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**Enhanced pause correlates with airway neutrophils and airway-epithelial injury in
in asthmatic mice treated with dexamethasone**

SHORT TITLE: Neutrophilia and epithelial injury increase Penh

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ABBREVIATIONS

Airway hyperresponsiveness (AHR)

Aluminum (alum)

Broncho-alveolar lavage (BAL)

Dexamethasone (Dex)

Enhanced pause (Penh)

Hematoxylin & eosin (H&E)

House dust mite (HDM)

Hypodermic injection (i.h.)

Intraperitoneal injection (i.p.)

Ovalbumin (OVA)

Phosphate-buffered saline (PBS)

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INTRODUCTION

Asthma is an airway disease that occurs when airflow is reversibly obstructed, leading to wheezing, coughing, and shortness of breath. The global burden of patients with asthma symptoms has increased by almost 30% over the past 20 years, and large numbers of asthma patients still suffer from airway hyperresponsiveness (AHR) despite regular treatment with glucocorticoids [1].

AHR is an exaggerated obstructive airway response to various pharmacological, chemical, and physical stimuli including methacholine, histamine, sulphur dioxide, fog, and cold air [2]. The mechanisms that contribute to AHR are not definitively known. The development of AHR is a primary indicator of asthma progression during asthma treatment [3]. AHR is usually related to the subsequent decline in lung function in asthma and predicts the future development of asthma [4].

Glucocorticoids have been recommended as first-line drugs for asthma treatment for over 20 years; however, glucocorticoid treatment can change the proportions of airway inflammatory cells [5]. Moreover, we have previously reported that glucocorticoid dexamethasone failed to relieve AHR due to inhibition of the proliferation and migration of airway epithelial cells, and might actually cause harm to airway epithelial integrity in asthma [6, 7]. We suggest that the alteration in airway inflammation and airway epithelial injury might be involved in AHR pathology, weakening the ability of glucocorticoids to treat asthma.

In the present study, we used an asthmatic mouse model treated with dexamethasone to measure enhanced pause (Penh) and count airway inflammatory cells and shedding of airway

epithelial cells in broncho-alveolar lavage (BAL) fluid, and subsequently investigated the correlations between each factor. Despite the controversy, Penh is still considered suitable for the diagnosis of lower airway disease in animal models [6, 8, 9]. The present study might illuminate novel risk factors associated with AHR during asthma treated with glucocorticoids.

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MATERIALS AND METHODS

Mice

The present study was approved by the Ethics Committee of Chongqing Medical University (reference number: 2012023) and complied with the ARRIVE guidelines. Female BALB/c and C57BL/6 mice (6- to 8-weeks old) were purchased from the Experimental Animal Center of Chongqing Medical University. The BALB/c mice were housed under specific pathogen-free conditions with ovalbumin (OVA)-free diets.

Allergen sensitization and airway challenge

We followed the OVA-induced mouse model of asthma that has been established in previous reports [10-12], with minor modifications (Fig. S1A). The BALB/c mice were sensitized with 50 µg OVA (Sigma-Aldrich, Saint Louis, MO, USA) plus 1 mg aluminum hydroxide (alum; Sigma-Aldrich, Saint Louis, MO, USA) via intraperitoneal (i.p.) injection and 50 µg of OVA plus 1 mg of alum via hypodermic injection (i.h.) at the roots of the extremities on days 0, 7 and 14. The BALB/c mice were subsequently challenged with 1% OVA aerosol for 30 min after day 21 for 10 days.

A second asthmatic mouse model was established according to previous reports [13-15], with minor modifications (Fig. S2). Female C57BL/6 mice were primed intranasally with 20 µg house dust mite (HDM, Greer, Lenoir, NC, USA) on days 1 and 14, and subsequently challenged intranasally with 20 µg HDM on days 21, 23, 25, 27 and 29. Healthy naïve mice were sensitized and challenged with phosphate-buffered saline (PBS).

Within 24 hours of the final challenge, all mice were examined for Penh and sacrificed

under anesthesia (10% chloral hydrate) via cervical dislocation. Following euthanization, bronchoalveolar lavage (BAL) fluid and lung tissue were collected for pathological analysis.

