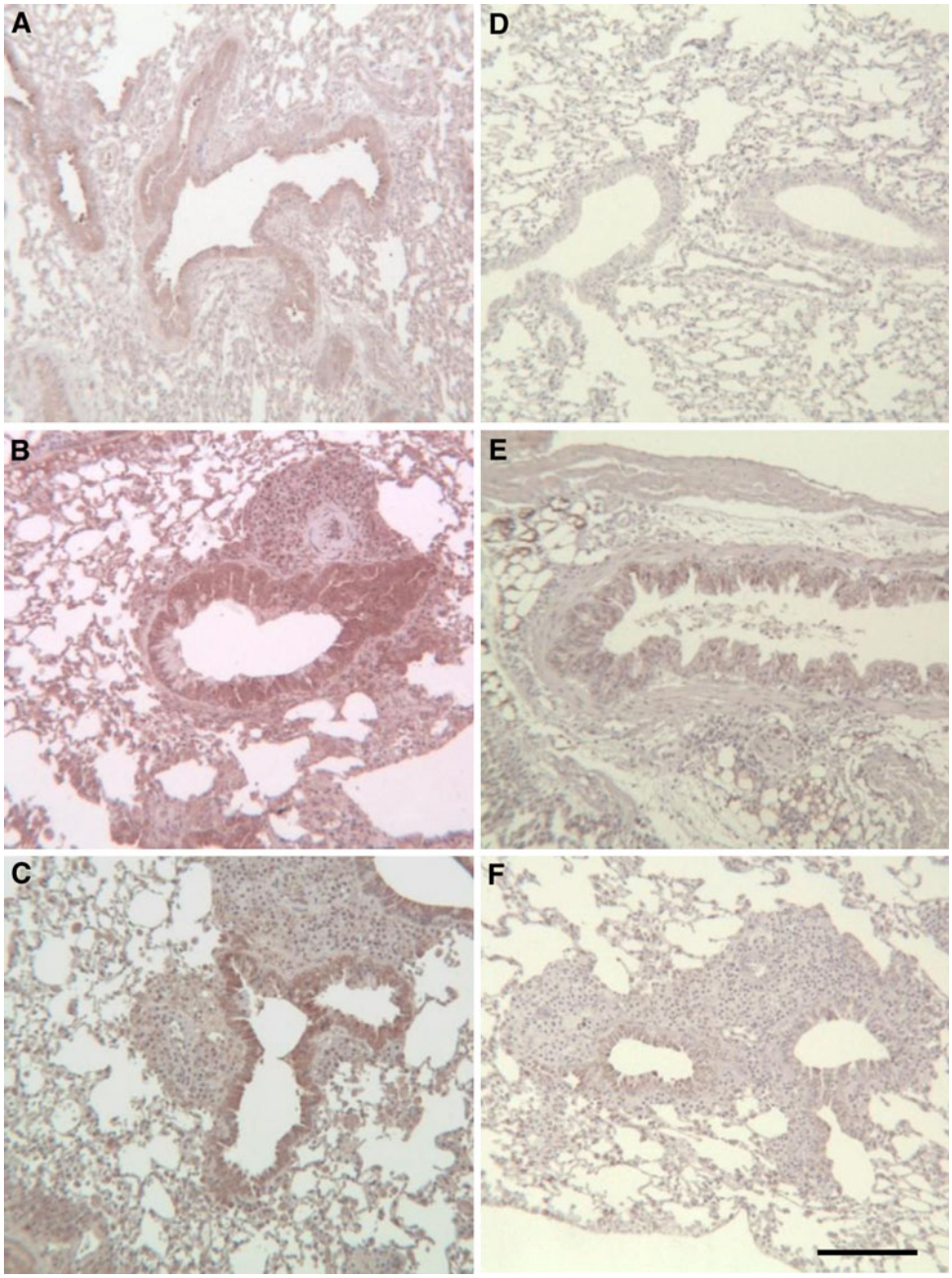

Fig. 8 Immunohistochemical analysis of TGF-β1 and MMP-9 expression. TGF-β1 and MMP-9 were investigated by immunohistochemistry in formalin-fixed, paraffin-embedded lung tissue sections. Little staining for TGF-β1 was observed in the saline-sensitized and -challenged control mice (**a**). In the lungs of mice receiving chronic OVA sensitization and challenge, staining for TGF-β1 was markedly increased (**b**). Treatment with TSA did not affect staining for TGF-β1 in the lung tissue (**c**). Little staining for MMP-9 was observed in the saline-sensitized and -challenged control mice (**d**). In the lungs of mice receiving chronic OVA sensitization and challenge, staining for MMP-9 was markedly increased (**e**). Treatment with TSA did not affect staining for MMP-9 in the lung tissue (**f**). *Bar* 200 μm

currently not achieved by steroid treatment. In conclusion, our experimental findings reveal that TSA may assist in reducing AHR, targeting airway inflammation, but more importantly remodeling in AAD. Overall, HDACi drugs require further experimental analysis before their use in humans but have shown promising effects in AAD therapy to date.

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Conflict of interest The authors declare that they have no conflict of interest.

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