Drug treatments

A group of OVA-induced BALB/c mice were administered different concentrations (0, 1, or 5 mg/kg) of dexamethasone 21-phosphate disodium salt (dexamethasone; Sigma-Aldrich, Saint Louis, MO, USA) via intraperitoneal injection on day 28 for 3 days (Fig. 1A). Healthy naïve mice were injected intraperitoneally with PBS.

A group of HDM-induced C57BL/6 mice were administered 2 mg sivelestat (a specific neutrophil elastase inhibitor; MedChemExpress, New Jersey, USA) via intraperitoneal injection following each HDM challenge. Another group of HDM-induced C57BL/6 mice were administered PBS via intraperitoneal injection.

Non-invasive measurement of enhanced pause

Penh was measured within 24 hours after the final OVA or HDM airway challenge. The mice were placed into a whole-body plethysmograph (EMKA Technologies, Paris, France) and exposed to increasing concentrations (0, 3.125, 6.25, 12.5, 25 and 50 mg/ml) acetyl- β -methylcholine chloride (methylcholine; Sigma-Aldrich, Saint Louis, MO, USA). Penh was subsequently recorded according to previously described methods [6, 9]. The airflow obstruction index is expressed as Penh and is related to the airway responsiveness [6, 8].

The Penh algorithm was based on the procedure developed by Hamelmann [16] with the

following equations:

$$\text{Pause} = \frac{T_e - T_r}{T_r} \quad (1)$$

$$\text{Penh} = \text{Pause} \times \frac{PEP}{PIP} \quad (2)$$

where T_e represents the expiratory time, T_r represents the relaxation time, PEP represents the peak expiratory pressure, and PIP represents the peak inspiratory pressure.

Broncho-alveolar lavage fluid assays

Following sacrifice, the trachea of each mouse was cannulated and the BAL fluid was collected by six injections of 0.5 ml PBS into the lungs.

Cells were collected from the BAL fluid by centrifugation, treated with red blood lysis buffer to remove the red blood cells, and counted by microscopy using a cell hemocytometer. Differential cell counts were performed based on standard morphological and staining characteristics of at least 200 cells per sample [15]. The remaining BAL was centrifuged and the collected supernatant was analyzed for cytokines.

Cytokine analysis

Commercially available enzyme-linked immunosorbent assay kits of IL-5 (≥ 8 pg/ml; 4A Biotech, Beijing, China), IL-17 (≥ 7 pg/ml; Neobioscience, Shenzhen, China) and IFN- γ (≥ 8 pg/ml; Neobioscience, Shenzhen, China) were used to measure the concentrations of these cytokines in the BAL fluid.

Histological analysis

Immediately following euthanization, the tissues were harvested and fixed in 4% buffered formaldehyde, dehydrated in a graded alcohol series, cleared with dimethylbenzene, and embedded in paraffin. The paraffin blocks were serially sectioned at 4- μ m thickness and stained with hematoxylin & eosin (H&E) using standard techniques.

Statistical analysis

The GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA) was used for the statistical analysis. Data are expressed as the mean $\pm$ standard error (SEM). Statistical analyses were performed for Penh with a two-way ANOVA and for cell counts with a one-way ANOVA or a Student's *t*-test. Correlations were performed using Pearson's rank correlation coefficients. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Identification of the ovalbumin-induced mouse model of asthma

The OVA-induced mouse model of asthma was identified by H&E staining of lung tissue (Fig. S1B), Penh values (Fig. S1C), and representative airway inflammatory cells (Fig. S1D) and cytokines (Fig. S1E) in the BAL fluid.

Eosinophils were significantly increased in the BAL fluid of the OVA-induced mouse model of asthma (Fig. S1D), indicating that the model had airway allergy inflammation. Moreover, both neutrophils (Fig. S1D) and IL-17 (Fig. S1E) were significantly increased in the BAL fluid of the model, which typically occurs in asthma mediated by both the Th2 and Th17 inflammatory responses [11, 17]. Further, it could be a severe subtype of asthma that has concurrent increases in airway eosinophils ($\geq 2\%$) and neutrophils ($\geq 40\%$) [18], similar to the situation in our OVA-induced asthmatic mouse model (eosinophils $2.3 \pm 0.3\%$; neutrophils $65.5 \pm 5.8\%$).

Dexamethasone minimally relieved airway hyperresponsiveness in ovalbumin-challenged mouse model of asthma

Penh, which indicates AHR [6, 8], was measured in mice after the last challenge. There was no statistical difference in the baseline measurement among the groups ($P > 0.05$). At methylcholine concentrations of 12.5, 25 and 50 mg/ml, Penh values showed significant differences between the naïve mice and the OVA-challenged mice treated with PBS ($P < 0.01$). At methylcholine concentrations of 3.125, 6.25, 12.5, 25 and 50 mg/ml, Penh values showed no significant differences among the OVA-challenged mice treated with PBS, 1 mg/kg

dexamethasone (Dex 1), or 5 mg/kg dexamethasone (Dex 5) ($P>0.05$; Fig. 1B).

Among the OVA-challenged mice treated with dexamethasone, Penh 50 (the Penh values in mice challenged with 50 mg/ml methacholine) showed a more significant gradient change and much smaller standard errors than other Penh values (Fig. 1B); thus, Penh 50 was chosen as representative data for correlation analysis.

Dexamethasone significantly decreased airway inflammation in ovalbumin-induced mouse model of asthma

H&E staining of lung tissues and differential cell counts in BAL fluid were evaluated to show the suppression of asthma airway inflammation by dexamethasone administration.

The morphological alterations, as assessed by H&E staining, revealed airway inflammation infiltration around the bronchi and blood vessels in OVA-challenged mice treated with PBS; however, this inflammation was greatly reduced in OVA-challenged mice treated with dexamethasone (Fig. 1C).

To quantitatively analyze airway inflammation, the cells in the BAL fluid were counted. Generally, the OVA-challenged mice treated with PBS had the most eosinophils, neutrophils, and lymphocytes in the BAL fluid; however, eosinophils and neutrophils were both significantly decreased in the BAL fluid of OVA-challenged mice treated with dexamethasone (Fig. 2A).

Correlation analysis between inflammatory cells and enhanced pause

The correlations between inflammatory cells and Penh were analyzed to investigate the

roles that inflammatory cells play in AHR pathology of asthma treated with glucocorticoids. Penh 50 was used as a typical indicator of AHR.

A positive correlation was found between Penh 50 and the total cell number in BAL fluid ($r=0.48$, $P=0.028$). We also found a strong positive correlation between Penh 50 and neutrophil levels in BAL fluid ($r=0.67$, $P<0.01$), indicating that airway neutrophils might be a risk factor for AHR development in OVA-challenged mice treated with dexamethasone. However, Penh 50 had no significant correlation with the levels of eosinophils ($r=0.06$, $P=0.82$), lymphocytes ($r=-0.02$, $P=0.93$), or macrophages ($r=-0.07$, $P=0.78$) in BAL fluid, indicating that these inflammatory cells likely have minimal effects on AHR in OVA-challenged mice treated with dexamethasone (Fig. 2B).

Correlation analysis between shedding of airway epithelial cells and enhanced pause

The shedding of airway epithelial cells is a criterion for determining airway epithelial injury in asthma [19]. In the present study, shedding of airway epithelial cells was the least in naïve mice and the most in OVA-challenged mice treated with PBS. The shedding of airway epithelial cells in OVA-challenged mice treated with dexamethasone was significantly less than that found in OVA-challenged mice treated with PBS, suggesting that dexamethasone decreased the shedding of airway epithelial cells. OVA-challenged mice treated with dexamethasone still showed greater shedding of epithelial cells than the naïve mice, indicating that excessive shedding of airway epithelial cells were in OVA-challenged mice group treated with dexamethasone (Fig. 3A).

To investigate the effect of airway epithelial injury on AHR in OVA-challenged mice

treated with dexamethasone, we analyzed the correlation between the shedding of airway epithelial cells and Penh 50 (an indicator of AHR). The results showed that the numbers of shed airway epithelial cells were strongly correlated with Penh 50 ($r=0.79$, $P<0.01$), demonstrating that excessive airway epithelial shedding might be a contributory factor in the promotion of AHR development (Fig. 3B).

Correlation analysis between the number of airway inflammatory cells and shed airway epithelial cells

To investigate the potential effect of airway inflammatory cells on airway epithelial injury in asthma treated with dexamethasone, we analyzed the correlation between the number of airway inflammatory cells and shed airway epithelial cells. The results showed that there was a positive correlation between total cell number in BAL fluid (mostly airway inflammatory cells) and the shedding of airway epithelial cells ($r=0.39$, $P=0.04$), indicating that airway inflammation might be a risk factor for the promotion of airway epithelial injury in asthma treated with dexamethasone. Moreover, a positive correlation was found between neutrophil levels and the shedding of airway epithelial cells in BAL fluid ($r=0.70$, $P<0.01$), indicating that airway neutrophil infiltration might be a risk factor for airway epithelial injury in OVA-induced asthmatic mice treated with dexamethasone. Further, there was no significant correlation between the shedding of airway epithelial cells and the number of eosinophils ($r=0.07$, $P=0.78$), lymphocytes ($r=-0.15$, $P=0.54$), or macrophages ($r=-0.20$, $P=0.40$) in BAL fluid, indicating that these cells have little impact on airway epithelial injury in OVA-induced asthma treated with dexamethasone (Fig. 4).

Sivelestat decreased enhanced pause, airway inflammatory cell number, and the shedding of airway epithelial cells in the house dust mite-induced C57BL/6 asthmatic mouse model

To show the reliability of Penh as a measure of AHR in rodents, an HDM-induced C57BL/6 mouse model of asthma was created. Following exposure to increasing concentrations (0, 3.125, 6.25, 12.5, 25 and 50 mg/ml) of methylcholine, Penh in the HDM-induced C57BL/6 asthmatic mouse model showed a trend similar to that in the OVA-induced BALB/c asthmatic mouse model, showing the reliability of Penh in different asthma models.

To investigate the effect of neutrophils on Penh, we treated HDM-induced C57BL/6 asthmatic mice with sivelestat, a specific neutrophil elastase inhibitor, and subsequently analyzed Penh. At a methylcholine concentration of 50 mg/ml, Penh values were significantly different between the HDM-induced asthmatic mice treated with PBS and those treated with sivelestat, indicating possible alleviation of Penh by suppression of neutrophil function (Fig. 5A).

To investigate the effect of neutrophils on airway inflammation and epithelial cell shedding, BAL fluid was collected and differential cell counts were performed. We found that the levels of eosinophils, lymphocytes, macrophages, and shed airway epithelial cells were decreased in the airways of HDM-induced asthmatic mice treated with sivelestat, indicating possible alleviation of airway inflammation and epithelial cell shedding by suppression of neutrophil function (Fig. 5B).

DISCUSSION

Airway hyperresponsiveness (AHR) is a fundamental abnormality in asthma and can often persist in asthma treated with glucocorticoids [20-22]. Glucocorticoids such as dexamethasone, budesonide, beclomethasone, and triamcinolone can change the proportions of airway inflammatory cells and may cause harm to airway epithelial integrity in asthma [6, 7, 23]. We found that alterations in airway inflammation and airway epithelial injury might be involved in AHR, which might further weaken the efficacy of glucocorticoids in the treatment of asthma.

Airway neutrophilia frequently occurs in acute exacerbations and persistent asthma [22, 24]. Neutrophils were believed to be short-lived cells *in vivo*; however, a recent study has shown that the average circulatory lifespan of neutrophils is up to 5.4 days and 12.5 hours in humans and mice, respectively, indicating that neutrophils might play a more important role in asthma than previously predicted [25]. Neutrophils have recently been found to promote AHR by increasing oxidative stress and occupying the luminal space in large airways [24]. Moreover, we found that AHR had a strong positive correlation with airway neutrophil levels, but not with the levels of eosinophils, lymphocytes, or macrophages. Further, HDM-induced asthmatic mice treated with sivelestat, a specific neutrophil elastase inhibitor, showed a decrease in AHR, suggesting that neutrophils are important and might even be the main cell type involved in AHR in certain types of asthma.

Airway epithelium is a physical and mechanical barrier representing the first line of defense against pathogens, antigens and other inhaled materials. Some researchers believe that airway epithelial injury is a necessary precursor to the onset of asthma [26]. Airway

epithelial injury is common even in the mildest forms of asthma [26], and the shedding of epithelial cells in BAL fluid is regarded as a criterion for determining airway epithelial injury in asthma [19]. Excessive shedding of airway epithelial cells may lead to a broken airway epithelial barrier. In the present study, we found that shedding of airway epithelial cells correlated positively with AHR, suggesting that excessive shedding of airway epithelial cells might be both an indicator of and a risk factor for AHR in asthma treated with glucocorticoids.

Neutrophils have been shown to augment lung damage in the pathogenesis of acute respiratory distress syndrome [27], and this may also occur in asthma. We found that airway neutrophil levels, but not the levels of eosinophils, lymphocytes, or macrophages, are positively correlated with shedding of airway epithelial cells in BAL fluid. Moreover, HDM-induced asthmatic mice treated with the neutrophil elastase inhibitor, sivelestat, showed less epithelial cell shedding, implying that airway neutrophils might be the primary inflammatory cells that augment epithelial damage in asthma treated with glucocorticoids. Neutrophils have been reported to release proteases, such as neutrophil elastase and matrix metalloproteinase 9, to degrade extracellular matrix molecules [28], and might further damage the attachment of columnar epithelium to the basement membrane, causing excessive epithelial shedding.

Eosinophils have been considered the central cells of allergic asthma [26]; therefore, many scholars assume that the number of eosinophils represents the greatest number of airway inflammatory cells in allergic asthma. However, we have found that eosinophils were fewer than neutrophils in the airways of OVA-challenged mice, which is in accordance with

recent studies in asthma patients [5, 29] and asthma animal models [11, 30]. Moreover, the neutrophil elastase inhibitor, sivelestat, decreased eosinophil infiltration in HDM-induced asthmatic airways, indicating that neutrophils might regulate eosinophil infiltration in asthma. Recently, the primacy of eosinophils in the inflammatory milieu of asthma has been challenged. In clinical studies, anti-eosinophil treatment has resulted in a dramatic reduction in airway eosinophil levels, but paradoxically has a minimal effect on AHR in asthma patients [26]. Previous studies have found that sputum eosinophil levels are not related to the degree of AHR, as assessed in terms of a 20% decrease in FEV1 [31]; and here we found that eosinophil levels in BAL fluid did not relate to AHR, as assessed in terms of Penh 50, indicating that the number of airway eosinophils might be an imperfect index for AHR.

Eosinophils appear to have a minimal impact on epithelial damage in the acute phase of asthma. We found that airway eosinophil levels did not correlate with the shedding of airway epithelial cells in asthmatic mice treated with dexamethasone. Moreover, Yoshihara [32] showed that airway epithelial damage does not correlate with eosinophil activity during acute exacerbations of pediatric asthma. Conversely, eosinophils have been found to promote epithelial damage in chronic asthma [33]. We suggest that these diverse results regarding the effects of eosinophils on airway epithelial cells might be due to the different phases (chronic or acute) of asthma. Firstly, eosinophils might cause little injury to airway epithelial cells, since fewer eosinophils infiltrate the airways in acute exacerbations of asthma [17]. Secondly, eosinophils are usually sensitive to treatment with glucocorticoids, and glucocorticoids might decrease eosinophils and further attenuate the damage to airway epithelial cells.

We found that shedding of epithelial cells was not related to the levels of airway

lymphocytes or macrophages, and similar results have been found in other studies regarding both acute [32] and chronic [33] asthma. Moreover, we found that Penh did not correlate with the levels of airway lymphocytes or macrophages. From our results, it can be concluded that lymphocyte or macrophage infiltration might have a slight role in airway epithelial shedding and AHR pathology in asthma treated with glucocorticoids.

At the present time, there is no gold standard for measuring lung function in mice, since neither invasive parameters (such as pulmonary resistance and dynamic compliance) nor non-invasive parameters (such as Penh) are optimal in all regards [34]. Penh is a calculated index involving expiratory time, relaxation time, and peak inspiratory and expiratory flow of an animal kept in a chamber [35], and it does not represent direct changes in airway diameter or resistance [36]. Nevertheless, Penh is still considered to be correlated with airway resistance and to be repeatable with respect to measuring airway reactivity in animal models [37, 38]. Moreover, Penh calculation is almost unaffected by environmental changes such as methylcholine challenge, box temperature changes, and relative humidity changes during normal testing [39]; therefore, Penh can, at least to a certain extent, reflect pulmonary mechanics [36, 39].

CONCLUSIONS/KEY FINDINGS

Airway neutrophil levels, but not the levels of eosinophils, lymphocytes, or macrophages, have positive correlations with Penh 50 and the shedding of airway epithelial cells. Moreover, HDM-induced asthmatic mice treated with the neutrophil elastase inhibitor, sivelestat, showed decreased Penh 50 and less epithelial cell shedding, indicating that neutrophils might

be the key inflammatory cells that cause AHR and augment airway epithelial injury in asthma. Further, the shedding of airway epithelial cells correlated positively with Penh 50, suggesting that excessive shedding of airway epithelial cells might be both an indicator of and a risk factor for AHR in asthma treated with glucocorticoids.

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AUTHOR CONTRIBUTIONS

All authors participated in the writing, review, and editing of this manuscript.

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Figure Legends

Figure 1. Penh and lung hematoxylin & eosin staining of OVA-induced BALB/c asthmatic mice treated with dexamethasone.

(A) Schematic of the effects of dexamethasone in an OVA-induced BALB/c mouse model of asthma. (B) Penh in mice treated with different concentrations of dexamethasone (n=7, using a two-way ANOVA). ** vs. OVA+PBS, $P<0.01$. (C) Representative H&E staining of lung tissues from OVA-induced BALB/c asthmatic mice treated with dexamethasone. Airway inflammation infiltrated around the bronchi and blood vessels in OVA-challenged mice treated with PBS, Dex 1, and Dex 5 (black arrows). Magnified, $\times 100$. Dex 1, 1 mg/kg dexamethasone; Dex 5, 5 mg/kg dexamethasone.

Figure 2. Correlation between Penh 50 and airway inflammatory cells in broncho-alveolar lavage fluid from OVA-induced BALB/c asthmatic mice treated with dexamethasone.

(A) Quantitative analysis of airway inflammatory cells in BAL fluid from mice treated with different concentrations of dexamethasone (n=6, using a one-way ANOVA). # vs. naïve, $P<0.05$; ## vs. naïve, $P<0.01$. * vs. OVA+PBS, $P<0.05$; ** vs. OVA+PBS, $P<0.01$. Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes; Mac, macrophages; Dex 1, 1 mg/kg dexamethasone; Dex 5, 5 mg/kg dexamethasone. (B) Correlation between Penh 50 and airway inflammatory cells in BAL fluid from OVA-induced BALB/c asthmatic mice treated with different concentrations of dexamethasone (using Pearson's correlation coefficient). The

data (Penh values and airway inflammatory cells in BAL fluid) were collected from naïve mice and OVA-induced asthmatic mice treated with 0, 1 and 5 mg/kg dexamethasone.

Figure 3. Correlation between Penh 50 and shedding of airway epithelial cells in OVA-induced BALB/c asthmatic mice treated with dexamethasone.

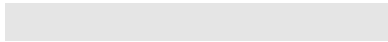
(A) Representative images of shed airway epithelial cells in BAL fluid from mice treated with different concentrations of dexamethasone (n=6, OVA+PBS vs. naïve, using a one-way ANOVA; OVA+Dex 1 vs. naïve, using a Student's *t*-test). ## vs. naïve, $P < 0.01$. Dex 1, 1 mg/kg dexamethasone; Dex 5, 5 mg/kg dexamethasone. **(B)** Correlation between Penh 50 and shed airway epithelial cells in BAL fluid from OVA-induced BALB/c asthmatic mice treated with dexamethasone (using Pearson's correlation coefficient). The data (Penh values and shed airway epithelial cells in BAL fluid) were collected from naïve mice and OVA-induced asthmatic mice treated with 0, 1 and 5 mg/kg dexamethasone.

Figure 4. Correlation between shedding of airway epithelial cells and airway inflammatory cells in bronchoalveolar fluid from OVA-induced BALB/c asthmatic mice treated with dexamethasone.

The number of cells in BAL fluid collected from naïve mice and OVA-induced asthmatic mice treated with 0, 1 and 5 mg/kg dexamethasone were compared (using Pearson's correlation coefficient). Eos, eosinophils; Lym, lymphocytes; Neu, neutrophils; Mac, macrophages; Shed Epith, shed airway epithelial cells.

Figure 5. Correlation between Penh and airway inflammatory cells in bronchoalveolar lavage fluid from HDM-induced C57BL/6 asthmatic mice treated with sivelestat.

(A) Penh in mice treated with different concentrations of sivelestat (n=6, using a two-way ANOVA). * vs. HDM+PBS, $P<0.05$; ** vs. HDM+PBS, $P<0.01$. (B) Quantitative analysis of airway inflammatory cells in BAL fluid from mice treated with different concentrations of sivelestat (n=5, using a one-way ANOVA). ## vs. naïve, $P<0.01$. * vs. HDM+PBS, $P<0.05$; ** vs. HDM+PBS, $P<0.01$. Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes; Mac, macrophages; SVT, sivelestat.



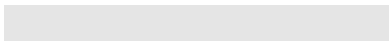
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CONFLICTS OF INTEREST

None of the authors have any conflicts of interest related to this study.

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Figure 1

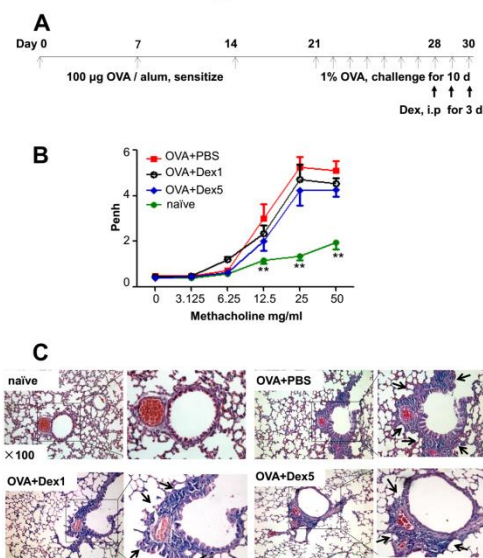


Figure 2

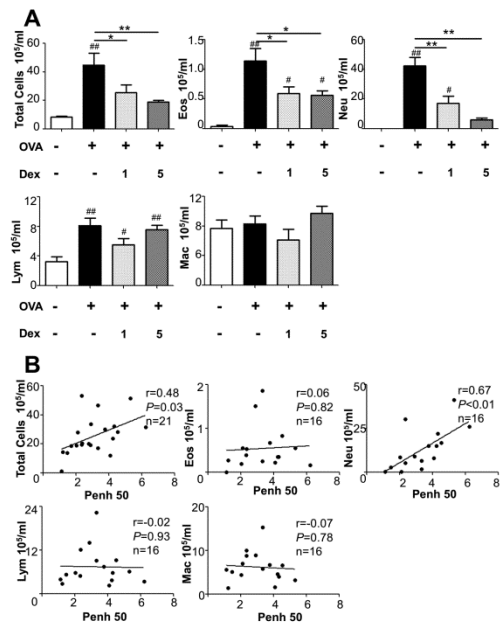


Figure 3

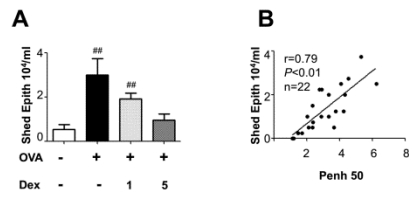


Figure 4

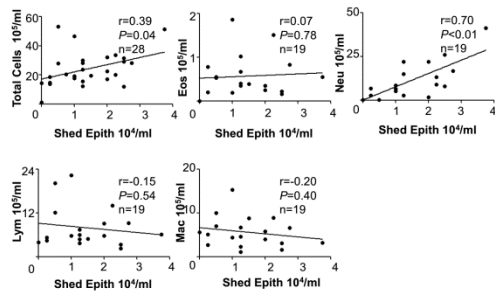


Figure 5

